A Role for the C2 Domain of the Ubiquitin-Protein Ligase Nedd4

by

Pamela J. Plant

A thesis submitted in conformity with the requirements of the Degree of Doctor of Philosophy,
Graduate Department of Biochemistry
University of Toronto

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A Role for the C2 Domain of the Protein Ubiquitin Ligase Nedd4

By Pamela Plant, for the Degree of Doctor of Philosophy, Department of Biochemistry,
University of Toronto, 2000

Abstract

Neural precursor cell-expressed developmentally downregulated 4 (Nedd4) is a ubiquitin-protein ligase that has an N-terminal conserved region 2 of PKC (C2) domain, three or four WW domains and a C-terminal homologous to E6-AP C-terminus (HECT) domain which possesses ubiquitin ligase activity of the third enzyme of the ubiquitination cascade (E3). Nedd4 was found to bind to the epithelial Na⁺ channel (ENaC), via its WW domains, and to be an important regulator of ENaC stability and function.

C2 domains are small (~130 amino acid) Ca²⁺- and lipid-binding domains that are found in a number of signalling molecules and many have been shown to mediate Ca²⁺-dependent membrane/protein association. The focus of this work was to determine a role for the C2 domain of Nedd4 in the functioning of this enzyme. It was found that Nedd4 displays a Ca²⁺-dependent membrane association, and was demonstrated that this phenomenon is due to the action of the C2 domain. In particular, the C2 domain mediates the redistribution of cytosolic Nedd4 to the apical region of polarized epithelia in response to increased cytosolic Ca²⁺ concentrations. This preference for the apical region may be mediated by an association of the C2 domain with the apically targeted molecule annexin XIIIb. The C2 domain interacts with annexin XIIIb in a Ca²⁺-dependent manner and co-localizes with it in cells and in isolated apical rafts in a Ca²⁺-dependent fashion. When annexin XIIIb expression is stimulated using an inducible system, it targets Nedd4 to the plasma membrane in response to increased cytosolic Ca²⁺. In the absence of annexin XIIIb overexpression, Nedd4 remains in the cytosol.

A novel interaction between the Nedd4-C2 domain and the SH2 domain of Grb10 was characterized, which may serve to target Nedd4 to the IGF-I receptor, a potential substrate,
within the cell. This interaction is non-phosphotyrosine-dependent and non-Ca\textsuperscript{2+}-dependent as well. We have shown that the IGF-I receptor is ubiquitinated in cells, which may be the ultimate consequence of the C2 association with SH2-Grb10.

Thus, the study of the role of the C2 domain of Nedd4 has provided evidence that this domain is serving to target Nedd4 to distinct locations within the cell, where it can interact with its substrates. This specialized distribution is mediated via specific protein-protein and protein-lipid interactions. The work on the underlying mechanisms of Nedd4 function has provided an insight into the more general issues of regulation of protein stability and cellular functioning.
Acknowledgments

Jean-Paul Sartre observed that individuals create their own living hell out of the people around them (l'en fer c'est les autres). Throughout the course of this degree I have observed differently....

I give many thanks to my supervisor, Daniela Rotin for her dogged initiative, untiring dedication to science and ceaseless ability to let me explore, uninhibited, the intellectual world around me. It was truly an honor to observe and work with an internationally recognized scholar and zealot of scientific precision and quality, attributes that naturally diffuse to those around her. To have observed these qualities, and see them extending into other aspects of her life, was inspirational.

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My immensely knowledgeable graduate committee, comprised of Sergio Grinstein, Reinhart Reithmeier and Mike Moran, has helped me steer my ship through the daunting seas of signalling and cellular biology and in particular, thanks to Sergio for buoying me up when my
ship appeared to be sinking. The administrative support I have received throughout my degree has been critical and I am truly appreciative of the efforts of Anna Vanek in the Biochemistry graduate office and Marjorie Samuel in Lung Biology at Hospital for Sick Children. I also acknowledge Peter Lewis, the Chairman of the Biochemistry Department for truly caring about the graduate students in the department and his effectiveness as a leader.

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# Table of Contents

Abstract       i  
Acknowledgments iii  
Table of Contents vi  
List of Figures x  
List of Tables xii  
Abbreviations xiii  

## Chapter One: Introduction

I. Overview 2  
II. Protein Degradation 3  
III. Components of the Ubiquitin Cascade 5  
   A. Ubiquitin 5  
   B. Ubiquitin-like Proteins 13  
   C. The Enzymes of Ubiquitin-Protein Degradation 18  
   D. Deubiquitination 22  
   E. Mechanisms of Degradation of Ubiquitinated Proteins 23  
IV. Biological Functions of the Ubiquitin System 27  
   A. General 27  
   B. Diseases Associated with the Ubiquitination Pathway 31  
V. E3 Enzymes 33  
   A. Classes of E3 enzymes 33  
   B. Recognition Sequences for E3 Mediated Ubiquitination 40  
VI. Ubiquitination and Endocytosis of Plasma Membrane Proteins 46  
   A. General 46  
   B. Ubiquitination of Yeast Plasma Membrane Proteins 49
C. Ubiquitination of Mammalian Plasma Membrane Proteins

VII. Nedd4

A. Nedd4 Physiological Function

B. Nedd4 Homologues and Nedd4-like Proteins

C. The Basis of Nedd4 Specificity: WW domains and their Interaction with PY Motifs

D. The Basis of Nedd4 Enzymatic Activity: The HECT Domain

VIII. C2 Domains

General Information

A. Primary and Tertiary Structure

B. Mechanism of Ca\(^{2+}\)-binding to the C2 Domain

C. Mechanism of C2 Membrane Interaction

D. Function of C2 Domains in Signalling Proteins

IX. Annexins

X. Summary

XI. Rationale and Goals of Research

Chapter Two: The C2 Domain of Nedd4 Mediates Ca\(^{2+}\)-dependent Membrane Association

I. Summary

II. Introduction

III. Experimental Procedures

IV. Results

A. Ca\(^{2+}\)-dependent Association of Endogenous Nedd4 with Cellular Membranes

B. The Nedd4 C2 Domain is Sufficient to Mediate Ca\(^{2+}\)-dependent Membrane Association in Vitro

C. Deletion of the C2 Domain Abrogates Ca\(^{2+}\)-dependent Membrane Association of Nedd4 In Vivo

V. Discussion
Chapter Three: Apical Membrane Targeting of Nedd4 is Mediated by an Association of its C2 Domain with Annexin XIII

I. Summary
II. Introduction
III. Experimental Procedures
IV. Results
   A. Identification of Annexin XIII as a Binding Partner for the Nedd4-C2 Domain
   B. Annexin XIII/a/b and Nedd4-C2 Domain Interact In Vitro and in Living Cells
   C. Ca\(^{2+}\)-dependent Distribution of Nedd4 in MDCK Cells Expressing Annexin XIIIb
   D. Subcellular Localization of Nedd4 in the Apical Pathway of MDCK Cells
   E. Annexin XIIIb Expression Enhances the Association of Nedd4 with Rafts
V. Discussion

Chapter Four: Summarizing Discussion and Future Directions

I. Summarizing Discussion
II. Future Directions
   A. A Role for the C2 Domain of Nedd4 in Endocytosis
   B. A Role for the C2 Domain of Nedd4 in Regulation of ENaC Activity
   C. A Role for the C2 Domain of Nedd4 in Mediating Targeting of Nedd4 to Potential Cellular Substrates
   D. Summary and Conclusions

Appendix I: The C2 Domain of Nedd4 Mediates a Phosphotyrosine-independent Interaction with the SH2 Domain of Grb10

I. Summary
II. Introduction
III. Experimental Procedures
List of Figures

Chapter One: Introduction

Figure 1-- Sequence Alignment of Ubiquitin 7
Figure 2-- Structure of Ubiquitin 9
Figure 3-- Schematic Representation of Various Protein-Ubiquitin Linkages 12
Figure 4-- Sequence Alignment of Ubiquitin-like Proteins 15
Figure 5 -- The Ubiquitination Cycle 20
Figure 6-- Structure of the 20S and 26S Proteasome 25
Figure 7-- Different Types of E3 Enzymes of E3 Enzyme Complexes 36
Figure 8-- HECT Domain Containing Proteins of the Nedd4/Nedd4-like Family 42
Figure 9-- Modular Representation of the Ubiquitin-Protein Ligase Nedd4 57
Figure 10-- Modular Representation of the Functional Domains of Proteins Containing C2 Domains 66
Figure 11-- Multiple Sequence Alignment of Various C2 Domains 69
Figure 12-- Schematic Representation of the Two Prototypical C2 Domain Topologies 72
Figure 13-- Model of the Bi-partite Ca²⁺-binding Motif of the C2A Domain of Synaptotagmin I 74
Figure 14-- A Comparison of the Structures for the C2 domains of Topology I, II (Ca²⁺-dependent and non-Ca²⁺-dependent) 77

Chapter Two: The C2 Domain of Nedd4 Mediates Ca²⁺-dependent Membrane Association

Figure 2-1 -- Ca²⁺-induced Association of Nedd4 with Crude Membranes of MDCK Cells 103
Figure 2-2 -- Confocal Micrographs of Ca²⁺-dependent Apical and Lateral Membrane Localization of Nedd4 in Polarized MDCK Cells 106
Figure 2-3 -- Time Course of Ca²⁺-dependent Apical and Lateral Membrane Association of Nedd4 in Polarized MDCK Cells 110
Figure 2-4 -- Ca²⁺-dependent Binding of GST-Nedd4-C2 to MDCK Crude Membranes 113
Figure 2-5 -- Ca²⁺-dependent Association of GST-Nedd4-C2 with Phospholipids 115
Figure 2-6-- Epitope-tagged Nedd4 in Transfected MDCK Cells Displays Ca^{2+}-dependent Membrane Association 118

Figure 2-7-- Lack of Ca^{2+}-induced Membrane Localization of C2-deleted Nedd4 Stably Expressed in MDCK Cells 121

Chapter Three: Apical Membrane Targeting of Nedd4 is Mediated by an Association of its C2 Domain with Annexin XIIIb

Figure 3-1-- Annexin XIIIb is Identified as an Interacting Protein of Nedd4-C2 Domain 137

Figure 3-2-- Ca^{2+}-dependent Co-precipitation of Nedd4-C2 Domain and Annexin XIIIa and b In Vitro 142

Figure 3-3-- Ca^{2+}-dependent Co-precipitation of Nedd4-C2 Domain and Annexin XIIIa and b from Mammalian Cells 145

Figure 3-4-- Annexin XIIIa and b but not Isolated Endonexin Folds Bind GST-Nedd4-C2 Directly In Vitro 148

Figure 3-5-- Ca^{2+}-dependent Distribution of Nedd4 and Annexin XIIIb in MDCK Cells 150

Figure 3-6-- Co-localization of Annexin XIIIb and Nedd4 in MDCK Cells 153

Figure 3-7-- Nedd4 Localization in Apical Exocytic Carriers 155

Figure 3-8-- Ca^{2+}- and Annexin XIIIb-dependent Raft-association of Nedd4 158

Chapter Four: Summarizing Discussion and Future Directions

Figure 4-1-- Proposed Model of Nedd4 Targeting and Regulation of ENaC Activity 166

Appendix I: The C2 Domain of Nedd4 Mediates a Phosphotyrosine-independent Interaction with the SH2 Domain of Grb10

Figure A-1-- mGrb10 Interacts with mNedd4 in the Two-hybrid System 180

Figure A-2-- Grb10 Co-immunoprecipitates with Nedd4 In Vivo 183

Figure A-3-- Grb10 Co-immunoprecipitates a weakly tyrosyl-phosphorylated Nedd4 and the Interaction is Ca^{2+}-independent 186

Figure A-4-- Effect of IGF-I and Insulin on Grb10/Nedd4 Binding 189

Figure A-5--Lack of In Vivo Ubiquitination of Grb10 Protein 191
List of Tables

Chapter One: Introduction

Table I: Ubiquitination Motifs of Yeast and Mammalian Proteins  43
Table II: Plasma Membrane Proteins that Undergo Ubiquitin-dependent Endocytosis  48
List of Abbreviations

APC -- anaphase promoting complex

C2 domain -- conserved region 2 of Ca$^{2+}$-dependent isoforms of PKC, a known Ca$^{2+}$ and lipid/membrane binding domain

CaLB -- Ca$^{2+}$-lipid binding domain (a subregion of the C2 domain)

CFTR -- cystic fibrosis transmembrane conductance regulator

DAG -- diacylglycerol

DUB -- Deubiquitinating enzyme

EGFR -- epidermal growth factor receptor

ENaC -- the epithelial Na$^{+}$ channel

GST -- gluthathione S-transferase

HECT -- homologous to E6-AP (E3 ubiquitin ligase) domain

IGF-IR -- insulin-like growth factor receptor I

IP$_3$ -- inositol 1,4,5 triphosphate

MDCK -- Madin Darby canine kidney

Nedd4 -- neural precursor cell-expressed developmentally-downregulated 4

PDGFR -- platelet-derived growth factor receptor

PH domain -- pleckstrin homology domain

PIP$_2$ -- phosphatidylinositol-4,5-bisphosphate

PKC -- protein kinase C

PLC -- phospholipase C

pSer, pThr, pTyr -- phosphoserine, threonine and tyrosine, respectively

RF-RING finger domain

RUB1 -- related to ubiquitin
SCF -- Skp1p-Cdc53p (Cullin)-F-box complex

SH2 domain -- Src homology 2 domain

SUMO-1 -- small ubiquitin-related modifier

TCR -- T-cell receptor

Ub -- ubiquitin

UBA -- ubiquitin activating enzyme

UBC -- ubiquitin conjugating enzyme

UBP -- ubiquitin-specific processing protease
Chapter One:
Introduction
Chapter 1: Introduction

I. Overview

In the following chapters, the identification, characterization and mechanism of action of the Ca$^{2+}$-lipid binding (CaLB or C2) domain of the protein ubiquitin ligase, Nedd4, will be presented. The studies were all performed with the rat isoform of Nedd4 (rNedd4). The thesis is organized into the following chapters:

(1) An introduction to protein degradation with a focus on the ubiquitination cascade, ubiquitination of plasma membrane receptors, E3 enzymes, Nedd4 and finally C2 domains, with a focus on the C2 domain of Nedd4.

(2) The characterization of the C2 domain of rNedd4, describing the involvement of the C2 domain in mediating the Ca$^{2+}$-dependent membrane association of rNedd4. The results of this chapter also reveal the apical membrane preference of the rNedd4 C2 domain in response to elevated intracellular Ca$^{2+}$.

(3) The characterization of the interaction between the C2 domain of rNedd4 and the apically targeted annexin XIIIb. These results may underlie the phenomena of apical membrane preference described in Chapter 2.

(4) A summary of the work in the previous three chapters in addition to a discussion on future research directions.

(Appendix I) The characterization of interactions between the C2 domain of Nedd4 and the SH2 domain of the adaptor molecule mGrb10. The significance of these interactions with respect to the ubiquitination of plasma membrane receptors is discussed.

Appendix II includes additional, unrelated work that was done during my first year as a Ph.D. student in the Department of Biochemistry, in another laboratory.

This chapter is an introduction to the basic principles of protein degradation, specifically, the process of ubiquitination. The role of ubiquitination in the degradative processes of intracellular
proteins and, in particular, plasma membrane proteins, will be discussed, as well as how this relates to cellular functioning. A description of E3 enzymes and their role in protein degradation will be discussed with a particular focus on Nedd4 and its involvement in regulation of the activity of the epithelial Na⁺ channel (ENaC), a protein with which it interacts. The chapter will conclude with an introduction to the features of C2 domains and the role these domains may be playing in cellular functioning.

II. Protein Degradation

The amount of a given protein present in the cell is controlled by its rate of synthesis and therefore, level of expression, balanced with its rate of degradation (or secretion). The rate of degradation of a protein is controlled by various proteolytic pathways. A number of diverse cellular processes are controlled by degradative pathways and proteolytic mechanisms within the cell. These processes include apoptosis, antigen processing in the immune response, cell-cycle progression, transcription and intracellular signalling. Many proteins involved in these processes are present as large multi-subunit complexes. The biosynthesis and activity of the complex may, in part, be regulated by the relative amounts of the individual component proteins. It is essential that the cell maintain the levels of uncomplexed proteins, which can interfere with normal functioning of complete complexes. One function of cellular proteolytic machinery is to recognize and degrade these unassembled proteins. In addition, proteolysis can serve as cellular “quality control” by disposing of misfolded or irreversibly impaired proteins that may arise through mutation, transcriptional or translational errors or chemical damage (Beckman et al., 1990).

Finally, cellular proteolysis also acts to confer short half-lives to proteins whose concentrations must change rapidly in response to cellular signals or alterations in the cellular milieu. Such proteins can be rapidly degraded either constitutively or at certain times in the life of the cell, thereby temporally affecting cellular processes. A model scenario is the cell cycle, where the
protein composition of the cell is rapidly changing as it undergoes cell-cycle transitions or as it differentiates from one cell type to another.

The proteolysis of intracellular and membrane bound proteins is a highly complex and tightly regulated process. The intracellular pathways that are responsible for protein degradation have classically been divided into two main groups: lysosomal and non-lysosomal. Lysosomes contain high concentrations of proteases and are sites of degradation for many targeted intracellular and extracellular soluble proteins as well as transmembrane proteins (reviewed in Dunn, 1994). Nonlysosomal systems include cytosolic and organelle specific proteases. Protein degradation by these nonlysosomal systems is also highly selective as only targeted proteins are degraded.

One degradative system in the cytosol is calpain. Calpain (CANP) is a calcium activated cysteine protease which exists as two forms μ and m reflecting their differences in Ca\(^{2+}\) concentration (μM and mM) requirements for in vitro activation (reviewed in Molinari and Carafoli, 1997; Suzuki and Sorimachi, 1998). CANP activation requires its association with the plasma membrane and interestingly, its association with the membrane is Ca\(^{2+}\)-dependent (Molinari et al., 1994). The activation of CANP at the membrane may be facilitated by the increased local Ca\(^{2+}\) concentration at the membrane. The concentration of divalent cations at the membrane is approximately 20 fold the concentration 0.5 nm from the membrane (Barber, 1980).

The main nonlysosomal proteolytic system is the proteasome, which recognizes multi-ubiquitinated proteins. The ubiquitination pathway represents a cascade of enzymes that display specificity towards numerous substrates within the cell. The ultimate consequence of this pathway is the covalent attachment of a single or multiple ubiquitin moieties to proteins which tags them for degradation by either the 26S proteasome or by internalization and subsequent degradation in the endosome/lysosome. Ubiquitin mediated degradation of regulatory proteins is critical to the control of several cellular processes such as cell-cycle progression, development and transcriptional regulation.
III. Components of the Ubiquitin Cascade

The categorical factor which determines that a cellular or membrane protein will be degraded by the proteasome is its modification by ubiquitin or ubiquitin-like moieties. Although the proteasome is not the absolute fate of all ubiquitinated proteins, the majority of cytosolic proteins that undergo this modification are destroyed by the proteasome.

III.A. Ubiquitin

Ubiquitin is a 76 residue (Fig. 1) widely expressed protein that is found in the free, unbound state or covalently attached to a variety of intracellular and transmembrane proteins as well as to itself. The amino acid sequence of ubiquitin is one of the most highly conserved; its amino acid sequence is identical amongst animals and the sequence of the yeast ubiquitin differs from that of mammals by only 3 amino acids (Fig. 1) (Ozkaynak et al., 1987). There are two critical regions within the primary sequence, one being the C-terminal glycine of ubiquitin that serves as an attachment site to the e-amino groups of lysine residues in the target proteins. Another critical site within the sequence is Lys-48 (Fig. 1) which, in most cases, serves as an acceptor site for other ubiquitin moieties. The ligation of subsequent ubiquitins leads to the formation of poly-ubiquitin chains. It has been shown in yeast (Arnason and Ellison, 1994) that ubiquitin molecules can be linked together through Lys-29 and Lys-63 residues as well.

The crystal and NMR solution structures of human ubiquitin demonstrate that the protein folds as a five-stranded β-sheet (Fig. 2), a three and one-half alpha helical turn and seven reverse turns (Vijay-Kumar et al., 1985, 1987; Weber et al., 1987). At the C-terminus, only four amino acids are unstructured. They protrude from the tight folds of the globular domain and form the site for protein-ubiquitin conjugation. The highly conserved Lys-48, involved in multi-ubiquitin chain formation, is located within a turn that is part of a highly contorted turn-rich region of the protein beginning at Phe-45 and ending at Ser-65. It has been demonstrated that, apart from the Lys-48
Figure 1. **Sequence alignment of ubiquitins.** The lysine residues (K) at positions 48 and 63, which are important for polyubiquitination are highlighted. Conserved residues are marked "*"*, while conservative substitutions marked ":\" and semi-conservative substitutions marked ",\". Fly, *Drosophila melanogaster*; dictyos, *Dictyostelium discoideum*; c.elegen, *Caenorhabditis elegans*; yeast, *Saccharomyces cerevisiae*. Sequence alignment was done with ClustalX 1.7.
Ubiquitin Alignment

CLUSTAL W (1.74) multiple sequence alignment

fly-ub
human-ub
dictyos-ub
c.elegan-ub
yeast-ub

MQIFVKTLYGKTITLEVEPSDVNVAIQDKEGIPDDQQLIFAGQLEDGRTLSDEY
MQIFVKTLYGKTITLEVEPSDVNVAIQDKEGIPDDQQLIFAGQLEDGRTLSDEY
MQIFVKTLYGKTITLEVEPSDVNVAIQDKEGIPDDQQLIFAGQLEDGRTLSDEY
MQIFVKTLYGKTITLEVEPSDVNVAIQDKEGIPDDQQLIFAGQLEDGRTLSDEY
MQIFVKTLYGKTITLEVEPSDVNVAIQDKEGIPDDQQLIFAGQLEDGRTLSDEY

***************

↑

Lys48

fly-ub
human-ub
dictyos-ub
c.elegan-ub
yeast-ub

IQKESTHLVLRLRRG
IQKESTHLVLRLRRG
IQKESTHLVLRLRRG
IQKESTHLVLRLRRG
IQKESTHLVLRLRRG

***************

↑

Lys63
Figure 2. Structure of ubiquitin. Secondary structural elements include a five-stranded β-sheet (shown in blue), a three and one-half alpha helical turn and seven reverse turns (shown in orange/yellow). At the C-terminus, the unstructured four amino acids that protrude from the folds of the globular domain (shown in black) form the site for protein-ubiquitin conjugation. The figure was produced using the program MOLMOL.
and residues near or at the C-terminus, no single residue has been shown to be essential to ubiquitin function (Monia et al., 1990; Wilkinson 1988). The sequence conservation may be based on selection for a tightly packed, protease-resistant structure in the globular domain of ubiquitin (Finley and Chau, 1991). The rigid structure may decrease the likelihood of degradation by the proteasome, where ubiquitin-protein conjugates are targeted for degradation and ubiquitin is recycled (Finley and Chau, 1991). Ubiquitin may be ligated to substrate proteins in various configurations, depending on the number of ubiquitin groups and whether multi-ubiquitin chains are formed (Fig. 3). Substrates are most rapidly degraded when they are conjugated to multiple molecules of ubiquitin (Hershko et al., 1984; Hough and Rechsteiner, 1986; Haas et al., 1990; Gregori et al., 1990). A substrate bearing a Lys-48 linked chain of 8 to 12 ubiquitins is degraded ~10 times more rapidly than a substrate bearing a single ubiquitin at the same position (Chau et al., 1989). In fact, the subunit 5a (S5a) of the regulatory complex of the 26S proteasome binds Lys-48-linked multiubiquitin chains of equal to or greater than 4 chains while it has low affinity for monoubiquitin and chains of \( n \leq 3 \) (Deveraux et al., 1994). This may result from the requirement of the proteasome complex for the packing order and symmetry of the multiubiquitin chain.

While a significant fraction of proteasome-mediated degradation occurs through conjugated intermediates bearing Lys-48-linked multiubiquitin chains, evidence does exist for multiubiquitin chains possessing linkage specificities distinct from Lys-48 (Haas et al., 1991; Spence et al., 1995; Baboshina and Haas, 1996). In particular, stable multiubiquitin chains with the linkage site at Lys-63 have been observed in yeast, requiring the ubiquitin conjugating (E2) enzyme RAD6 (UBC2) and this is proposed to account in part for the DNA repair function of this E2 isoform (Spence et al., 1995). The yeast uracil permease, Fur4p (see below), has been observed to be modified by short Lys-63-linked chains as well, and it has been shown that conjugation of these molecules onto the two lysine acceptor sites (Lys-48 and Lys-63) on the transporter is necessary for Fur4p endocytosis (Galan and Hagenauer-Tsapis, 1997).
Figure 3. Schematic representation of various protein-ubiquitin linkages. The target protein is shown as a dark line with amino termini (N) and carboxyl termini (C) indicated. Ubiquitin is represented by the lollipop; A, monoubiquitinated protein; B, ubiquitin-protein fusion; C-E, multiubiquitinated proteins; C, protein modified at different sites by ubiquitin, D, protein carrying multi(poly)ubiquitinated chain, as the term is used in the text and E, protein modified by ubiquitin with Lys48 and Lys 63 linkages. From Finley and Chau, 1991.
III.B. Ubiquitin-like Proteins

The covalent attachment of other ubiquitin-related modifiers also serves to regulate protein function and stability. One such modifier, SUMO-1 (Small Ubiquitin-related Modifier), which is 18.4% identical (44.7% similar) to ubiquitin (Fig. 4), was discovered by several independent groups working on nuclear processes, indicating the involvement of SUMO-1 in regulating nuclear events. Amongst others, SUMO-1 associates with the human DNA repair proteins Rad51 and Rad52 (Shen et al., 1996) as well as the GTPase activating protein of Ran, Ran GAP1 (Matunis et al., 1996; Mahajan et al., 1997). Ran, a small GTPase, and RanGAP1 are both required for functional nuclear transport (reviewed in Gorlich et al., 1996). Conjugation of SUMO-1 to RanGAP1 targets an otherwise cytosolic RanGAP to the nuclear pore complex (NPC) located on the cytosolic side of the pore (Matunis et al., 1996; Mahajan et al., 1997; Saitoh et al., 1996), an action which is essential for nuclear transport (Mahajan et al., 1997). There is no evidence, however, suggesting that conjugation of SUMO-1 targets RanGAP1, or any other interacting proteins, for destruction. In fact, following SUMO-1 conjugation, there is no significant proteolysis of RanGAP1 (Matunis et al., 1996; Mahajan et al., 1997; Saitoh et al., 1997a). Moreover, no RanGAP species containing multiply conjugated SUMO-1 molecules have been observed and the conserved lysines of ubiquitin that are utilized for the production of multi-ubiquitin chains are not conserved in SUMO-1 (Fig. 4). This, together with the fact that proteins conjugated with a single ubiquitin monomer do not display the instability that proteins modified with ubiquitin-polymers do, might suggest that SUMO-1 conjugation may not subject the target protein to rapid degradation (with the assumption that SUMO-1 and ubiquitin are analogous) (Saitoh et al. 1997b).

RUBL is another example of a novel protein modification in the degradation pathway. RUBL, a protein involved in yeast protein degradation, has 53% amino acid identity to ubiquitin (Fig. 4) (Liakopoulos et al., 1998). RUBL utilizes a conjugation cascade, similar to that described for ubiquitin, whereby the ubiquitin-activating (E1)-like proteins (UBA1 and UBA3) are required
Figure 4. Sequence alignment of ubiquitin-like proteins. Sequence alignment of ubiquitin with other small ubiquitin-like proteins. The Lys48 of ubiquitin important for polyligation is highlighted (K). Conserved residues (representing residues present in at least 50% of the sequences indicated) are bolded on the consensus line, while conserved substitutions are marked “:” and semi-conserved substitutions are marked “.”. Sequence alignment was done with ClustalX 1.7.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>RUB-1</td>
<td>----------------------------</td>
</tr>
<tr>
<td>nedd8</td>
<td>----------------------------</td>
</tr>
<tr>
<td>human-Ub</td>
<td>----------------------------</td>
</tr>
<tr>
<td>gdx</td>
<td>----------------------------</td>
</tr>
<tr>
<td>FUB-1</td>
<td>----------------------------</td>
</tr>
<tr>
<td>UCRP</td>
<td>----------------------------</td>
</tr>
<tr>
<td>Elongin-B</td>
<td>----------------------------</td>
</tr>
<tr>
<td>smt-3</td>
<td>----------------------------</td>
</tr>
<tr>
<td>SUMO-1</td>
<td>PVAVGILAOEVKLSPDFHNNFFFDGMKNGIVECLGPK</td>
</tr>
</tbody>
</table>
for its activation and the E2-related enzyme UBC12 is necessary for its conjugation. Although conjugation of RUB1 is not essential in yeast, several key regulators of cell-cycle progression, namely CDC53/cullin, a subunit of the SCF ubiquitin ligase complex (described below), are major substrates for RUB1 ligation (Liakopoulos et al., 1998). It is likely that conjugation of this modifier may be playing a regulatory role in this cascade by influencing the activity of SCF complexes or by affecting SCF specificity towards its different substrates.

Elongin B is another protein containing a ubiquitin-like domain (Fig. 4) and is 58% identical (79% similar) to ubiquitin. Elongin B is part of a functional complex and together with elongins A and C stimulates transcriptional elongation in vitro (Aso et al., 1996). The tripartite complex is loosely analogous to the SCF complex configuration (see below) and elongin B is believed to bind directly to elongin C to promote the assembly and stability of the complex (reviewed in Shilatifard 1998; Patton et al., 1998). The elongin B and C complex can also form a separate complex with the von Hippel Lindau (VHL) tumor supressor gene product (Duan et al., 1995). This interaction is believed to be essential for VHL tumor supressor activity since VHL mutants from VHL tumors and carcinomas display substantially reduced binding to the elongin BC complex (reviewed in Shilatifard 1998).

The existence of many other small proteins with a high degree of sequence identity to ubiquitin has been reviewed elsewhere (Hochstrasser 1996; Ciechanover 1998). These proteins are likely to be involved in post-translational modifications serving non-proteolytic roles. One example of these ubiquitin-like modifiers is UCRP (Fig. 4), an interferon inducible 15 kDa protein that is similar to two tandem repeats of ubiquitin and may be involved in targeting proteins to the cytoskeleton (Loeb and Haas, 1994). Because of the high evolutionary conservation of the ubiquitin sequence, the discovery of these related proteins will likely continue to grow.
III.C. The Enzymes of Ubiquitin-Protein Degradation

The ubiquitination pathway is comprised of three enzymes (E1-E3) which act in concert to covalently attach single or multiple ubiquitin molecules onto the target protein (Fig.5) (reviewed in Hershko and Ciechanover 1998). The tagged protein is subsequently degraded via the 26S proteasome or the lysosomal network. In the ubiquitination system, the C-terminal glycine residue of ubiquitin is activated in an ATP-requiring step by a specific activating enzyme, E1 (Step 1). This step consists of an intermediate formation of ubiquitin adenylate, with the release of PPi followed by the binding of ubiquitin to a cysteine residue of E1 via a thioester linkage, with the release of AMP. Activated ubiquitin is then transferred to a cysteine residue of a ubiquitin-conjugating enzyme, E2 (Step 2). The final step is catalyzed by a ubiquitin ligase or E3 enzyme, whereby ubiquitin is linked via its C-terminus in an amide isopeptide linkage to an ε-amino group of the substrate protein’s lysine residues (Step 3). The transfer of ubiquitin to the substrate is governed by at least two different mechanisms. With the HECT-domain family (see below) of ubiquitin ligases (E3 enzymes), ubiquitin is first transferred from an E2 to an active site cysteine residue of the E3 enzyme. This E3-ubiquitin thioester is the donor for amide bond formation with the protein substrate. In the other family of E3 enzymes (i.e. the SCF and APC complexes), the formation of the E3-ubiquitin thioester has not been demonstrated. In these cases it is likely that these E3 enzymes mediate direct transfer of ubiquitin from the E2 to the substrate. In both cases, a polyubiquitin chain is usually formed following linkage to the substrate protein. As mentioned above, this results from the C-terminus of each ubiquitin moiety being linked to the specific Lys-48 of the previous ubiquitin. The ultimate consequence of the E3 addition of ubiquitin to target proteins is branding them for degradation by the proteasome (Chau et al., 1989) or by internalization and degradation (see below).

While there few E1s (only one identified in mammals and three in yeast), there are many different E2s and E3s (likely more to be identified in the future). In fact, the components of the ubiquitin-system are all known for the yeast S. cerevisiae as its genome has been sequenced in its
**Figure 5.** The Ubiquitination Cycle. Ub is synthesized as oligomers or as N-terminal fusions with other proteins (X) and is co-translationally cleaved by peptidases. Ub is activated by E1 in an ATP-dependent fashion. A cascade of thiol intermediates involving E1, E2 and E3 culminate in an isopeptide linkage between Ub and the ε-amino group of a Lys residue on a bound substrate (S), or in a similar linkage with Lys48 of substrate bound Ub to form (or elongate) a poly-Ub chain. In certain instances, E2s mediate monoubiquitination of a substrate independent of E3. Polyubiquitinated substrates are targeted to the 26S proteasome, where they are subject to ATP-dependent proteolysis, with the resultant generation of peptides (shown as small ovals) and free branched poly-Ub chains. Alternatively, poly- or mono-Ub may be cleaved from proteins by isopeptidases (de-ubiquitinating enzymes). Branched poly-Ub chains are disassembled to their constitutive components by isopeptidases. From Weissman, 1997.
entirety. In this organism, there are three E1 (ubiquitin-activating) enzymes encoded for, while there are thirteen E2 (ubiquitin-conjugating) enzymes and seven E3 enzymes (that act directly on their substrates) (Hochstrasser, 1996). E3s contain binding sites for E2 enzymes as well as for their substrates (Reiss et al., 1989; Reiss and Hershko, 1990; Hatakeyama et al., 1997). Evidence has suggested that the selective physical interaction between E2 and E3 enzymes form the basis of specificity for functionally distinct E2:E3 combinations (Nuber et al., 1996; Kumar et al., 1997b). While the interaction between an E3 and its target protein may be either direct or indirect, via an adaptor protein, it is believed that these interactions comprise one of the main mechanisms which underlie the selectivity of ubiquitin-protein ligation; by specific E3s binding to specific recognition signals within their substrates. The other mechanism is through modifying enzymes and ancillary proteins that play an important role in the recognition process. Many target proteins must undergo post-translational modifications such as phosphorylation (see below; Recognition Sequences for E3-mediated Ubiquitination), or associate with ancillary proteins such as molecular chaperones prior to recognition by the appropriate ligase (reviewed in Ciechanover, 1998).

The existence of an “E4” co-factor has recently been demonstrated (Koegl et al., 1999) although it is not known whether it represents a globally functioning family of co-factors or whether it functions within a very specific and limited scope. This E4 co-factor has been shown to be vital to efficient multiubiquitination needed for recognition by the proteasome. In the absence of E4, ubiquitination of a substrate is initiated but ligation of only up to 3 ubiquitin molecules ensues and degradation via the proteasome is not signaled (Koegl et al., 1999) as recognition by the proteasome requires at least four ubiquitin molecules (Deveraux et al., 1994). Members of the E4 family include the yeast protein UFD2, the developmental regulator NOSA from *Dictyostelium* and two human homologues identified from database searches (accession numbers Q14139 and AB014584). It was shown that in yeast, UFD2 binds to ubiquitin moieties of preformed conjugates and works in concert with E1, E2 and E3 to catalyze ubiquitin chain assembly (Koegl et al., 1999), and that this activity is linked to cell survival under stress conditions.
III.D. Deubiquitination

De-ubiquitinating (DUB) enzymes play a central regulatory role in controlling turnover of intracellular proteins. The enzymes achieving this task are often classified into two families: (1) those that only cleave ubiquitin from peptides and small adducts, such as the yeast Yuh1, and (2) those that cleave a broader range of protein substrates. Small adducts include the products of proteasomal proteolysis while other protein substrates originate from free and/or substrate linked polyubiquitin chains, prior to proteasomal processing, and/or polyubiquitin fusion proteins (Hochstrasser, 1996). The latter family of enzymes, the UBP class (Ubiquitin-specific processing protease) is largely divergent, but all contain similar residues in a region believed to be the active site. The family membership is large, with over 16 UBP enzymes in yeast alone (exceeding the number of E2 enzymes in the same organism) raising the possibility that specific protein turnover rates might be differentially regulated by these enzymes (Hochstrasser, 1996). This notion is also supported by the finding that when the Drosophila deubiquitinating enzyme, Fat facets, is mutated in the conserved region, the enzyme is non-functional and abnormal eye development ensues, in addition to female infertility (Huang et al., 1995). Interestingly, the loss of Fat facets is suggested to promote protein degradation as the eye phenotype is partially suppressed by reducing the levels of a component of the proteasome. It was proposed that Fats facets, under normal conditions, prevents a specific protein from becoming degraded by either deubiquitinating and returning a protein to its normal state, or by altering its ubiquitin modification such that its conformation is no longer favorable for degradation.

Not all de-ubiquitinating enzymes will act as negative regulators of the ubiquitin system, like Fat Facets. Isopeptidases (i.e. isoT) are another class of UBP's that are localized free in the cytosol or are associated with the 26S proteasome (Lam et al., 1997). These proteasome-associated isopeptidases can function as regulatory restraints on the action of the 26S complex. Their ability to deubiquitinate a proteasome-targeted ubiquitinated substrate can modulate the rate of degradation of the substrate (Lam et al., 1997). There is evidence that the yeast isopeptidase Doa4 works in
conjunction with the 26S proteasome (Papa and Hochstrasser, 1993). Disruption of the Doa4 gene in yeast leads to the accumulation of free polyubiquitin chains or increased levels of ubiquitinated proteolytic remnants of the 26S proteasome action. This leads to the depletion of cellular monoubiquitin pools, resulting in defects in cellular ubiquitin-mediated processes (Papa and Hochstrasser, 1993). Thus, Doa4-mediated de-ubiquitination is necessary to replenish the cellular pool of ubiquitin. Another yeast isopeptidase, Ubp14, a functional homologue of the human isoT, mediates the disassembly of unanchored ubiquitin chains and facilitates 26S degradation by preventing these chains from competitively inhibiting poly-ubiquitinated substrates to bind to the 26S complex (Americk et al., 1997; Piotrowski et al., 1997).

III.E. Mechanisms of Degradation of Ubiquitinated Proteins

The degradation of ubiquitinated proteins is a multistep process involving distinct enzymes. Proteins ligated to polyubiquitin chains are commonly degraded by the multisubunit complex, the 26S proteasome, in a process which requires ATP hydrolysis. The S. cerevisiae and Xenopus 26S proteasome include a 20S catalytic core composed of 28 subunits which are structured as a hollow cylinder (Peters, 1993, 1994; Groll et al., 1997) (Fig. 6A). This cylinder is essentially a stack of four rings, each containing seven distinct subunits, with the general architecture \( \alpha_1 \beta_1 \gamma \beta_1 \alpha_1 \). The \( \beta \) subunits contain active sites of proteases with various specificities (Fig. 6B) (reviewed in Rubin and Findley, 1995; Groll et al., 1997; Hershko and Ciechanover, 1998; Bochtler et al., 1999). The mouth of the cylinder is narrow (1.3 nm) and is capped at both ends with another complex of subunits, the 19S particle (Fig. 6C). The S5a subunit of this 19S regulatory proteasome cap is believed to be the binding site for the polyubiquitin chain (Devaurex et al., 1994; van Nocker et al., 1996). Entry into the 20S proteasome can only occur following substantial structural rearrangement of the substrate protein, a process which likely requires passage through the 19S particle where the energy provided by the ATPase activity would facilitate protein unfolding.
Figure 6. Structure of the 20S and the 26S proteasome. (A) Topology of the 28 subunits of the yeast 20S proteasome drawn as spheres. (B) Cut-away view of the proteasome molecule along the cylinder axis. Three of the six calpain inhibitor molecules bound to β1'/PRE3, β2'/PUP1 and β5'/PRE2 are shown as space filling models in red (From Groll et al., 1997). (C) Ultrastructure of the 26S proteasome as a correlation average of a side-on view of the Xenopus 26S proteasome. Note that the 19S cap complexes of presumably regulatory function are attached to the 20S core particle in opposite orientations. From Peters, 1994a.
The products of hydrolysis by the proteasome are de-ubiquitinated peptides, short peptides linked to ubiquitin via their lysine residues and polyubiquitin chains. Isopeptidases, such as proteasome associated UBPs and C-terminal hydrolases, such as isoT act in concert with the 26S proteasome to free ubiquitin from the bound substrate into reusable forms. Following release from the substrate by the action of UBPs and hydrolases, the poly-ubiquitin chains are broken down into mono-Ub by non-proteosomal forms of UBPs which show greater affinity for free poly-Ub chains than ubiquitinated substrates (Wilkinson, 1995a/b). These mono-Ub moieties are then reused in the cascade.

The human oncogene tre-2 (Nakamura et al., 1992) which is an inactive isopeptidase, is tumorigenic (Papa and Hochstrasser, 1993). The inactive protein may act in a dominant-negative fashion, interfering with tre-2 enzyme-mediated degradation of positive regulators of cell proliferation (i.e. the G1 cyclins). The tre-2 oncogene does this by inhibiting the flux through the 26S structure by preventing cleavage of proteasome-bound peptide-poly-Ub conjugates (Papa and Hochstrasser, 1993).

It appears that transmembrane proteins that are tagged by ubiquitin are usually not targeted for degradation by the proteasome. In fact, most membrane proteins are removed from the cell surface via endocytosis and often degraded in the lysosome. In some cases, multimeric complexes are degraded not as a whole, but in individual components with fractions of the protein being recycled to the plasma membrane and the rest being delivered to the lysosome (vacuole in yeast) for degradation by lysosomal/vacuolar proteases. Although extensive studies have been performed on these processes in S. cerevisiae (see below in Ubiquitination of Membrane Proteins) it is still unclear as to why the polyubiquitin chain of ubiquitinated plasma membrane proteins signals endocytosis and not 26S proteasomal degradation.
IV. Biological Functions of the Ubiquitin System

IV.A. General

Ubiquitin-dependent pathways play major roles in numerous biological processes in addition to the elimination of short-lived or improperly folded proteins. Some of these processes include cell differentiation, cell cycle progression, antigen presentation, development, apoptosis, signal transduction, transcriptional regulation, stress response, maturation of autophagic vesicles, functions of the nervous system, muscle atrophy and transmembrane and vesicular transport amongst others (for a more thorough review see Finley and Chau 1991; Hochstrasser, 1996 and Hershko and Ciechanover 1998). In many cases the evidence of the involvement of the ubiquitin pathway in these processes is still circumstantial, and neither the cellular targeted substrates nor the underlying mechanisms involved have been determined. A few of the better documented examples are discussed briefly below.

Cell Cycle progression: The selective proteolysis of a number of regulatory proteins is critical to the control of the cell cycle (reviewed in Hershko, 1997). The progression of the cell cycle is driven by the oscillations in the expression/activities of cyclin-dependent kinases (Cdk). The Cdk activity is regulated by the temporally regulated synthesis and degradation of positive regulatory subunits, cyclins, as well as fluctuations in levels of negative regulators, Cdk inhibitors (Ckis), in addition to phosphorylation (Hershko and Ciechanover, 1998). The accumulation of the various cyclins (specific for the G1, S- or M-phases of the cell cycle) activates Cdks at appropriate times during the cell cycle and are subsequently degraded, causing kinase inactivation. Similarly, levels of some Ckis, which specifically inhibit certain cylin/Cdk complexes, increase and decrease at certain times during the cell cycle. It is the selective ubiquitin-mediated degradation of the cyclins and Ckis as well as other regulators that play vital roles in the control of the cell-cycle (Hershko and Ciechanover, 1998). In budding yeast, proteolytic events have been shown to underlie the major cell cycle transitions: (1) the G1-S phase transition depends on the degradation of the Cdk inhibitor
Sic1 (Schwob et al., 1994), (2) the degradation of anaphase inhibitors to promote sister chromatid separation at the onset of anaphase (Holloway et al., 1993; Yamano et al., 1996; Funabiki et al., 1996) and, (3) the exit from anaphase depends on the degradation of mitotic cyclins (Surana et al., 1993). For a further description of these processes, see Classes of E3 Enzymes.

**DNA repair.** One of the predominant E2 ubiquitin conjugating enzymes in yeast is RAD6/UBC2 (Jentsch et al., 1987). When RAD6 is mutated, this confers hypersensitivity to environmental stress such as ultraviolet light, X-rays and chemical mutagens as well as an inability to repair DNA lesions (Jentsch et al., 1987). Consistent with this is the observation that RAD6 expression is induced in response to DNA damage and is maintained at a high basal level throughout the mitotic cell cycle (Madura et al., 1990). Mutations in the conserved Cys88, the site of ubiquitin linkage, behave as null alleles in vivo (Sung et al., 1990) suggesting that the diverse functions of RAD6 are mediated by its ubiquitin-conjugating activity. It was found that RAD6 is involved in the in vitro polyubiquitination of several mammalian histones (Jentsch et al., 1987; Sung et al., 1988; Haas et al., 1990). Accordingly, it has been proposed that the DNA repair function of RAD6 is mediated by ubiquitination of chromosomal proteins that flank a site of DNA damage thereby altering the chromatin structure of the damaged site or by marking the site for localized binding of repair enzymes (Finley and Chau, 1991).

**Ribosome Biogenesis.** Ubiquitin conjugation has roles in addition to mediating protein degradation: a non-proteolytic, chaperonin function of ubiquitin has been described (Finley et al., 1989). An example of this is provided by ribosomal subunits that are co-translationally synthesized as ubiquitin conjugates. A single copy of ubiquitin is fused in frame to a tail protein that is either 52 aa (Ub52) or 76-81 (Ub80) in length (Finley et al., 1989; Redman and Rechsteiner, 1989). The ubiquitin fusion proteins become incorporated into the ribosome, and the ubiquitin portion is removed during the process. The ubiquitin moiety acts as a chaperone to facilitate the tail proteins incorporation into ribosomes (Planta and Raue, 1988; Finley et al., 1989). If the ubiquitin coding element is deleted from the UBI3 gene, a deficiency of small ribosomal subunits is observed (Finley et al., 1989) and similar results were obtained when the human homologue of UBI3 was expressed.
in yeast (Monia et al., 1990). The UBI3 subset (1-3) of genes defines a class of genes in which an element encoding either ubiquitin or a protein similar to ubiquitin is fused to a downstream coding sequence (Finley and Chau, 1991). Many loci encoding these ubiquitin conjugates have been found in vertebrates and the ubiquitin sequence is fully conserved in the case of ribosomal fusions, perhaps because the ribosomal genes are expressed at levels that are great enough to serve as a bulk source of ubiquitin (Finley and Chau, 1991).

Degradation of Proteins Retained in the Endoplasmic Reticulum: Recent findings support a role for the ubiquitin system in the degradation of proteins retained in the endoplasmic reticulum (ER) (reviewed in Bonifacino and Klausner, 1994; Brodsky and McCracken, 1997; Bonifacino and Weissman, 1998). Secretory and/or transmembrane proteins are discharged into the ER lumen or inserted into the ER membrane, respectively, during biosynthesis. Many of these proteins are capable of being retrotranslocated from these locations in the ER back into the cytosol, through the same machinery by which they were introduced into the ER, in a process known as dislocation or retrotranslocation (Wiertz et al., 1996; Plemper et al., 1997). This machinery, capable of bidirectional translocation, is known as the translocon which is composed of the trimeric ER membrane protein Sec61, the heterodimeric signal-sequence recognition particle (SRP) receptor, and in some cases the translocating chain-associating membrane (TRAM) protein (Gorlich and Rappaport, 1993; Pilon et al., 1997; Plemper et al., 1997) and requires the additional action of the ER membrane or lumenal chaperones Cnelp/calnexin and Kar2p/BiP, respectively (McCracken and Brodsky, 1996; Plemper et al., 1997). Degradation of the retrotranslocated proteins occurs through the ubiquitin/proteasome system (Sommer and Jentsch, 1993; Fenteany et al., 1994; Ward et al., 1995; Biederer et al., 1996, Hiller et al., 1996; Werner et al., 1996). Some of the ER membrane proteins found to be degraded via this system include subunits of the T-cell receptor (TCR) (Huppa and Ploegh, 1997; Yu et al., 1997; Yang et al., 1998), the cystic fibrosis transmembrane regulator (CFTR) (Ward et al., 1995; Jensen et al., 1995), unassembled Na/K ATPase α subunits (Coppi and Guidotti, 1997), MHC class I heavy chains (Hughes et al., 1997), mutant Sec61 subunits (Sommer and Jentsch, 1993; Biederer et al., 1996) and HMG-CoA reductase, carboxypeptidase Y
and proteinase A (Hampton et al., 1996; Hiller et al., 1996). With most of these examples in yeast, the degradative process has been found to require specific E2s.

Antigen Processing: An important part of the immune response is the action of cytotoxic lymphocytes (CTLs), that respond to linear peptide sequences presented on the cell surface of major histocompatibility complex (MHC) class I molecule expressing cells. The peptides that are presented are generated through limited proteolysis of antigenic proteins, a process which occurs in the cytosol by the ubiquitin-proteasome pathway (Rock et al., 1994; Groettrup et al., 1996; Rock, 1996; Rock and Goldberg, 1999). The resulting peptides are transported across the membrane of the ER by the TAP transporter, a member of the ABC family of membrane transporters. On the lumenal side of the ER, the peptides bind to newly synthesized MHC (class I) molecules which follow the transport pathway from the ER to Golgi apparatus and onto the plasma membrane (Germain and Margulies, 1993; York and Rock, 1996). The cytokine γ-interferon, which stimulates antigen presentation, leads also to induction and exchange of three proteasomal subunits in human cells (Gaczynska et al., 1993; Driscoll et al., 1993). These exchanges lead to alteration in the cleavage site preferences of the proteasome: the tryptic- and chymotryptic-like activities, which are mostly the result of the newly incorporated subunits (Ortiz-Navarrete et al., 1991; Martinez and Monaco, 1991; Kelly et al., 1991). The changes result in peptides that terminate mostly with basic or hydrophobic residues, similar to the vast majority of known peptides presented on MHC class I molecules (Rock and Goldberg, 1999). These C-terminal residues may be required for selective uptake by the ER TAP transporter (Heemels et al., 1993) and for better binding to the MHC molecule (Rammensee et al., 1993).

Development: Several lines of evidence have suggested a regulation of development by the ubiquitin system. The Drosophila bendless gene (ben) is an E2 whose expression is restricted to the CNS during development. Mutations in this gene lead to morphological deficits, particularly in synaptogenesis (Oh et al., 1994). UbcD1 is another Drosophila E2, implicated in eye development. This E2 interacts with a component (SINA) of a complex that antagonizes a transcriptional factor (TTK88) whose expression represses neuronal cell fate determination in the developing eye (Tang
This repression is brought about by targeting the transcriptional factor for degradation, as it has been shown by Li and colleagues (1997) that SINA promotes ubiquitination of TTK88, which is then rapidly degraded by the proteasome. Other proteins that are components of the ubiquitin system implicated in regulation of development include: a de-ubiquitinating enzyme from the UBP family, fat facets, involved in eye development in Drosophila, the E3 enzymes, E6-AP involved in human brain development and Su(dx) (Suppressor of deltex) a regulator of Notch signalling and finally, a human E2 enzyme HR6B involved in spermatogenesis.

IV.B. Diseases Associated with the Ubiquitination Pathway

Due to the involvement of ubiquitin in an extensive array of cellular processes, mutations in the protein cascades governing ubiquitination have been implicated in the pathogenesis of many diseases. These diseases include a vast number of malignancies, neurodegenerative diseases, muscle wasting and immune and inflammatory responses. A more comprehensive overview of these diseases can be found elsewhere (Ciechanover, 1998; Schwartz and Ciechanover, 1999). This section will provide a brief overview of several diseases including those linked to mutations in E3 enzymes or the recognition/targeting of their substrates.

The expression of the tumor suppressor p53 is tightly regulated by protein stability. The human papilloma virus is implicated in the pathogenesis of cervical cancers and specifically targets p53 for ubiquitin-regulated proteolysis by encoding a protein, E6, that binds to p53 and another cellular protein, E6-AP, which is an E3 enzyme (Scheffner et al., 1993). The interaction between the three serves to ubiquitinate p53, thereby degrading it and maintaining very low levels within the cell, resulting in unregulated transcriptional activity and malignant transformation.

In some cases of Angelman syndrome, a rare inherited disorder that is characterized by mental retardation and seizures, the disease is caused by a mutation in the gene for the E3 enzyme, E6-AP (Kishino et al., 1997). Although the target substrate for E6-AP in these cases has not been identified, these findings implicate a critical role for E3 in human brain development.
In many neurodegenerative diseases there is an observed increase in the accumulation of ubiquitin conjugates in the pathologic lesions such as in the brainstem Lewy bodies in Parkinson’s disease (Spillantini et al., 1997). Recently, a German family with a missense mutation in the ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1) gene has been described (Leroy et al., 1998). UCH-L1 belongs to a family of deubiquitinating enzymes, and is believed to cleave polymeric ubiquitin to monomers and to hydrolyse bonds between ubiquitin molecules and small adducts (Larsen et al., 1998). The mutation was mapped to an isoleucine (93) that is highly conserved evolutionarily, from human to mouse, rat, yeast and Arabidopsis (Leroy et al., 1998).

Greater than 600 distinct mutations in the cystic fibrosis transmembrane regulator (CFTR) are known to cause cystic fibrosis. CFTR is a multi-spanning membrane protein localized to the apical surface of epithelia where it functions as a chloride channel. The most commonly occurring mutation is a deletion of F508 in the cytosolic region. Although ΔF508 CFTR maintains its normal function as a chloride channel, its targeting from the endoplasmic reticulum (ER) is impaired and it is unable to reach the cell surface. The pathogenesis of the disease is contributed to by the retention of the CFTR protein in the ER and failure to properly mature (Ward et al., 1995). It has been proposed that, as CFTR is normally ubiquitinated, the ΔF508 mutation may affect the rate of ubiquitination and, hence, degradation of the channel. This hypothesis is supported by the findings that inhibitors of proteosomal action stabilize the CFTR (ΔF508) protein within the ER (Ward et al., 1995; Jensen et al., 1995).

Roest and colleagues have shown that knockout mice with inactivation of the gene encoding a ubiquitin conjugating enzyme mHR6B (whose homologue in yeast, UBC2/RAD6, has been shown to be involved in DNA repair) leads to male sterility (Roest et al., 1996). In these mice, the spermatogenic epithelium displayed several errant qualities; there was increased apoptosis in the cells and the spermatozoa were abnormal with aberrant head morphology and impaired motility. It was proposed that mHR6B is involved in the polyubiquitination and degradation of histones, a process that is critical for postmeiotic chromatin remodeling during spermatogenesis (Roest et al., 1996).
Liddle’s syndrome is a hereditary form of hypertension that is directly linked to the deletion or mutation of the proline rich (PY motif) sequences in the C-terminal regions of the β and γ subunits of the epithelial Na⁺ channel (ENaC). It has been shown that these regions of the channel are responsible for binding to the WW domains of Nedd4 (Staub et al., 1996). This association leads to suppression of ENaC activity by reducing the number of channels at the cell surface, a suppression requiring functional ubiquitin ligase Nedd4-HECT domain (Goulet et al., 1998; Abriel et al., 1999). Accordingly, ENaC numbers and function are regulated by ubiquitination (Staub et al., 1997). It is believed that the mutations found in Liddle’s syndrome patients may result in ENaC stabilization, excessive resorption of Na⁺ and water in the distal nephron and, consequently may be involved in the development of hypertension (Firsov et al., 1996; Abriel et al., 1999).

V. E3 Enzymes

As the focus of my project was the role of the C2 domain of Nedd4, which is an E3 enzyme, the remainder of this chapter will focus on the characteristics of E3 enzymes. In particular, I shall describe the various classes of E3 enzymes and what signals they respond to for mediating ubiquitination of cytosolic and membrane-bound proteins in yeast and mammals and some of the roles ascribed to the functional domains of the Nedd4 HECT E3 enzyme.

V.A. Classes of E3 enzymes

Ubiquitin protein-ligases play a pivotal role in conferring specificity of ubiquitin-mediated protein degradation, through the recognition of their substrates and in transfer of ubiquitin from an E2 to their substrates. There are various mechanisms by which an E3 can promote ubiquitin-protein ligation. In some cases, the substrate interacts directly with the E3 while in other cases, the E3 is associated with its substrate as part of a complex with an adaptor molecule. Moreover, the
mechanisms of transfer of an activated ubiquitin from a thioester intermediate to the acceptor amino group of the substrate lysine(s) differ with various E3s. In some cases, the E3 accepts the activated ubiquitin from an E2 and binds it as a thioester intermediate prior to the transfer to the substrate, yet in other cases, the E3 ligase may serve merely to aid in transfer of the ubiquitin directly from an E2 to the protein by tight binding of the E2 and the substrate. E3 enzymes are, therefore, generally defined as enzymes that bind, directly or indirectly, to specific protein substrates and promote the transfer of ubiquitin, directly or indirectly, from a thioester intermediate to amide linkages with proteins or polyubiquitin chains (reviewed in Ciechanover 1998). Governed by this definition, E3 enzymes have been categorized into five groups (Fig. 7): (1) N-end rule E3 enzymes, (2) the cyclosome or anaphase promoting complexes (APCs), (3) SCF (Skp1p-Cdc53p (cullin)-E-box protein) complexes, (4) RING-finger E3s and (5) HECT containing E3s.

_N-end Rule Ubiquitin Ligases_

The archetype of _N-end rule_ E3 enzymes, E3α (also called the N-recognin and whose yeast counterpart is UBR1), is a 200 kDa protein that binds to destabilizing N-terminal amino acids (Bachmair _et al._, 1986; Varshavsky, 1992; Varshavsky, 1996) as described below. Although the E3 enzymes involved in this pathway are well characterized, their physiological substrates are still relatively unknown. Some examples of substrates include Cup9p, a transcriptional repressor of the _S. cerevisiae_ peptide transporter Pdr2p, implicating a role for UBR1 in peptide transport. In addition, UBR1 has been ascribed a role in control of osmoregulation in yeast by involvement in controlling the Snlp-dependent phosphorylation cascade (Varshavsky 1997). Some substrates of E3α do not adhere to the N-end rule and are capable of binding to this enzyme via an undefined region known as the “body” site (Fig. 7) although this phenomena has not been well characterized.

_SCF Ubiquitin Ligase Complex_

A multisubunit complex that has been shown to have E3 activity is the SCF _complex_ (also known as phosphoprotein-ubiquitin ligase complexes or PULC). This complex is responsible for the degradation of other cell-cycle regulators such as the Cdk inhibitor, Sic1p (Schwob _et al._,
Figure 7. Different types of E3 enzymes or E3 enzyme complexes.

C2 domain, conserved region 2 (Ca$^{2+}$-lipid binding) domain; HECT, Homologous to E6-AP C-terminal protein-ubiquitin ligase domain; APC, anaphase promoting complex; DB, destruction box; Cdc, cell division cycle proteins; Ub, ubiquitin; SH2, src-homology 2 domain. Modified from Hersko and Ciechanover, 1998.
A. N-end rule E3 (E3α)

B. Hect-domain E3 (Rsp5p)

C. Cyclosome/APC

D. Phosphoprotein-ubiquitin ligase complexes

E. RING finger E3 (c-Cbl)
1994) or the G1 cyclins. The G1-S transition of the cell cycle depends on the degradation of these G1 cyclins, which are not only temporally but spatially regulated. Loss of several of the cell division cycle (Cdc) proteins (i.e. Cdc34p, Cdc53p) that are integral components of the SCF complex, causes the yeast to arrest in the G1 phase because of a failure to degrade the G1 cyclins Cln1p and Cln2p (Deshaies et al., 1995; Barral et al., 1995; Willems et al., 1996). Cyclin binding regulates the activity of cyclin-dependent kinases (Cdks) which induce downstream processes by serine/threonine phosphorylation of selected proteins. It is the cyclic assembly, activation and rapid disassembly of multiprotein complexes involving Cdks and cyclins that drives the cell-cycle. The complex itself interacts with a number of adaptor molecules that are responsible for the recruitment of different binding partners through specific protein-protein interaction domains such as WD40 motifs (Neer et al., 1994) and leucine-rich repeats (Kobe and Deisenhofre, 1994). One of these adaptor subunits is an F-box protein which links the core catalytic complex (comprised of Skp1, Cdc53 or CUL1, and the E2 enzyme Cdc34) through the F-box motif (a degenerate 40 residue motif named for cyclin F) which binds to Skp1 (Bai et al., 1996). Skp1 therefore plays a role in multiple degradation pathways owing to its ability to assemble complexes with different F-box proteins, which recruit specific substrates for degradation. Through interactions with other sequences (i.e. WD40 motifs and leucine-rich repeats) in the protein, the F-box containing protein brings substrates in proximity to the core complex for transfer of ubiquitin from Cdc34 (reviewed in Tyers and Willems, 1999). This entire process is dependent on phosphorylation of the putative substrates which facilitates interaction with F-box containing proteins.

In addition to Skp1, the F-box proteins also bind Rbx1 which, in turn, interacts with Cdc53. Rbx1 is a RING finger (RF) containing protein, a RING finger being a small Cys-rich, metal binding domain (Saurin et al., 1996) that is found in an number of E3 ubiquitin ligases (Lorick et al., 1999). Rbx1 function in yeast was found to be essential to SCF-mediated ubiquitination of the CDK inhibitor Sic1 (Kamura et al., 1999) and the G1 cyclin Cln1 (Skowyra et al., 1999). Whether Rbx1 functions as a ubiquitin ligase within the SCF context is yet to be determined.
Interestingly, the SCF system is capable of targeting proteins for destruction that are part of heteromeric complexes which include subunits that are not short-lived. The regulation of transcriptional activity by NF-κB serves as a salient example of such a degradative process. The transcriptional regulation by NF-κB1 is involved in regulation of development and differentiation as well as the modulation of immune and inflammatory responses. Its activation is a two step proteolytic process: NF-κB1 is synthesized as a precursor protein (p105) and limited proteolysis releases an intact N-terminus, p50 (Palombella et al., 1994; Orian et al., 1995), which can associate with p65, another cellular protein, to generate the active heterotrimeric NF-κB1 factor. The C-terminal domain, remaining after p105 proteolysis, is degraded (Blank et al., 1991; Fan et al., 1991). Under resting conditions in the cell, the p50/p65 complex associates with its inhibitor IκBα which acts to sterically hinder the nuclear localization of the complex due to the obstruction of a nuclear localization signal within p65, and consequently is retained in the cytosol (Henkel et al., 1992; Latimer et al., 1998). Following extracellular signalling, IκBα is phosphorylated (Brown et al., 1995), signalling it for rapid degradation by the SCF complex (Chen et al., 1995, 1996; Scherer et al., 1995; Alkalay et al., 1995; Sears et al., 1998; Maniatis, 1999). This enables the rapid translocation of the active NF-κB1 complex into the nucleus where it can exert its transcriptional control.

The Cyclosome

The cyclosome (APC) is a high molecular weight complex which has ubiquitin ligase activity specific for cell-cycle regulatory proteins that contain a destruction box (the degenerate motif: R(A/T)(A)L(G)X(I/V)(G/T)(N); described below). The complex activity is cell cycle-regulated; it is inactive in interphase but becomes active at the end of mitosis when mitotic cyclins (cyclin B) are degraded. The APC is a multimeric complex that has 8-12 subunits (in Xenopus and yeast, respectively reviewed in Patton et al., 1998; Tyers and Willems, 1999) that is reminiscent of the complex architecture of the SCFs described above. Cyclosome activity is dependent on phosphorylation by Cdc2 kinase in clam egg extracts and can be inhibited by an okadaic-acid sensitive phosphatase (Lahav-Baratz et al., 1995). Cdc2 kinase activity is dependent on the
heterodimeric formation with B cyclins, and cyclosome activation eventually leads to the ubiquitination and degradation of these cyclins, thereby acting as a auto-inhibitory feedback due to the eventual inactivation of Cdc2 kinase and itself. Generally, the specific cyclosome subunits involved in ubiquitin ligase function, the specificity of binding for destruction box-containing substrates and binding to E2s are yet to be known. A subunit of the APC, Apc2, was found to be a homologue of Cdc53 from SCF complexes (Zachariae, 1998; Yu, 1998) and Apc11 was found to be a homologue of Rbx1, the RING-finger containing protein, promoting the notion that there is a relationship between the APC and SCF complexes.

RING Finger Ubiquitin Ligases

Interestingly, c-Cbl, a protein that becomes tyrosine phosphorylated by interacting with tyrosine kinase receptors and is involved in several signalling pathways, is also a RING-finger containing protein that is capable of accelerating ligand bound, activated epidermal growth factor receptor (EGFR) ubiquitination and subsequent degradation through endocytosis (Levkowitz et al., 1998). It was shown that critical residues within the RF are responsible for control of ubiquitination, implying a direct role of this region in the ligation of ubiquitin to the receptor (Waterman et al., 1999). Recently, the RF of c-Cbl was expressed as a GST fusion protein and was shown to mediate direct ubiquitination of 125I-labeled ubiquitin to substrate proteins (Joazerio et al., 1999) suggesting that RF is an E3. The c-Cbl protein was shown to act as an E3 that recognizes tyrosine phosphorylated substrates through its SH2 domain, that recruits and activates an E2 ubiquitin-conjugating enzyme through its RING domain (Joazerio et al., 1999). Several other RING containing proteins have also been shown to facilitate E2-dependent ubiquitination, including the breast cancer susceptibility gene (BRCA1), an MHC class II gene repressor (NF-X1), kf-1 a protein that may be involved in membrane protein sorting, TRC8 a multispanning membrane protein and finally A07 and Praj1, proteins with undefined functions (Lorick et al., 1999). It is interesting to speculate that the RF of Rbx1 and Apc11 may also be serving a similar role and that the RF plays a universal role in ubiquitin ligation.
The HECT Family of Ubiquitin Ligases

The HECT (Homologous to E6-AP C-terminal) domain containing E3 enzyme family shares a common 350 amino-acid C-terminal region homologous to that of E6-AP and includes Nedd4 and the family of Nedd4-like proteins (Fig. 8). E6-AP was the first family members to be studied. E6-AP is a 100 kDa protein that has a function, with the papilloma virus E6 oncoprotein, in the ubiquitination and degradation of the tumor suppressor protein, p53 in reticulocyte lysates (Scheffner et al., 1993). All HECT domains contain a conserved Cys residue near the C-terminus that has been shown (for E6-AP, Nedd4 and Rsp5) to be capable of forming a thiolester bond with activated ubiquitin, thereby serving as an intermediary between an E2 enzyme and the protein substrate (Scheffner et al., 1995). The homology between the HECT containing proteins does not extend beyond the C-terminal regions and the differences in the upstream sequences are likely linked to conferring specificity in recognition of protein substrates (Huibregste et al., 1995). In addition to E6-AP, other well characterized HECT containing E3s include Rsp5 (S.cerevisiae), its homologues Pub1 (S.pombe), Nedd4 (rat, mouse, human, Xenopus, fly) and Smurf1 (human and Xenopus) which are described below.

V.B. Recognition sequences for E3 mediated ubiquitination

Various ubiquitin-protein ligases recognize specific motifs in their target substrates providing specificity for ubiquitination. The signals on the potential substrates may vary but are generally primary, secondary or post-translational modifications (Summarized in Table I: Ubiquitination motifs of yeast and mammalian proteins). Possession of any of these motifs does not imply constitutive ubiquitination, however. In many cases, misfolding of the protein or association with ancillary proteins may expose an otherwise masked ubiquitination motif. In addition, masking of the degradation signal may result from association of other subunits or, as in the case of the heterodimeric yeast transcriptional factor MATa2-MATa1, following heterodimerization (Johnson et al., 1998).
Figure 8. HECT Domain Containing Proteins of the Nedd4/Nedd4-like Family.

Schematic representation of the domain architecture of Nedd4 and Nedd4-like E3 ubiquitin ligases. The name designation and organism are given for each protein. Members of this family are characterized by an N-terminal C2 domain (circle), multiple WW domains (squares) located in the middle part of the protein and a ubiquitin-protein ligase domain (black octagon) at the extreme C-terminus. Some family members are predicted proteins derived from DNA database searches (accession numbers are indicated). The sequence of WWP1/AIP5/AIP4 is incomplete at the N-terminus as indicated (......). Modified from Harvey and Kumar (1999).
HECT Domain Containing Proteins of the Nedd4/Nedd4-like family

<table>
<thead>
<tr>
<th>Protein</th>
<th>C2 domain</th>
<th>WW domains</th>
<th>HECT domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nedd4 (rat/mouse)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rsp5p/Npi1p (S. Cerevisae)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pub1p (S. Pombe)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nedd4 (human/Xenopus)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Itch (mouse)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Su(dx) (fly)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smurf1 (Xenopus)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WWP2/AIP2 (human)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WWP1/AIP5/AIP4 (human)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KIAA0439 (human)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KIAA0322 (human)</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>
Table I: Ubiquitination motifs of yeast and mammalian proteins

<table>
<thead>
<tr>
<th>PROTEIN</th>
<th>SEQUENCE</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ste2p (truncated)</td>
<td>SINNDAKSS (C-t)</td>
<td>Hicke et al., 1998</td>
</tr>
<tr>
<td></td>
<td>Includes a critical lysine</td>
<td></td>
</tr>
<tr>
<td>Ste3p</td>
<td>large PEST-like (58AA)(C-t)</td>
<td>Roth et al., 1998</td>
</tr>
<tr>
<td>Ste6p</td>
<td>large acidic A-box (52AA) including a DAKTI signal (internal linker region)</td>
<td>Kolling and Losko, 1997</td>
</tr>
<tr>
<td>Fur4p</td>
<td>PEST-like (20AA) (N-t) Preceded by two target lysines</td>
<td>Marchal et al., 1998 Marchal et al., in preparation</td>
</tr>
<tr>
<td>Tat2p</td>
<td>31AA (N-t) including critical lysines</td>
<td>Beck and Hall, 1999</td>
</tr>
<tr>
<td>Zrtlp</td>
<td>A critical lysine (AA 195)</td>
<td>Eide, personal communication</td>
</tr>
<tr>
<td>Mitotic Cyclins (Cyclins A,B)</td>
<td>Destruction box (R (A/T) (A) L (G) X (I/V) (G/T) (N))</td>
<td>Glotzer et al., 1991 Klotzbucher et al., 1996</td>
</tr>
<tr>
<td>Various proteins (type I/II)</td>
<td>(basic/hydrophobic) N-terminal amino acids</td>
<td>Varshavsky 1992 ;1996</td>
</tr>
<tr>
<td>Mammalian:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>αβγENaC</td>
<td>xPPxY (PY motif) (Nedd4-WW domain binding sites)</td>
<td>Staub et al., 1996</td>
</tr>
<tr>
<td>GHR</td>
<td>DSWVEFIELD (UbE motif)</td>
<td>Govers et al., 1999</td>
</tr>
<tr>
<td>PDGFR</td>
<td>C terminal Tyr-phosphorylation sites (c-Cbl-SH2 domain binding sites ?)</td>
<td>Mori et al., 1992;1993 Levkovitz et al.,1998 Waterman et al., 1999 Joaseiro et al., 1999 Lee et al., 1999</td>
</tr>
<tr>
<td>EGFR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSF1-R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c-Jun</td>
<td>N-terminal region (27AA)</td>
<td>Trier et al.,1994</td>
</tr>
<tr>
<td>IκBα</td>
<td>Ser32/36 phosphorylation</td>
<td>Chen et al., 1995 ;1996</td>
</tr>
<tr>
<td>β-catenin</td>
<td>Ser phosphorylation (DSGIHS)</td>
<td>Aberle et al.,1997</td>
</tr>
</tbody>
</table>

Modified from: Rotin and Hagenauer-Tsapis, 1999
As described above, the N-end rule relates the half-life of a protein to the identity of its N-terminal residues. The N-recognin (mouse E3α or yeast UBR1) has at least two substrate binding site types, type 1 and type 2 (Varshavsky 1997) whereby type 1 sites recognize basic residues (Arg, His, and Lys) and type 2 sites are specific to bulky hydrophobic residues (Phe, Leu, Trp, Tyr and Ile) (reviewed in Hersko and Ciechanover 1992, 1998; Varshavsky 1996). The destabilizing amino acids of the N-terminal regions of N-end rule substrates are also further categorized by their recognition by distinct targeting complexes (Varshavsky, 1996).

Many recent findings (Yaglom et al., 1995; Lanker et al., 1996; Kornitzer et al., 1994; Won and Reed, 1996; Diehl et al., 1997) have suggested that phosphorylation of proteins targets them for degradation and moreover, these phosphorylations may occur within so-called PEST sequences. PEST sequences are regions enriched in Pro (P), Glu (E), Ser (S) and Thr (T) residues (Rogers et al., 1986; Rechsteiner and Rogers, 1996) that are present in many rapidly degraded proteins. PEST sequences are also enriched in S/TP sequences which are the minimum consensus phosphorylation sites for Cdk's and other protein kinases (Yaglom et al., 1995). In particular, the NF-κB inhibitor, IκBα, is phosphorylated at two specific sites, Ser32 and Ser36, in response to stimuli that induce NF-κB activation. This modification is required for ubiquitin ligation and degradation because if either of these residues is mutated, the inhibitor does not become phosphorylated and is not degraded. As a result, NF-κB can not be activated by a broad array of stimuli (Brown et al., 1995; Brockman et al., 1995). The c-Cbl protein acts as an E3 that can recognize tyrosine-phosphorylated substrates, such as the activated platelet-derived and epidermal growth factor receptors, through its SH2 domain (Joazeiro et al., 1999). This represents a distinct mechanism for substrate targeting in the ubiquitin system. It has been suggested that phosphorylated tyrosine can also serve as a targeting signal for the ubiquitination pathway by mediating interactions with SH2 containing proteins that may in turn recruit E3s, as may occur with Grb2, a major Cbl associated protein (Joazeiro et al., 1999).

Another important moiety in signalling ubiquitination in mitotic cyclins and other cell-cycle regulators is the "destruction box". This 9 amino acid degenerate motif is located approximately 40
amino acids from the N-terminus of mitotic cyclins and is necessary for their ubiquitination and degradation in extracts of *Xenopus* oocytes (Glotzer *et al.*, 1991). From a compilation of destruction boxes from 40 cyclins from various organisms (Madura and Varshavsky, 1994) destruction boxes have the general sequence: R (A/T) (A) L (G) X (I/V) (G/T) (N). Amino acids that appear in parentheses occur in greater than 50% of known destruction sequences. These destruction sequences can act as a transferable degradation signal; fragments of the destruction box-containing N-terminal sequences of the cyclins fused to reporter proteins conferred cell-cycle-stage-specific degradation (Glotzer *et al.*, 1991; King *et al.*, 1996; Brandeis *et al.*, 1996). Although most destruction box-containing proteins are ubiquitinated by the cyclosome/APC, which is activated during late mitosis, there are some proteins that are degraded at different times during the cell-cycle which would indicate an additional level of regulation.

The sequences SINNDAKSS (Hicke *et al.*, 1998), and DAKTI (Kolling and Losko, 1997) were identified in the yeast Ste2p receptor and Ste6p receptor respectively and found to function as ubiquitination motifs. Phosphorylation of the Ser residues of SINNDAKSS was found to be required for sufficient and necessary for endocytosis of Ste2p and the lysine proved to be critical for ubiquitination (Hicke *et al.*, 1998).

The N-terminal region of c-Jun contains a δ-domain which is a sequence of 27 amino acids that is a transferable ubiquitination signal. Deletion of this domain confers stability on the protein (Treier *et al.*, 1994). Interestingly, mutation of all lysines within the δ-domain did not affect the stability of the protein and lysines in other regions of the protein were not essential for ubiquitination. Whether there are homologous sequences in other proteins and whether this represents a global signal for ubiquitination and degradation is unknown.

Ubiquitin-dependent internalization of the growth hormone receptor (GHR) has been shown to depend on a ten residue sequence known as the UbE (ubiquitin-dependent endocytosis) motif; DSWVEFIELD (Govers *et al.*, 1999). This sequence is homologous to sequences in other proteins, some of which are known to be ubiquitinated (i.e. c-erbB-2 and PDGFR) (reviewed in Strous and Govers, 1999). This domain likely served as a binding site for the ubiquitin conjugation
system, directly or via adaptor molecule(s), and when all cytoplasmic lysines were mutated to arginines, internalization proceeded only when the UbE domain was intact (Govers et al., 1999). It was proposed that GHR internalization requires the recruitment of the ubiquitin conjugation machinery to the UbE motif rather than the conjugation of ubiquitin to the GHR itself.

WW domains are present in multiple copies in some HECT containing E3 enzymes, particularly RSP5 (S.cerevisiae), Pub1 (S. pombe) and their mammalian homologue Nedd4 (Hein et al., 1995; Nefsky and Beach, 1996; Staub et al., 1996). The WW domains are highly conserved 40 amino acid protein-protein interaction modules, and many have been shown interact with several different proline-rich sequences, one which conforms to the minimal consensus of XPPXY (PY motif) (Chen and Sudol, 1995; reviewed in Staub and Rotin, 1996). Several ubiquitinated proteins possess PY motifs, including the β and γ subunits of the epithelial Na⁺ channel (ENaC). These regions of the channel mediate Nedd4 binding and are necessary for Nedd4-mediated regulation of channel stability (Abriel et al., 1999). It is likely that the specificity of the WW domain-PY motif interaction plays a role in conferring substrate specificity for this family of E3 enzymes (see below for further details: WW domains and their interaction with PY motifs).

VI. Ubiquitination and Endocytosis of Plasma Membrane Proteins

VI.A. General

In many cases, the activity of proteins present at the plasma membrane is controlled by mechanisms affecting their stability. For example, the activity of many signal transducing receptors and ion channels is regulated by altering their presence at the membrane itself; proteins can be removed quickly by endocytosis into the cell. Proteins can then be rapidly degraded by transport from the endosomal compartments to the lysosome or can be recycled back to the plasma membrane. In the case of signalling receptors, these fates would allow the cell to return to a basal state following receptor stimulation. The importance of endocytosis is highlighted by the fact that
several diseases ensue when the internalization and downregulation of some membrane proteins falters. For example, mutations that block the downregulation of the epithelial Na\textsuperscript{+} channel causes Liddle’s Syndrome and failure to downregulate the epidermal growth factor receptor, results in an oncogenic phenotype.

The regulation of many plasma membrane proteins is due, in part, to their internalization via clathrin mediated endocytosis. The presence of motifs in the cytosolic domains of proteins may signal their rapid internalization by interacting with components of clathrin coats (for review see Kirchhausen et al., 1997). These motifs may consist of sequences such as the tyrosine based (YXX\(\Omega\) where \(\Omega\) is a hydrophobic amino acid or NPXY), di-leucine based, acidic cluster-based or dilysine based signals (Kirchhausen et al., 1997). Alternatively, stimulation of plasma membrane receptors by hormone/ligand binding could alter the oligomeric state of the proteins (i.e. dimerization), thereby exposing multiple signals for clustering within coated pits and regulating receptor concentration for internalization. Receptor activation could also result in other modifications such as ubiquitination which could expose otherwise “hidden” internalization signals by inducing conformational changes. This would signal the protein for internalization and degradation.

Although it was long ago suspected that ubiquitin was serving a role beyond proteolysis, it is the recent data on ubiquitination of plasma membrane proteins that has provided strong evidence that the modification of proteins by ubiquitin can result in a fate other than direct targeting to the 26S proteasome. In these cases, ubiquitination ultimately leads to degradation of the target protein but this mechanism is indirect. The number of plasma membrane proteins whose ubiquitination has been demonstrated continues to grow (Table II) and indeed, it is now evident that the modification of several yeast and mammalian membrane receptors and ion channels by ubiquitin is somehow linked to their internalization and degradation. Interestingly, there is no established pattern for the general mechanism underlying this regulation; in yeast, ubiquitination is a signal for internalization via the endocytic pathway, while in animal cells, this modification may signal degradation through the proteasome and/or lysosomal pathways. In the case of the growth hormone receptor, is evident
### Table II: Plasma Membrane Proteins that Undergo Ubiquitin-dependent Endocytosis

<table>
<thead>
<tr>
<th><strong>A. Yeast</strong></th>
<th><strong>B. Mammalian</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ABC transporters</strong></td>
<td><strong>ENaC</strong></td>
</tr>
<tr>
<td>Ste6p (α-factor)</td>
<td>Kölling and Hollenberg (1994)</td>
</tr>
<tr>
<td></td>
<td>Loayza and Michaelis (1997)</td>
</tr>
<tr>
<td>Pdr5p (multidrug transporter)</td>
<td>Egner et al. (1995)</td>
</tr>
<tr>
<td></td>
<td>Egner and Küchler (1996)</td>
</tr>
<tr>
<td><strong>G-receptors</strong></td>
<td><strong>PDGFR</strong></td>
</tr>
<tr>
<td>Ste2p (α-factor)</td>
<td>Hicke and Riezman (1996)</td>
</tr>
<tr>
<td></td>
<td>Terrel et al. (1998)</td>
</tr>
<tr>
<td></td>
<td>Dunn and Hicke (personal communication)</td>
</tr>
<tr>
<td>Ste3p (α-factor)</td>
<td>Davis et al. (1993)</td>
</tr>
<tr>
<td></td>
<td>Roth and Davis (1996)</td>
</tr>
<tr>
<td><strong>MFS transporters</strong></td>
<td><strong>EGFR</strong></td>
</tr>
<tr>
<td>Gap1p (general amino acid permease)</td>
<td>Hein et al. (1995)</td>
</tr>
<tr>
<td></td>
<td>Springael and André (1998)</td>
</tr>
<tr>
<td></td>
<td>Springael et al. (1999)</td>
</tr>
<tr>
<td>Put4p (proline permease)*</td>
<td>Grenson (1992)</td>
</tr>
<tr>
<td>Dal5p(allantoate permease)*</td>
<td></td>
</tr>
<tr>
<td>Gnp1p(glutamine permease)*</td>
<td></td>
</tr>
<tr>
<td>Tat2p (tryptophan permease)</td>
<td>Beck and Hall (1999)</td>
</tr>
<tr>
<td>Hxt6p/Hxt7p (glucose transporters)</td>
<td>Krampe et al. (1998)</td>
</tr>
<tr>
<td>Gal2p (galactose permease)</td>
<td>Horak and Wolf (1997)</td>
</tr>
<tr>
<td>Mal61p (maltose permease)</td>
<td>Medintz et al. (1996;1998)</td>
</tr>
<tr>
<td>Mal11p</td>
<td>Lucero and Lagunas (1997)</td>
</tr>
<tr>
<td></td>
<td>Riballo et al. (1995)</td>
</tr>
<tr>
<td>Fur4p (uracil permease)</td>
<td>Volland et al. (1994)</td>
</tr>
<tr>
<td></td>
<td>Galan et al. (1996;1997)</td>
</tr>
<tr>
<td>Fui1p (uridine permease)</td>
<td>Volland (personal communication)</td>
</tr>
<tr>
<td>Zrt1p (Zn2+ transporter)</td>
<td>Gitan et al. (1998)</td>
</tr>
<tr>
<td>itr1p (inositol permease)</td>
<td>Lai et al. (1995)</td>
</tr>
<tr>
<td></td>
<td>Robinson et al. (1996)</td>
</tr>
<tr>
<td>Can1p (arginine permease of <em>Candida albicans</em> expressed in <em>S. cerevisiae</em>)</td>
<td>Matejckova-Forejtova et al. (1999)</td>
</tr>
</tbody>
</table>

*deduced from protection against ammonium-induced catabolite inactivation

Modified from Rotin and Haguenauer-Tsapis, 1999
that the ubiquitination machinery itself is required for internalization of the ubiquitinated receptor but ubiquitin attachment itself is not sufficient for internalization and degradation.

VI.B. Ubiquitination of Yeast Plasma Membrane Proteins

Seminal work on the role for ubiquitination in regulating membrane protein internalization and degradation was carried out on the yeast Ste2p and Ste6p plasma membrane proteins. Ste6p, a yeast plasma membrane protein that is responsible for transporting the mating pheromone α-factor, was one of the first plasma membrane proteins reported to be ubiquitinated in yeast. This receptor was found to accumulate in high-molecular weight forms in strains that are deficient in endocytosis (Kolling and Hollenberg, 1994). In yeast that carry mutations in several of the ubiquitin conjugating enzymes, ubc4ubc5 double mutants, as well as in strains with protease-deficient vacuoles, the half-life of cell-surface associated Ste6p was markedly prolonged (from 13 to 41 min. in ubc4ubc5). A region of the transporter, called the D-box, was found to be critical to ubiquitination and turnover of the receptor (Kolling and Hollenberg, 1994). When this region, containing the motif DAKTI, was removed it correlated with decreased ubiquitination and diminished endocytosis. Mutation of the Lys to Arg in the sequence only modestly reduced endocytosis and did not affect the ability of the transporter to become ubiquitinated (Kolling and Losko, 1997). Indeed, transfer of this D-box region to an otherwise stable membrane protein, Pma1p, resulted in ubiquitination, endocytosis and reduced half-life. When only the DAKTI sequence alone was transferred, there was little effect on the stability of the protein suggesting that while this region is important in the process of ubiquitination and degradation of yeast membrane proteins, it is not alone sufficient to induce them (Kolling and Losko, 1997).

The G protein-coupled receptor Ste2p binds to the yeast pheromone, α-factor, and has seven transmembrane spans and a C-terminal cytosolic region that is subject to serine phosphorylation. The receptor is constitutively ubiquitinated, yet in response to α-factor binding, the receptor is further ubiquitinated which stimulates rapid internalization and degradation (Hicke
and Reizmann, 1996). When yeast that are defective in endocytosis \((end4)\) are stimulated with \(\alpha\)-factor, Ste2p accumulates at the cell surface in high-molecular weight ubiquitinated forms. In addition, \textit{ubc1ubc4} and \textit{ubc4ubc5} mutants, show a marked reduction in the internalization time (5- to 15-fold slower than wild-type) of Ste2p ubiquitinated forms in response to ligand binding. Degradation of Ste2p in yeast possessing a mutation in vacuolar protease function \((\textit{pep4prb1})\) showed similar results while mutants of the proteasome \((\textit{pre1pre2}, \textit{yta5}, \textit{yta1})\) showed little effect on the degradation of the receptor (Hicke and Reizmann, 1996). Not only did these results form a link between ubiquitination, internalization and degradation of the receptor but, they also showed that degradation of this plasma membrane protein was not affected by the proteasome.

The region of the receptor that seemed to be playing a role in these processes is a C-terminal sequence \textit{SINNDAKSS} (amino acids 333-339 of the C-terminal tail), with the last five amino acids reminiscent of the Ste6p \textit{DAKTI} sequence. When the three Ser residues were mutated to Ala, and the Lys residue mutated to Arg in a truncated version of the receptor, ubiquitination and endocytosis were both impaired (Hicke and Reizmann, 1996). Unlike the Ste6p \textit{DAKTI}, mutation of the Lys in the Ste2p \textit{SINNDAKSS}, proved sufficient for obliterating receptor ubiquitination and degradation, proving that ubiquitination was a requirement for receptor degradation and that the ligation of ubiquitin occurred on this lysine residue. It is likely that phosphorylation on the flanking Ser residues is necessary for ubiquitination of the Lys within this sequence.

The yeast \(\alpha\)-factor receptor (Ste3p) is a member of the G protein-coupled family of receptors. As with Ste2p, ligand binding enhances the rate of receptor internalization yet, constitutive endocytosis and degradation are dependent on ubiquitination as well (Roth and Davis, 1996). When endocytosis is blocked in \textit{end} mutant strains, there is an increase in the levels of ubiquitinated species and internalization is impaired in \textit{ubc4ubc5} mutants as well, together indicating that ubiquitination is required for constitutive internalization by endocytosis and subsequent degradation (Roth and Davis, 1996). A similar conclusion was made for ligand stimulated endocytosis and degradation, by repeating the same experiments using a mutant form of Ste3p that was defective in constitutive endocytosis.
The yeast general amino acid permease (GAP1p) (Springael and Andre, 1998), uracil permease (Fur4p) (Galan et al., 1996) and multidrug transporter (Pdr5p/Sts1p) (Egner and Kuchler, 1996) are also well documented examples of ubiquitin-mediated endocytosis/vacuolar degradation of plasma membrane proteins in yeast. All of these transporters have been shown to accumulate as ubiquitinated species in end mutant strains. Fur4p also contains a destruction box (RXXLXXXX(N)) and a PEST sequence near its amino terminus. A point mutation (R294A) within the destruction box renders the permease resistant to stress-induced degradation (Galan et al., 1994). As well, mutations of Ser to Ala within the PEST sequence of Fur4p results in reduced ubiquitination and degradation. This effect was likely due to reduced phosphorylation on these Ser residues since mutations of these to Glu, which mimics the negative charge of a phosphate residue, reverses the inhibitory effect on ubiquitination and endocytosis (Marchal et al., 1998).

It appears that both the number of ubiquitin moieties and the type of ubiquitin-ubiquitin (Ub-Ub) linkages in polyubiquitin moieties attached to membrane proteins determines the nature and efficiency of their degradation. Overexpression of a mutant ubiquitin having all Lys substituted with Arg had no effect on the internalization of Ste2p, suggesting that ligation of only one ubiquitin molecule is sufficient for endocytosis/degradation of this receptor (Terrell et al., 1998). This finding supports the lack of a role for the proteasome in the degradative process since monoubiquitinated proteins are not substrates for the 26S proteasome which requires attachment of at least four ubiquitin moieties for recognition (Chau et al., 1989; Deveraux et al., 1994). In addition, when a ubiquitin molecule was fused in frame to the Ste2p receptor, it was able to serve as an effective internalization signal in the absence of other ubiquitination events, indicating that the type of ubiquitin linkage to the receptor was not important (Terrell et al., 1998). This conclusion does not hold true for the uracil permease, however. Although mono-ubiquitination has been shown to be sufficient for Fur4p internalization, the attachment of short polyubiquitin chains with Lys-63 Ub-Ub linkages accelerates the internalization of the permease and its subsequent degradation (Galan and Hagnuener-Tsapis, 1997). It has been proposed that multiubiquitin chains may create steric hinderance of critical protein-protein interactions necessary for proper internalization of
membrane proteins. As well, short-ubiquitin linkages would eliminate the possibility of proteasomal degradation, thereby protecting the internalization-containing cytoplasmic domains of membrane proteins (Hicke 1999).

VI.C. Ubiquitination of Mammalian Plasma Membrane Proteins

In mammalian cells one of the most extensively studied ubiquitinated proteins is the growth hormone receptor (GHR). Binding of the growth hormone induces receptor ubiquitination on multiple lysine residues (Govers et al., 1997) and its subsequent internalization and degradation within the lysosome. As with the yeast plasma membrane receptors, mutation or elimination of components of the ubiquitin-conjugation system affect the stability of the receptor. When a temperature-sensitive E1 enzyme expressed in CHO cells is inactivated at the restrictive temperature, ubiquitinated GHR accumulates at the plasma membrane (Strous et al., 1996). Furthermore, when the GHR UbE motif (DSWVEFIELD, described above) is interrupted by truncation (at amino acid 330), leaving three potential di-leucine internalization sequences in the C-terminal region, these are not sufficient for receptor internalization and degradation (Govers et al., 1998). Interestingly, a truncation further downstream (at amino acid 349) activates a latent di-leucine endocytosis signal (DTDRLL) and the UbE motif is no longer necessary for ligand-induced internalization (Govers et al., 1998). These results may suggest that specific ubiquitin-dependent proteolysis of the C-terminal tail by the proteasome is taking place such that the degradation of the GHR is a gradual process, co-ordinated by the activity of the proteasome and the lysosome (Govers et al., 1999). This hypothesis is supported by the finding that proteasome inhibitors are capable of blocking both internalization and degradation of the GHR (reviewed in Strous and Govers, 1999).

Recent observations suggest that ubiquitin attachment, itself, to the receptor is not necessary for internalization (Govers et al., 1999). Instead, it is proposed that recruitment of the ubiquitination system to the GHR, and to the UbE motif in particular, is required. This is in contrast to the findings for the yeast α-factor receptor (Terrell et al., 1998) and for the epithelial Na⁺ channel
(Staub et al., 1997; see below). The authors propose that the ubiquitination machinery may be playing the role of adaptor for clathrin-mediated endocytosis and that ubiquitination is a consequence of this recruitment (Strous and Govers, 1999).

The octameric T-cell receptor (TCR) complex (αβγδεζζ,) is assembled in the endoplasmic reticulum and the number of correctly assembled receptor complexes is limited by the availability of the individual subunits. Only complete complexes are targeted to the plasma membrane as partial complexes and unassembled subunits are rapidly destroyed (Huppa and Ploegh, 1997; Yu et al., 1997; Yang et al., 1998). While TCRs lacking the ζ subunit exit the ER and are degraded in the lysosome (Sussman et al., 1988), other partial receptors are retained in the ER and are degraded by a non-lysosomal pathway (Klausner et al., 1990; Bonifacino and Lippincott-Schwartz, 1991) which is largely unknown. Ligand binding to the T-cell receptor results in receptor ubiquitination, with multiple TCR subunits (particularly the ζ subunit) ubiquitinated on cytoplasmic lysine residues (Cenciarelli et al., 1992). Lysines that were introduced into non-native positions in the ζ cytoplasmic tail were similarly modified (Hou et al., 1994). Modification by ligand-induced ubiquitination requires phosphorylation of the cytosolic portion of the ζ subunit by the tyrosine kinase p56

Currently, it is not known whether the ligand-induced ubiquitination of the TCR plays a role in down-regulation by targeting to the 26S proteasome or by internalization. Lysosomal inhibitors such as monensin and ammonium chloride are effective in abrogating receptor degradation (reviewed in Bonifacino and Weissman, 1998) while inhibitors of the proteasome have an insignificant effect on the stability of the ubiquitinated, ligand-bound receptor. The TCR is a long-lived protein, however, and ubiquitinated TCRs are detected for several hours following receptor activation which might suggest that the selective ubiquitination of a few of its subunits may play a role in modulating its activity.
Similar to the TCR and the EGFR (described above), the platelet-derived growth factor receptor (PDGFR) also becomes ubiquitinated in response to ligand binding (Mori et al., 1992) and this requires the kinase activity and the carboxyl-terminal 98 amino acids of the receptor (Mori et al., 1992). It was found that autophosphorylation of tyrosine residues in the cytosolic tails of the receptor following ligand binding is required for interaction with the SH2 domain of c-Cbl (Bonita et al., 1997; Lupher et al., 1996). These interactions may also take place indirectly through the adaptor molecule Grb2 which binds to the proline rich region of Cbl via its SH3 domains and to autophosphorylated receptors via its SH2 domains (Meisner et al., 1997; Kazlauskas, 1994; Bazenet et al., 1996). NIH 3T3 cells expressing oncogenic forms of Cbl resulted in a hyperphosphorylated PDGFR receptor and an upregulation of signalling downstream the receptor (Bonita et al., 1997). The effects of the oncogenic mutants of Cbl on the receptor reflected a reversal of the negative regulatory role of wild-type Cbl. Cbl was found to promote ubiquitination and ligand-induced degradation of PDGFR which results in the downregulation of the receptor action (Miyake et al., 1998). Stimulation of PDGFR induced Cbl association with the receptor and overexpression of the wild-type Cbl in NIH3T3 cells led to an enhancement of the ligand-dependent ubiquitination and degradation of PDGFR (Miyake et al., 1999). A mutation in the N-terminal SH2 domain that eliminates binding to tyrosine phosphorylated residues of the receptor, abrogated the ability of Cbl to enhance the ligand-induced ubiquitination and degradation of the PDGFR and inhibit PDGF-dependent cell proliferation (Miyake et al., 1999).
VII. Nedd4

VII.A. Nedd4 physiological function

Another well detailed example of ubiquitination functioning in mammalian plasma membrane protein stability is the epithelial Na⁺ channel, which will be described in the context of Nedd4, the E3 ubiquitin ligase found to function in this process.

Neuronal precursor cell-expressed developmentally downregulated 4 (Nedd4) is a multimodular ubiquitin protein ligase (E3) comprised of a C2 domain, 3 (or 4) WW domains, and a C-terminal ubiquitin protein ligase HECT domain (Fig. 9). Nedd4 was originally identified as a partial cDNA clone from mouse neural precursor cells whose respective mRNA levels were down-regulated during development of the mouse brain (Kumar et al., 1992). Most work done on the characterization of Nedd4 has been in the context of the epithelial Na⁺ channel (ENaC), as rat Nedd4 was isolated as an interacting protein with the proline-rich regions of ENaC in a two-hybrid screen (Staub et al., 1996). It was found that Nedd4-ENaC interactions occur by binding of the WW domains of Nedd4 to the highly conserved proline-rich PY motifs in the C-termini of the α,β and γ subunits of ENaC (PPPAY, PPPNY, and PPP(R/K)Y, respectively) (Staub et al., 1996). Deletions or mutations of these PY motifs in β and γ ENaC cause Liddle's syndrome (Shimkets et al., 1994; Hansson et al., 1995a,b; Tamura et al., 1997; Inoue et al., 1998), a hereditary form of hypertension characterized by an abnormal increase in the activity of ENaC in the distal nephron (Schild et al., 1995; Schild et al., 1996; Snyder et al., 1995). Other mutations (PPPNY to either PPANY or PPPNA) within the second βrENaC proline rich sequence led to abolishment of the rNedd4-WW binding in the two-hybrid assay (Staub et al., 1996) and a parallel increase in ENaC activity (Schild et al., 1996).

These findings, suggesting that Nedd4 was a putative (down)regulator of ENaC activity, were later supported by several lines of evidence. (1) Immunocytochemical analysis of rNedd4 in the fluid-absorbing epithelia of the kidney and lung, showed that Nedd4 displayed similar
Figure 9. Modular representation of the protein-ubiquitin ligase Nedd4. Nedd4 possesses an N-terminal C2 or Ca$^{2+}$-lipid binding domain, three (rat) or four (mouse) tandem copies of the WW domain, an E2 binding region and a C-terminal HECT domain.
Nedd4
(Neural precursor cell Expressed Developmentally Downregulated 4)
(Staub et al., 1997a). (II) ENaC was shown to have a short half-life and that the channel was ubiquitinated in cells on the α and γ subunits but not the β subunit. The major site of ubiquitin conjugation was determined to be a cluster of lysine residues at the N-terminus of the γ subunit. Furthermore, the consequence of mutating these residues to arginine was the elimination of ubiquitination as well as increased channel activity caused by an increase the number of channels present at the plasma membrane (Staub et al., 1997b). (III) Overexpression of wild-type Nedd4 in Xenopus oocytes co-expressing ENaC inhibited channel activity, whereas, a catalytically inactive Nedd4 (with a mutation of the critical cysteine in the HECT domain preventing ubiquitin conjugation) increased channel activity (Goulet et al., 1998; Abriel et al., 1999). This effect was dependent on the PY motifs of ENaC, as mutations in these regions, such as those associated with Liddle’s syndrome, diminished Nedd4-mediated regulation of ENaC. The action of Nedd4 on ENaC was through an effect on channel numbers and not channel properties since overexpressed Nedd4 decreased the number of ENaC channels at the cell surface. (IV) Nedd4 mediated the ubiquitin-dependent down-regulation of Na⁺ channel activity in response to increased intracellular Na⁺ (Dinudom et al., 1998).

VII.B. Nedd4 Homologues and Nedd4-like Proteins

At present, several orthologues of Nedd4 have been described in yeast, mouse, rat and human. The yeast, rat, mouse and fly proteins all share a similar domain architecture while human and Xenopus Nedd4 possesses an additional WW domain (Fig. 9). The yeast homologues of Nedd4, in both Saccharomyces cerevisiae and Schizosaccharomyces pombe, have been characterized and studied extensively. RSP5p/Npi1p, the S. cerevisiae protein, was originally identified as a suppressor of mutations in the SPT3 gene which codes for a transcriptional factor which interacts with the TATA-box binding protein (TBP) (Eisenmann et al., 1992). It has been shown to be essential for ubiquitin-mediated turnover of several membrane proteins; GAP1 (Hein et al. 1995), Fur4 (Hein et al. 1995; Galan et al. 1996; Galan and Hageunauer-Tsapis 1997), the
maltose transporter (Lucero and Lagunas 1997) and the α-factor receptor (Dunn and Hicke, 1999). Rsp5 was also shown to play a role in potentiation of human progesterone receptor and human glucocorticoid receptor transcriptional activity in yeast and in human cells, a function also described for human Nedd4 (Imhof and McDonnell, 1996). Rsp5p has been implicated in mediating ubiquitination of the large subunit of RNA polymerase II (Huibregtse et al., 1997) in addition to its DNA-damaged induced degradation (Beaudenon et al., 1999). Rsp5/Npi1 is essential in yeast and recent studies have shown that WW domains 2 and 3 as well as the HECT domain are indispensable (Wang et al., 1999). Pub1, the Nedd4 homologue in Schizosaccharomyces pombe, regulates the ubiquitination of the mitotic activating tyrosine phosphatase cdc25 (Nefsky and Beach, 1996) thereby controlling cell cycle progression. A functioning pub1 ubiquitin ligase was also found to be essential for tolerance of low pH in S. pombe and it was hypothesized that this might be due to affects on membrane transport processes, particularly the permeases which are mostly H+ symporters (Saleki et al., 1997).

The human and mouse isoforms of Nedd4 have been studied to a lesser extent. Interestingly, human and mouse Nedd4 was found to be a target of the caspases during apoptosis (Harvey et al., 1998). The cleavage of Nedd4 by the caspases is induced by a variety of stimuli and cleavage may occur through the action of several caspases. The cleavage of Nedd4 removes the N-terminal C2 domain from the rest of the protein leaving the WW domains and HECT domain undisturbed (Harvey et al., 1998). Removal of this region may have implications in structural or functional stability of the protein. Human and mouse Nedd4 also interact in vitro (via their WW domains) with the PY motifs present in the haemateopoietic transcription factor p45/NF-E2 and RNA polymerase II (Gavva et al., 1997).

Several other proteins have been identified that are closely related to Nedd4 (summarized in Fig. 8). These proteins are largely uncharacterized but one, Itch, has been shown to be an important regulator of the inflammatory response (Perry et al., 1998). Mutations in the Itch locus in mice results in development of a fatal disease characterized by inflammation of the stomach and the skin resulting in scarring due to constant itching, as well as many other inflammatory disorders (Perry et
Although the biochemical pathways affected by this ubiquitin ligase are not yet known, Itch may function to regulate some of the many cytokine receptors that are involved in inflammatory response which are thought to be regulated by the ubiquitination pathway (Perry et al., 1998). Other genes that show similarity to Nedd4 and are highly similar to each other are WWP1 and 2 and AIP4 (Pirozzi et al., 1997; Wood et al., 1998). WWP1 and WWP2 (WW domain-containing protein) were originally identified as binding to PY motif containing peptides (Pirozzi et al., 1997). Their WW domains bind with varying specificities to the PY motifs of several proteins such as rasGAP, AP-2, and β-dystroglycan (Pirozzi et al., 1997), although it is not known whether these proteins represent the physiological substrates of WWP1 and 2. In addition to AIP4, WWP1 and 2 were later identified as interacting with atrophin-1, a protein containing five PY motifs (Wood et al., 1998). One of the newly identified Nedd4-like E3 enzymes is Smurf1 which selectively interacts with TGF-β receptor-regulated SMAD1, specific for the BMP pathway (Zhu et al., 1999). Smurf1 was shown to trigger SMAD1 ubiquitination and degradation leading to their inactivation, which may serve to control many processes including embryonic development and a wide variety of cellular responses to TGF-β signals (Zhu et al., 1999). Su(dx), described above, was identified in a genetic screen for mutations that reversed deltex (a positive regulator of Notch) loss-of-function phenotypes (Lindsley and Zimm, 1992). The gene product, Su(dx) was characterized as a Nedd4-like E3 ubiquitin ligase containing a C2 domain, four WW domains and a C-terminal HECT domain (Cornell et al., 1999) and is involved in the downregulation of Notch receptor signalling. Other members of the Nedd4 family include several genes of unknown function such as KIAA0439, KIAA0322 and AC004893.

VII.C. The Basis of Nedd4 Specificity: WW Domains and their Interaction with PY Motifs

The WW (or WWP) domain itself, is a conserved region of approximately 40 amino acids and was so named due to two highly conserved tryptophans and an invariant proline (Bork and
Sudol, 1994; Hofmann and Bucher, 1995; Andre and Springael, 1994). As previously suggested, it is likely that the specificity of E3 enzymes, containing the structural architecture of Nedd4, is through the interaction of their WW domains with PY motif containing proteins. A growing number of signalling molecules contain these domains in multiple or single copies including: Nedd4, its yeast homologues Rsp5 and Pub1, YAP (Yes Associated Protein), Dystrophin, some formin binding proteins, Pin1 (a transcriptional regulator and possibly of the cell cycle) and others.

It is clear that there is a specific interaction between the WW domains of Nedd4 and the PY motifs of the ENaC subunits. WW domains have been shown to bind substrates other than PY motifs. At present, there have been three distinct proline-rich ligands identified for the WW domain; the PY motif, the sequence PPLP and the PGM motif. While the WW domains of Nedd4 and YAP bind to PY motifs (Staub et al., 1996; Chen and Sudol, 1995), the WW domains of the formin binding protein, FBP and the neural protein FE65 have been found to bind to the PPLP motif (Chan et al., 1996; Ermekova et al., 1997). Recently, the WW domains of the formin binding protein FBP21 have been shown to recognize a proline, glycine and methionine-rich (PGM) motif (Bedford et al., 1998). Interestingly, a fourth type of ligand for WW domains has recently been proposed; phosphoserine and phosphothreonine (Lu et al., 1999; Verdecia and Noel, 1999). The WW domains of both the peptidyl-prolyl isomerase, Pin1, and mNedd4 have been suggested to bind pSer and pThr containing sequences (Lu et al., 1999). However, a recent determination of the structure of the Pin1 WW domain interaction with a pSer containing peptide suggests that the selectivity for pSer/pThr is mediated primarily by an Arg(17) located on the tip of the Pin1 WW domain (Verdecia and Noel, 1999). The absence of this conserved Arg in the WW domains of mNedd4 and in other WW domain sequences suggests that this interaction is not likely to be as wide-spread as reported by Lu et al. (1999).

Two structures have now been solved for WW domains; the NMR structure of the WW domain of YAP in complex with its cognate peptide (Macias et al., 1996) and the crystal structure of the WW domain of the Pin1 protein (Ranganathan et al., 1997). These studies have shown that the WW domain forms a curved, three-stranded anti-parallel β sheet. The sheets form concave
surfaces on which there are several conserved hydrophobic amino acids which are thought to mediate ligand binding through contact with the essential proline and tyrosine residues of the PY motif (Macias et al., 1996). There was no NMR derived information on the structure of the peptide used in these studies; only distances between the termini of the peptide and the WW domain could be extracted. As a result, there is no experimental information on the basis of specificity for the WW domain and the peptide (Macias et al., 1996). It does appear, through binding studies, that the YAP WW domain prefers basic amino acids in the variable positions of the PY motif (Chen et al., 1997) and longer stretches of prolines preceding the PY motif are favorable in this particular interaction.

While it has been shown that the WW domains of Nedd4 are responsible for mediating interactions with PY-motif containing substrates, some of the substrates for the yeast homologues of Nedd4, Rsp5p and Pub1, do not contain PY motifs. As mentioned before, the S. pombe homologue, Pub1 has been implicated in the ubiquitination and degradation of the tyrosine phosphatase Cdc25 (Nefsky and Beach, 1996) and is required for low pH tolerance in this organism (Saleki et al., 1997). In addition, the S.cerevisiae homologue Rsp5p, is required for ubiquitin-mediated internalization of GAP1p and Fur4p (described above) (Springael and Andre, 1998; Galan et al., 1996). The peculiar observation is that neither Cdc25, Gap1p nor Fur4p possess (xPPxY) PY motifs. Thus, it is not clear whether Rsp5p can directly bind these proteins. One possibility is that there is another region, aside from the WW domains, in the E3s that is interacting with the substrates. If direct interactions are not taking place, it is likely that another PY motif containing protein is serving an intermediary between the permeases/receptors and the E3, thereby serving as a docking or adaptor molecule which serves to direct the E3s to their substrates.

**VII.D. The Basis of Nedd4 Enzymatic Activity: The HECT Domain**

As mentioned above, the HECT domain was originally identified in fourteen proteins that shared sequence similarity to E6-AP over the ~350 carboxyl terminal residues of each protein.
(Huibregtse et al., 1995). For this class of E3 enzymes, ubiquitin moieties bound to E3s through thiol-ester linkages represent the final intermediates in the ubiquitin cascade (Scheffner et al., 1995; Huibregtse et al., 1995). The minimal domain of E6-AP and Rsp5 required for ubiquitin thioester formation coincides with the size of the HECT domain (Schwarz et al., 1998).

The first evidence that Nedd4 functioned as a catalytically active E3 came when it was shown that a fusion protein of GST and amino acids 52-777 of Nedd4 catalyzed ubiquitination of bacterial cellular proteins in the presence of E1 and E2 (Hatakeyama et al., 1997). It was also shown that this activity was dependent on the presence of the E2 enzymes from the UbcH5 family members and the closely related AtUBC8 but not AtUBC1 (Hatakeyama et al., 1997). The molecular basis of these interactions was a conserved region in the C-terminal portion of Nedd4 (amino acids 423-583) that interacted with the C terminus of UbcH5B, an interaction that occurred independent of ubiquitin, an E1 or a complete HECT domain (Hatakeyama et al., 1997).

Huang and colleagues have recently solved the structure of the HECT domain of the E6-AP ubiquitin-protein ligase (Huang et al., 1999). The crystal structure of the catalytic HECT domain reveals a bilobal structure with a broad catalytic cleft at the junction between the two lobes. The cleft contains many conserved residues, among which are those that are mutated in Angelman syndrome, and the highly conserved cysteine necessary for ubiquitin transfer. The structure of the E6-AP HECT domain in complex with the E2 enzyme UbcH7, reveals a conserved Phe(63) of UbcH7 forming a critical contact with the HECT domain. This interaction may provide the specificity for interaction between the two enzymes and moreover, may be the primary determinant of the specificity of an E2 for the HECT family of E3s (Huang et al., 1999).

VIII. C2 Domains

The C2, protein-lipid and protein-protein binding domain, was originally identified as the second conserved (hence, C2) region in the amino-terminal regulatory half of conventional, Ca\textsuperscript{2+}-dependent isoforms of protein kinase C (PKC\textsubscript{α,β,γ}) (Coussens et al., 1986; Knopf et al., 1986;
Ono et al., 1986; Parker et al., 1986). Since non-classical or "atypical" isoforms of PKC, lacking the C2 domain, failed to exhibit Ca\(^{2+}\) regulation it was proposed that the C2 domain was responsible for the Ca\(^{2+}\) regulation of PKC. Since that time, single or multiple copies of the C2 domain have been found in a number of soluble and membrane proteins of diverse function, often alongside other protein modules such as SH2, SH3, WW, PTB, PH and PDZ domains (Fig 10). In synaptotagmin, an integral membrane protein involved in synaptic vesicle exocytosis (Perin et al., 1990), there are two tandem C2 domains in the cytosolic portion of the protein. As both PKC and synaptotagmin shared the properties of binding phosphatidylserine vesicles upon the addition of Ca\(^{2+}\) (Bazzi and Nelsestuen, 1987, 1990; Brose et al., 1992), it was implicit that the domain was mediating Ca\(^{2+}\)-dependent binding to acidic phospholipids. The C2 domains characterized to date display binding to a number of substrates and ligands in addition to Ca\(^{2+}\) and phospholipids, including inositol polyphosphates and numerous intracellular proteins. Although the domain was originally thought to function exclusively as a Ca\(^{2+}\)-lipid binding module, in several proteins it acts as a Ca\(^{2+}\)-dependent protein-protein interaction module and in some cases serves its function independent of Ca\(^{2+}\).

**VIII.A. Primary and Tertiary Structure of the C2 Domain**

The C2 domain represents a region spanning approximately 130 amino acids (Fig. 11) which includes a 43 amino acid core region of semi-conserved amino-acids, the CaLB (Ca\(^{2+}\)-lipid-binding) domain (Clark et al., 1991). The CaLB domain of the ras GTPase-activating protein, rasGAP, was found to mediate Ca\(^{2+}\)-dependent phospholipid binding when expressed as a GST-fusion protein (Gawler et al., 1995). The consensus sequence for the C2 domain includes five highly conserved aspartate residues (Fig. 11) which are responsible for the co-ordination of Ca\(^{2+}\) ions (see below). These residues are often found to be substituted with other oxygen containing side-chains (asparagine, glutamine) which would enable Ca\(^{2+}\) co-ordination (Fig. 11). All the C2
Figure 10. Modular representation of functional domains in proteins containing C2 domains. Shown are schematic domain maps for several different proteins containing C2 domains, grouped according to functional classes. C2 domains of type I topology are represented by filled circles while those of type II topology are hatched circles. This representation intends to indicate the relative positions, not the relative sizes of the domains and, hence, is not drawn to scale. TMS, transmembrane segment; Rab-BD, Rab binding domain; SH2 and SH3, Src homology-2 and -3 domains; PH, pleckstrin homology domain; GRD, GAP-related domain; EF, EF-hand Ca\(^{2+}\)-binding domain; G-protein, G-protein interaction domain; C1, phorbol-ester binding domain. Modified from Nalefski and Falke, 1996.
Others:

**Ubiquitin-Protein Ligation**

- WW
- WW
- WW
- HECT

Nedd4/Rsp5/Pub1

**Tyrosine Phosphatase**

- phosphatase

PTEN
Figure 11. Multiple sequence alignment of various C2 domains. Sequence alignment of 12 published C2 domains (also shown schematically in Fig 10.). The secondary structures of type I and type II topologies are schematically shown above and below the sequences, respectively, indicating β-strands (brackets). The five highly conserved aspartates are shown in bold (D). The consensus residues present in >50% of the sequences are indicated at the bottom of the alignment. The amino acids corresponding approximately to the CaLB domain are shown below the consensus. Dashes in sequences indicate gaps inserted to maximize the alignment; numbers in brackets ( ) inserted into the sequence indicate intervening residues omitted for clarity. Synaptotagmin is abbreviated syn, rabphilin as rab, protein kinase C as PKC, cytosolic phospholipase A2 as cPLA2, phospholipase C-δ as PLC-δ, phospholipase D as PLD and phosphoinositide-3-kinase-α as PI3Kα. Tandem repeats of C2 domains in a given protein are designated a or b. Genbank accession numbers for the sequences are indicated in parentheses.
Sequence Alignment of Various C2 Domains

| topology I | ...strI..... | ...strII..... | ...strIII..... | ...strIV..... | ...strV..... | ...strVI..... | ...strVII..... | ...strVIII..... |
| synI(a) | KKGKQYSLYDFDPNQULVGIQAEALPALDMGGTSDPYVKVFLLPE--K | | | | | | | |
| synI(b) | KKGKQYSLYDFDPNQULVGIQAEALPALDMGGTSDPYVKVFLLPE--K | | | | | | | |
| rab(a) | TLGALSLPDYDQNSLHHTTIEIKAAGKLPMDSNGLAGYVKHLHPGAS-K | | | | | | | |
| rab(b) | ERGKLVLSTMYQQGGLVIIGRCHVLAAMDANGYSDFPVKVLKFPDKM-G | | | | | | | |
| PKCa | KGRNLYKAEVADEK---LHVTVRDANKLPDMPNDSLPYVKLKLPFDPK-N | | | | | | | |
| rNedd4 | ...RRVVRKVIAGICLKDILDDASDPYVRVTLYDPMSGV | | | | | | | |
| cPLA2 | ...HKFVTVRDLTNTGAGDFDLTPYDVPFISTTP | | | | | | | |
| PLCδ | ...RKNIRVIGQQL(5)NNKIVPDVFVEIHHGSRD | | | | | | | |
| rasGAP | ...SLNLHIEBAHKLVP---KHTFYPQNYIYNSV | | | | | | | |
| PLD | ...GTHLVTIHVEKD(21)PGKVGSKLATIDKELKAR | | | | | | | |
| perforin | ...AQILEVTFIQAWSLGW-DFWTADAYKLFFGQG | | | | | | | |
| PI3Ka | ...RALRIGICATVNL-MRDIKIKVTGIRGYGQ | | | | | | | |
| consensus | ...LVI...A.L.D......DPV...L...... | | | | | | | |
| topology II | ...strI..... | ...strII..... | ...strIII..... | ...strIV..... | ...strV..... | ...strVI..... | ...strVII..... | ...strVIII..... |
| CaLB | | | | | | | | |
| topology I | ...strI..... | ...strII..... | ...strIII..... | ...strIV..... | ...strV..... | ...strVI..... | ...strVII..... | ...strVIII..... |
| synI(a) | KKKKTTTIKNLNPYNESFPE--VPFEIQKVQVTVTLYDK---IGK-N-----DA | | | | | | | |
| synI(b) | KKKKTTTIKNLNPYNESFPE--VPFEIQKVQVTVTLYDK---IGK-N-----DA | | | | | | | |
| rab(a) | SNKLRTTLRNPWNTTVYHGTIDMQRKTLRSVCEDKFGN-H-------EP | | | | | | | |
| rab(b) | KAKHTQIKKTLNPENFEFIFYYD-IHSDKLAKSLISVWYD---IGKSN--DY | | | | | | | |
| PKCa | ESKQTGKICTSLNPWNESTFK-LKPSKDRR-LSVEWIDR---TTR-N-----DF | | | | | | | |
| rNedd4 | ...LTSVQDITIKSLNKWKNEELIF---VLPQQTHLFEVEFDENR---LTR-D-----DF | | | | | | | |
| cPLA2 | ...DSRKRTHPHFINDPWNFETEF---LDNPQENVILELMBAMVY---VMD---ET | | | | | | | |
| PLCδ | ...ASQAVTITNNGPFWDTEFAFE---VVVPLALIRFLVEDYDA---SSK-N-----DF | | | | | | | |
| rasGAP | ...-QVAKTAREGQPVWSEEFYF-DLPPPNNRGSNIILTSLNK---KS-K---DP | | | | | | | |
| PLD | ...-VGRTRILENEQSNPRFTWESFHYV---CAHASNVITFVLDMP-IGA---TG | | | | | | | |
| perforin | ...-ELRTSTVDNPNNPWSVRLDGF-DVLLATGGPRLQWWDQ---GRD-T-----DL | | | | | | | |
| PI3Ka | ...CDVNTQVRPCS-NPRWNEVNYDIYIDPFLRAAERLCLSVCIVGERGKAEHECFLAWGN | | | | | | | |
| consensus | ...NP.WNE.F.........L.V.D.D........D........ | | | | | | | |
| topology II | ...strI..... | ...strII..... | ...strIII..... | ...strIV..... | ...strV..... | ...strVI..... | ...strVII..... | ...strVIII..... |
| CaLB | | | | | | | | |
| topology I | ...strI..... | ...strII..... | ...strIII..... | ...strIV..... | ...strV..... | ...strVI..... | ...strVII..... | ...strVIII..... |
| synI(a) | IGEFKVPMNTVFPGHTVTEERWRDQSAE--- (141-266) synI-a | | | | | | | |
| synI(b) | IDKVFPVGNYST---GAELRHWSDLAP--- (272-397) synI-b | | | | | | | |
| rab(a) | IGTEFRSLKLK-PNQKNRNICLERV--- (402-529) rab-a | | | | | | | |
| rab(b) | IGGCQLGISAK---GERLKHWECLKNK--- (560-685) rab-b | | | | | | | |
| PKCa | MGSLSFGVSELM----KMPASGWYKLLQE--- (158-281) PKCa | | | | | | | |
| rNedd4 | ...LGGQDVPYLPLPTENPRMERPYTFKDFVLFHPRSHKSRVKYGLRLKMTLPK (77-219) rNedd4 | | | | | | | |
| cPLA2 | ...LGTATFTVSSKM---VGKEKSVFPIFNVQY--- (18-141) cPLA2 | | | | | | | |
| PLCδ | ...IQGSTIPLSLKQ---GYRHVHLZKNGDIQPS---ATLVKISLQD--- (629-727) PLCδ | | | | | | | |
| rasGAP | ...ILFHRMLQSLQRKHHATDMEFLSHPLKGS---GSLRVARYSME (594-715) rasGAP | | | | | | | |
| PLD | ...IGRAYVFPYVEELDGEIDRWEVDLEDKPFHVS---SKHVILFYQVF (8-150) PLD | | | | | | | |
| perforin | ...LGTDQAPKSGS---HEVRCNLNH---GHLKFRYHARCL (415-526) perforin | | | | | | | |
| PI3Ka | ...INLFDTYDTLVSGLMANNLWVFPLGREDLN (13) ---PCLELFMDFSS (332-482) PI3Ka | | | | | | | |
| consensus | ...IG..---W.L....--- (629-727) perforin | | | | | | | |
domains identified to date conform to one of two topologies which differ in their \(\beta\)-stranded connectivity (Fig. 12) (Nalefski and Falke, 1996). The first \(\beta\)-strand of Topology One (I) type C2 domains, which include the synaptotagmin C2A domain, occupies the same structural position as the eighth \(\beta\)-strand of Topology Two (II) C2 domains (i.e. the phospholipase, PLC-61), thereby shifting the order of homologous strands in the primary sequence (Figures 11 and 12). At present, all the proteins identified with a C2 domain conform to either topology (approximately fifty percent aligned to each topology) the significance of these differing topologies is presently unknown.

While there is considerable variability in the conservation of primary sequence, the tertiary structure of the domain core is well conserved as shown by the structures of synaptotagmin and PKC (topology I), PLC\(\delta\), and cPL\(\alpha\) (topology II); all C2 domains form eight anti-parallel \(\beta\)-strands structured around a conserved 40 residue core known as the "C2-key" motif (Sutton et al., 1995; Shao et al., 1996; Essen et al., 1996; Grobler et al., 1996; Perisic et al., 1998; Xu et al., 1998; Pappa et al., 1998) (Fig. 13), which corresponds approximately to the CaLB domain. Aside from the five aspartates and surrounding residues (CBR1-3 regions) that are conserved in numerous C2 there is little conservation in the top and bottom loop regions of the \(\beta\)-sandwich core structure (Rizo and Sudhof, 1998) and this may represent functional specificity of the different C2 domains, variability in Ca\(^{2+}\) affinity or differences in protein associations.

While these structures establish a general paradigm for C2 domains, two recently solved structures of the tandem C2A and C2B domains of synaptotagmin III (Sutton et al., 1999) and of the PKC-\(\delta\) C2 (Pappa et al., 1998) provide models for understanding the disparate binding properties of non-Ca\(^{2+}\)-dependent C2 domains. A comparison of the structures of the C2A (Ca\(^{2+}\)-dependent) and C2B (non-Ca\(^{2+}\)-dependent) domains of synaptotagmin III exhibits differences in the shape of the Ca\(^{2+}\)-binding pocket, the electrostatic surface potential, and the stoichiometry of bound divalent cations for the two domains (Sutton et al., 1999). Similarly, the crystal structure of the PKC-\(\delta\) C2 domain (Pappa et al., 1998) has allowed for a comparison in structural features of
Figure 12. Schematic representation of the two prototypical C2 domain topologies. The fold of the synaptotagmin C$_2$ A domain is termed “topology I” while that of the phosphoinositide-specific phospholipase C-δ1 domain is designated “topology II”. From Nalefski and Falke, 1996.
Figure 13. Model of the bi-partite Ca\(^{2+}\)-binding motif of C2A domain of synaptotagmin.

(A) Ribbon diagram of the crystal structure of the C2A domain of synaptotagmin I (SynI) (Sutton et al., 1995) showing the regions with the largest Ca\(^{2+}\)-induced chemical shift changes (shaded black). These regions (residues 168-183, 198-202, and 227-240) include the loops labeled 1,2 and 3 that constitute the Ca\(^{2+}\)-binding region. (B) A picture of the motif of the C2A domain that binds to Ca\(^{2+}\) ions. The backbone of residues 170 -180 (loop 1), 198-202 (loop 2) and 229-240 (loop 3) is represented by a purple ribbon, the two Ca\(^{2+}\) ions by green spheres, and the Ca and side chain heavy atoms of the five aspartate residues participating in the Ca\(^{2+}\) binding by yellow (O) and magenta (C) spheres. (C) General model of Ca\(^{2+}\) binding by C2 motifs. The Ca\(^{2+}\) binding residues in loop 1 are labeled X1 and X7 and those in loop 3 are labeled Y1, Y3 and Y9; these residues are most often aspartates but may also be glutamates and sometimes asparagine. Circles represent residues between the aspartate residues. From Shao et al., 1996.
the C2 domain involved in Ca\(^{2+}\) co-ordination between Ca\(^{2+}\)-dependent and independent C2 domains. The structure of the PKC-\(\delta\) C2 domain revealed unique structural elements for this non-classical/Ca\(^{2+}\)-independent or "novel" isoform of PKC (Fig. 14). This C2 domain possesses a helix and a protruding \(\beta\) hairpin in the loop regions containing basic residues and, although they are not involved in Ca\(^{2+}\) co-ordination, they may contribute to membrane-interaction site. A phosphorylation site was identified in the Ca\(^{2+}\)-binding region, also known as CBR, and recent studies show that tyrosine phosphorylated PKC-\(\delta\) is localized in the cytosol whereas catalytically active PKC-\(\delta\) is membrane associated (Shanmugam et al., 1998) suggesting that tyrosine modification may alter PKC-\(\delta\) activity and membrane association. A comparison of the PKC-\(\delta\) C2 domain with the C2 domains of SynI and PLC-\(\delta\) (Fig. 14), indicates that the most striking difference between the Ca\(^{2+}\)-independent PKC-\(\delta\) C2 is in the CBR loops; all adopt quite different conformations and orientations (and are hence, known as CBR-like loops) and lack all but one of the five conserved Ca\(^{2+}\) co-ordinating sidechains present in the C2 domains of SynI and PLC-\(\delta\). (Pappa et al., 1998).

VIII.B. Mechanism of Ca\(^{2+}\) binding to the C2 domain

The interaction between the C2 domain and its Ca\(^{2+}\) ligand is apparent from the NMR and crystal structure of the first C2 domain of synaptotagmin, determined in the presence or absence of Ca\(^{2+}\) (Sutton et al. 1995; Shao et al. 1996) (Fig. 13). Most C2-domain containing proteins bind acidic phospholipids in a Ca\(^{2+}\)-dependent manner via the C2 key which folds into a four-stranded \(\beta\) sheet (described above). The loops that form the "mouth" of this region are lined with the five highly conserved aspartate residues (Fig. 13), which are parts of regions which form the CBRs (Fig. 14). The Ca\(^{2+}\) ions are also co-ordinated through carboxylate groups from sidechain and mainchain atoms of two binding loops, CBR1 (between \(\beta2\) and \(\beta3\), topology I; \(\beta1\)-\(\beta2\), topology II) and CBR3 (\(\beta6\)-\(\beta7\), I; \(\beta5\)-\(\beta6\); II) with a shorter CBR2 (\(\beta4\)-\(\beta5\), I; \(\beta3\)-\(\beta4\), II) playing a
Figure 14. A comparison of type I and type II topology C2 domain structure. (A) A comparison of the structures for the C2 domains of SynI C2A, topology I (cyan), PKC-δ C2, topology II and non-Ca\(^{2+}\)-dependent (green) and PLC-δ C2, topology II (magenta); β strands are numbered in white for comparison. (B) Comparison of the Ca\(^{2+}\)-binding loops of SynI C2A (cyan), PLC-δ C2 (magenta) and the structurally equivalent loops from PKC-δ C2 (green). The three Ca\(^{2+}\)-binding loops of PLC-δ C2 and SynI are labeled as CBR1, 2 and 3. Sidechains involved in Ca\(^{2+}\) coordination are shown for of SynI C2A and PLC-δ C2 as are the equivalent sidechains of PKC-δ C2. The corresponding PKC-δ C2 loops are described as CBR1-like and CBR3-like to indicate that although they are structurally equivalent, they do not bind Ca\(^{2+}\). The CBR2-like loop of PKC-δ C2 is much shorter and is shifted relative to the other CBR2 loops and is not represented in this figure. C, COOH-terminus; N, NH2-terminus. From Pappa et al., 1998.
supportive role (Sutton et al., 1995; Shao et al., 1996; Essen et al., 1996; Perisic et al., 1998; Pappa et al., 1998).

Several independent reports have suggested that the Ca\(^{2+}\)-induced membrane targeting is triggered by the co-operative binding of multiple Ca\(^{2+}\) ions (Davelto et al., 1993; Li et al., 1995a; Fukuda et al., 1996). The stoichiometry of the binding, however, remains controversial (Sutton et al., 1995; Shao et al., 1996; Essen et al., 1997; Nalefski et al., 1997; Perisic et al., 1998; Ubach et al., 1998). NMR studies which monitored the Ca\(^{2+}\) binding pockets of the synaptotagmin C2A and PKC-\(\beta\) domains revealed a biphasic titration curve saturated by at least two ions with two binding constants: the first exhibiting a dissociation constant of 60 \(\mu\)M and the second, 400 \(\mu\)M (Shao et al., 1996). Similar results were obtained with the C2 domain of cPLA2 using equilibrium binding and stopped-flow kinetic experiments: the C2 domain binds two Ca\(^{2+}\) ions with positive cooperativity (\(K_\text{d}\) of 10 \(\mu\)M and 56 \(\mu\)M), yielding a conformational change and membrane docking (Nalefski et al., 1997). The Ca\(^{2+}\) signalling cycle of the C2 domain passes through an active, membrane bound state possessing two occluded Ca\(^{2+}\) ions, one of which is essential for maintenance of the protein-membrane complex (Nalefski et al., 1997).

Recent evidence using NMR spectroscopy and site-directed mutagenesis suggests that the C2A domain of synaptotagmin I binds to three Ca\(^{2+}\) ions in a tight cluster at the tip of the module (Ubac et al., 1998). The binding involves one serine (Ser235) that is conserved amongst synaptotagmin family members and the five aspartates conserved amongst many C2 domains (Fig 11). All three Ca\(^{2+}\) ions are required for the interactions of the C2A domain with syntaxin and phospholipids (Ubach et al., 1998). The authors propose that the C2 domain functions as an electrostatic switch, whereby the \(\beta\)-sheets of the domain provide a fixed scaffold for the Ca\(^{2+}\) binding loops, and interactions with target molecules are triggered by a Ca\(^{2+}\)-induced switch in the electrostatic potential of the domain (Ubach et al., 1998). The Ca\(^{2+}\)-independent C2B domain of synaptotagmin III was found to bind only one divalent cation which would leave the CBRs with a residual negative charge relative to the C2A domain (Sutton et al., 1999). The C2A domain also had
a more uniform electrostatic surface potential than the C2B domain which may explain differences in interactions and membrane binding properties (Sutton et al., 1999).

Finally, it was demonstrated that Ca²⁺ is the only divalent metal that promotes binding to the C2 domain of cPLA₂ at physiological concentrations (Ca²⁺ was 50-fold greater than Sr²⁺ and 10,000 fold over Ba²⁺), thereby reinforcing the concept that the Ca²⁺-dependent C2 domains in general, link second messenger Ca²⁺ to protein function (Nalefski et al., 1998).

VIII.C. Mechanism of C2 domain Membrane Interaction

Although the resolution of the three dimensional structure of various C2 domains has clarified the mechanism of Ca²⁺-binding to the domain, the events involved in domain binding to phospholipids and/or membranes following Ca²⁺-binding are largely unknown and controversial. It was originally hypothesized that binding of Ca²⁺ to the C2 domain of various molecules would induce conformational changes, thereby exposing functional groups responsible for either phospholipid/membrane binding or binding to proteins present at the membrane (Newton, 1995). Functional groups may include hydrophobic residues that can insert into the bilayer or charged sidechains that can bind electrostatically to specific phospholipid headgroups (Newton, 1995). These may then act as effective membrane anchors serving to orient numerous basic residues present on the top face of the C2 domain to interact with acidic headgroups at the membrane (Newton, 1995; Sutton et al. 1995). Indeed, the CBR3 loop of SynI, PLC-δ and cPLA₂ has been implicated in membrane interactions and is oriented roughly perpendicular to the β sheets (Sutton et al. 1995; Essen et al., 1996; Perisic et al., 1998) believed to serve as an interface with the lipid environment of the membrane. The existence of a conformational change which occurs upon Ca²⁺-binding to the C2A domain of synaptotagmin I as evidenced by a decreased protease sensitivity during Ca²⁺-induced membrane binding (Davelov and Sudhof, 1994), supports this notion. In addition, the C2 domain of cPLA₂ undergoes a Ca²⁺- stimulated conformational change which has been detected directly by intrinsic fluorescence (Nalefski and Falke, 1998).
Several findings support a model in which, following Ca\(^{2+}\)-binding, the Ca\(^{2+}\)-binding regions lie near the membrane (Essen et al., 1996; Grobeler et al., 1996; Essen et al., 1997; Perisic et al., 1998; Xu et al., 1998; Ubach et al., 1998; Edwards and Newton, 1997; Medkova and Cho, 1998; Zhang et al., 1998; Chapman and Davis, 1998). This notion is supported by the observation that Ca\(^{2+}\) ions become occluded upon membrane docking of the C2 domain of cPLA2 (Nalefski et al., 1997). In this C2 domain, a Ca\(^{2+}\)-triggered conformational change has also been detected directly by changes in fluorescence of probes directed to the Ca\(^{2+}\) binding loops (Nalefski and Falke, 1998). The membrane docking surface was localized to the same surface that co-operatively binds a pair of Ca\(^{2+}\) ions and the three Ca\(^{2+}\)-binding loops themselves provide most or all of the membrane contacts for docking (Nalefski and Falke, 1998). The C2 domain of cPLA2 interacts primarily with the headgroups of phospholipids, implying that the domain does not insert itself into the bilayer (Nalefski et al., 1998). There was an observed selectivity amongst headgroups and yet no preference in the fatty acid chains or the linkages to the glycerol backbone of the phospholipid (Nalefski et al., 1998).

The translocation of proteins to the membranous or particulate (P100) cellular fraction via the C2 domain may reflect direct binding of the protein to lipids at the plasma membrane or interaction with proteins at the site of translocation. Extensive work on PKC has been done to address this very issue. Ron et al. (1995) showed that translocated PKC interacts with several proteins from the particulate fraction that will only bind in the presence of PKC activators. These proteins, coined as RACKs (receptors for activated C-kinase) are neither substrates nor inhibitors of PKC (Ron et al. 1995). Interestingly, peptides that mimic the binding site of PKC to RACK (which contained regions of the C2 domain: AA209-216, 186-198, 218-226) bound to RACK and inhibited PKC translocation. In addition, recombinant fragments of synaptotagmin containing the C2 region bind to RACKs and inhibit PKC translocation and association with RACKs (Mochly-Rosen et al. 1992). Recent work carried out by Davis et al. (1996) suggests a direct interaction between annexin VI and the CaLB domain of p120 GAP, thereby furthering the notion that C2/CaLB domains interact with membrane proteins as well. It is unknown whether these protein
interactions serve to tether the C2 domain containing proteins to the membrane or whether they serve in their Ca\textsuperscript{2+}-dependent membrane delivery.

### VIII.D. Function of C2 domains in signalling proteins

The C2 domain was shown to be a functional module which serves to bind Ca\textsuperscript{2+} and acidic phospholipids in PKC (Coussens et al. 1986) as well as in other signalling molecules such as p120 ras-GTPase activating protein (GAP), phospholipase C\textgamma (PLC\textgamma), cytoplasmic phospholipase A\textsubscript{2} (cPLA\textsubscript{2}), synaptotagmin and rabphilin-3A (Figure 1) (Trahey et al. 1988; Stahl et al. 1988; Clark et al. 1991; Perin et al. 1991; Shirataki et al. 1993). These molecules were found to display a Ca\textsuperscript{2+}-dependent localization/association with the plasma membrane, with intracellular membranes or purified phospholipids, and these actions may serve to localize the molecule to within the vicinity of its intracellular target(s).

**Protein Kinase C**

Protein kinases C (PKCs) are a family of serine/threonine kinases that are involved in transducing a myriad of signals that activate cellular functions and induce proliferation (reviewed in Nishizuka, 1988, 1992; Dekker and Parker, 1994; Newton, 1995). The family consists of 10 members, all containing amino-terminal regulatory domains and a carboxyl-terminal catalytic domain. Based on the structural differences in the regulatory region of the protein and their regulatory co-factors, PKCs are generally classified into three groups: conventional PKC (\(\alpha, \beta I, \beta II\) and \(\gamma\)), novel PKC (\(\delta, \epsilon, \eta\) and \(\theta\)) and atypical PKC (\(\zeta, \mu\) and \(\upsilon\)). Much is known of the various functions and regulatory mechanisms of PKC in various signalling pathways (reviewed in Nishizuka, 1995; Newton, 1995) yet, a role for the second conserved regulatory region (C2) is just beginning to emerge. Conventional PKC isoforms are activated by the Ca\textsuperscript{2+}-dependent translocation of the protein to membranes containing phosphatidylserine and by diacylglycerol (DAG). The C1 domain is involved in the binding of PKC to DAG and to its structural analogs, phorbol esters (Ono et al., 1989; Bell and Burns, 1991; Burns and Bell, 1991). Structural (Sutton and Sprang,
1998) and mutational studies (Edwards and Newton, 1997; Medkova and Cho 1998) have shown that the C2 domain of conventional PKC is responsible for the Ca\textsuperscript{2+}-dependent translocation of the protein to membranes. The C2 domain of conventional mammalian isoforms is predicted to have a type I topology whereas the novel mammalian isoforms are predicted to utilize the type II topology (Nalefski and Falke, 1996) and atypical PKC isoforms do not possess C2 domains. Moreover, the order of the conserved domains differs between the conventional (C1, C2, C3-C4) and the novel (C2-C1, C3-C4) isoforms containing C2 domains.

The isolated C2 domains of PKC-\(\alpha\) (Corbalan-Garcia et al., 1999) and PKC-\(\beta\) (Shao et al., 1996) have been shown to exhibit Ca\textsuperscript{2+}-induced binding to either pure phosphatidylserine vesicles or mixed vesicles containing phosphatidylserine and phosphatidylcholine. The PKC-\(\alpha\) C2 domain, expressed as a GST fusion protein, localized to the plasma membrane in a Ca\textsuperscript{2+}-dependent manner and this translocation was dependent on three of the five conserved aspartate residues (Corbalan-Garcia et al., 1999). The C2 domains of all classical, Ca\textsuperscript{2+}-regulated PKC isoforms possess all five of the conserved aspartates shown to be involved in Ca\textsuperscript{2+} co-ordination, yet, their novel Ca\textsuperscript{2+}-independent PKC counterparts lack at least two of these conserved residues (Nalefski and Falke, 1996). It has been proposed that basic substitutions at these co-ordinating positions substitute for bound Ca\textsuperscript{2+}, thereby enabling constitutive activation of the kinase domain or docking to a membrane or receptor protein (Newton, 1995).

Ca\textsuperscript{2+}-dependent membrane association of PKC\(\alpha\) occurs as a multistep process (Medkova and Cho, 1998). Binding of the first Ca\textsuperscript{2+} ion to the loop regions of the C2 key motif, was essential to initial binding of the protein to membrane surfaces, whereas binding of the second Ca\textsuperscript{2+} ion induces conformational changes which, in turn, triggers membrane penetration and activation of the kinase (Medkova and Cho, 1998). Recently, the functional relationship between the C1 and C2 domain of PKC\(\alpha\) has been determined (Medkova and Cho, 1999). While the C2 domain is responsible for the initial Ca\textsuperscript{2+} and phosphatidylserine-dependent electrostatic membrane interactions, the C1 domain is involved in subsequent membrane penetration and diacylglycerol binding which eventually leads to enzyme activation (Medkova and Cho, 1999).
Synaptotagmin

Synaptotagmin (Syn) is a type Ib membrane protein which plays a key role in vesicular transport (reviewed in Sudhof and Rizo, 1996; Rizo and Sudhof, 1998). It has a short intravesicular N-terminus, a single transmembrane span and a large cytoplasmic domain, most of which is composed of two C2-domains. It was shown that its C2 domains may serve independent roles. The first C2 domain (C2-A) binds Ca$^{2+}$ (Brose et al. 1992; Daveltov and Sudhof, 1994) as well as phospholipids (Brose et al. 1992; Daveltov and Sudhof 1993) and syntaxin (a protein with an essential function in exocytosis) in a Ca$^{2+}$-dependent manner (Li et al. 1995b). Point mutations in the Ca$^{2+}$-binding site of the C2A domain which eliminate Ca$^{2+}$-dependent phospholipid binding disrupt the interaction with syntaxin suggesting that binding occurs through a similar mechanism and that residues are critical for both interactions (Li et al., 1995b). The second domain (C2B) binds to phospholipids irrespective of the presence of Ca$^{2+}$ (Damer and Creutz, 1994; Fukuda et al., 1994) as well as proteins, including other synaptotagmin molecules thereby facilitating homo and heterodimerization (Chapman et al., 1996; Sugita et al. 1996; Fukuda et al., 1999) the adaptin molecule AP-2, which recruits clathrin to membranes for endocytosis (Zhang et al. 1994) and SNAP-25 (Schiavo et al., 1997) involved in synaptic vesicle fusion. As well, the C2B domain has been shown to interact directly (and independently of Ca$^{2+}$) with N- or P/Q-type voltage-gated Ca$^{2+}$ channels involved in triggering exocytosis in nerve terminals (Charvin et al., 1997; Sheng et al., 1997). It is believed that this interaction may serve to locate synaptotagmin within domains of elevated Ca$^{2+}$ to facilitate neurotransmission.

Synaptotagmin C2B has also been suggested to act as a bimodal Ca$^{2+}$ sensor, switching bound lipids during exocytosis (Schiavo et al., 1996). The binding of phosphoinositides to the second C2 domain has been shown to be a Ca$^{2+}$-independent process (Fukuda et al., 1994; 1995), yet, Ca$^{2+}$ ions were shown to switch the specificity of C2B binding from phosphatidylinositol-3,4,5-trisphosphate bound at resting Ca$^{2+}$ concentrations to phosphatidylinositol-3,4-bisphosphate bound at Ca$^{2+}$-concentrations required for transmitter release in nerve terminals (Schiavo et al., 1996). Interestingly, binding of inositol 1,3,4,5-tetrakisphosphate (IP$_4$) disrupts the Ca$^{2+}$-sensing function
of neuronal synaptotagmin α and β in exocytosis (Fukuda et al., 1995a/b) by blocking vesicle fusion. Further examination of this phenomenon showed IP₄ binding to the C2B domains of several non-neuronal synaptotagmins and that there was a phylogenetically distinct branch of synaptotagmins that did not bind to IP₄ (Ibata et al., 1998). This promotes the notion that IP₄ binding to the syt C2B domain regulates Ca²⁺-induced (neuronal) and constitutive (non-neuronal) vesicle exocytosis. As such, in addition to the Ca²⁺-dependent regulatory role of the C2A domain, the C2B domain could act as a Ca²⁺ sensor in nerve terminals to regulate neurotransmitter release.

Recent studies have shown that the recruitment of the synaptotagmin C2B domain to the AP-2 complex is enhanced in the presence of tyrosine based endocytic motifs YxxØ (where x is any amino acid and Ø amino acids have bulky, hydrophobic side-chains) (Haucke and De Camilli, 1999). It was proposed that binding of the tyrosine motif to AP-2 induces a conformational change in AP-2 that increases its affinity for synaptotagmin C2B. This recruitment was also enhanced in the presence of the synaptic vesicle transmembrane protein, SV2a, that possesses two tyrosine motifs in its C-terminus, YSRF and YRRI (Haucke and De Camilli, 1999). The binding of the C2B domain of synaptotagmin to the AP-2 complex was occurring through a mechanism distinct from the tyrosine based motifs; likely through a cluster of lysines residues, as was suggested earlier (Chapman et al., 1998).

Membrane binding by synaptotagmins displays phospholipid specificity towards synthetic vesicles containing phosphatidylserine or phosphatidylinositol but not phosphatidylcholine or phosphatidylethanolamine (Davelto and Sudhof 1993; Chapman and Jahn, 1994). There is also a preference of the C2 domain for divalent rather than monovalent cations with a preference order of Ca²⁺>Sr²⁺, Ba²⁺>Mg²⁺ (Davelto and Sudhof 1993; Li et al., 1995a). Other divalent cations fail to promote phospholipid binding and only Sr²⁺ and Ba²⁺ induce the decreased protease sensitivity of the C2 domain in the presence of phospholipid as observed for synaptotagmin (Davelto and Sudhof 1994).
Cytosolic Phospholipase A<sub>2</sub>

Cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) releases arachidonic acid from the sn-2 position of glycerophospholipids to initiate the production of leukotrienes and prostaglandins, which are potent mediators of inflammation (Clark <i>et al.</i>, 1995). The activation of cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) is, in part, due to rises in intracellular Ca<sup>2+</sup> and the subsequent Ca<sup>2+</sup>-induced membrane translocation mediated by its C2 domain. While the activity of the C-terminal catalytic domain of cPLA<sub>2</sub> is independent of Ca<sup>2+</sup>, the Ca<sup>2+</sup>-dependent membrane binding action of the N-terminal C2 domain brings the enzyme in close proximity to its substrates at the nuclear envelope and endoplasmic reticulum (Nalefski <i>et al.</i>, 1994). The C2 domain alone is responsible for this Ca<sup>2+</sup>-dependent translocation, and the CBR1 and CBR3, in particular, mediate this action by anchoring the domain to the target membrane (Perisic <i>et al.</i>, 1999; Bittova <i>et al.</i>, 1999). A recombinant cPLA2 lacking its C2 domain (cPLA2[ΔC2]) was incapable of binding to membranes in response to Ca<sup>2+</sup> although it retained its ability to hydrolyze monomeric lysophospholipids at a normal (~2.0 pmol/min) rate (Nalefski <i>et al.</i>, 1994). In addition, the subcellular localization of the mutant was different from the wild-type protein upon cell activation (Schievella <i>et al.</i>, 1995). Interestingly, the C2 domain of cPLA2 displays a dramatic difference in its preference for phospholipids; it displays a strong preference for neutral phospholipids containing phosphatidylcholine or phosphatidylethanolamine (Nalefski <i>et al.</i>, 1998). The preference for divalent cations (Ca<sup>2+</sup>&gt;Sr<sup>2+</sup>, Ba<sup>2+</sup>) is the same as synaptotagmin (Nalefski <i>et al.</i>, 1998). Similar to Nedd4-C2 domain, cPLA2-C2 domain conforms to a type II topology and contains four of the five acidic side chains that are implicated in the co-ordination of Ca<sup>2+</sup>, with the fifth conserved Asp substituted by an Asn (Fig. 11). Unlike SynI, it is not known whether the C2 domain of cPLA2 engages in any protein-protein interactions. Interactions with receptor proteins could play a role in targeting and may explain the specific intracellular localization of cPLA2 to the endoplasmic reticulum and nuclear envelope in response to Ca<sup>2+</sup> (Glover <i>et al.</i>, 1995; Scheivella <i>et al.</i>, 1995). This is the location of some of the downstream enzymes of the arachidonic acid pathway such as 5-lipoxygenase and cyclooxygenase.
An alternative proposal is that the accumulated cPLA2 substrate tethers the molecule at the appropriate membrane (Ghomasi et al., 1992).

**Phosphoinositol-specific Phospholipase C**

In response to mitogenic signals that increase intracellular Ca\(^{2+}\), phosphoinositol-specific phospholipase C (PLC) liberates diacylglycerol (DAG) and inositol (1,4,5)trisphosphate (IP\(_3\)) by catalyzing the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP\(_{2}\)). Whereas IP\(_3\) regulates Ca\(^{2+}\) release channels in the endoplasmic reticulum and further propagates elevation of intracellular Ca\(^{2+}\) (reviewed in Berridge 1993), the membrane resident DAG activates several protein kinase C (PKC) isoforms (Dekker et al., 1995). Three families of PLC isoforms have been described in mammals: PLC\(\beta, \delta, \gamma\) (reviewed in Rhee et al., 1989; Kriz et al., 1990). While the members within each family are highly similar to one another at the amino acid level, little identity exists between members of different families (Rhee et al., 1989) except in the modular domains: the C2 domain, the catalytic domain and the PH domain as well as in an EF-hand motif (Rhee and Bae, 1997; Essen et al., 1996). It is, therefore, likely that the family of enzymes is regulated by (I) Ca\(^{2+}\) binding to its EF hand and/or C2 domain (ii) binding of PIP\(_2\) and IP\(_3\) to its PH domain and finally (iii) membranes containing appropriate ligands (Rhee and Choi, 1992; Yagisawa et al., 1994; Ferguson et al., 1995; Lemmon et al., 1996). Indeed, studies have shown that the initial steps of activation of PLC\(\delta1\) is a translocation to the plasma membrane (via its PH domain) where it binds to the lipid interface, containing multiple phosphoinositides such as PIP\(_2\) and IP\(_3\) (Yagisawa et al., 1998; Lomasney et al., 1996; Lemmon et al., 1995; Hyvonen et al., 1995).

The C2 domain has been shown to be essential in catalysis as a partial deletion in this region results in an inactive enzyme (Yagisawa et al., 1994). Determination of the structure of the PLC\(\delta1\) has proposed a “tether and fix” model of PLC membrane binding and activation (Essen et al., 1996). In this model, the PH domain is thought to “tether” the protein to the membrane while the C2 domain “fixes” the catalytic domain in the appropriate conformation. Although there have been few detailed studies of any PLC C2 domain in isolation, mutational analysis of the PLC\(\delta1\) C2 domain and the affect of these mutations on enzymatic activity has led to a proposed model of C2
domain functioning (Lomasney et al., 1999). Deletion of a region of amino acids from the C2 domain of PLCδ1 impaired Ca²⁺ binding and reduced its stimulation and binding by phosphatidylserine. It has been proposed that phosphatidylserine stimulates the affinity of the enzyme (PLCδ1) for its substrates by binding to the C2 domain in a Ca²⁺-dependent manner; Ca²⁺ binding promotes the formation of an enzyme-PS-Ca²⁺ ternary complex which leads to activation of the enzyme via a 20 fold reduction in the $K_m$ for the substrate (Lomasney et al., 1999). In addition, PLCβ1 and β2 are regulated by the $G_q$ family of heterotrimeric G-proteins (Camps et al., 1992; Katz et al., 1992; Carozzi et al., 1993). Recently, it has been shown that the C2 domains of PLCβ1 and β2 are involved in selective interactions with activated $G_{αq}$ subunits of the $G_q$ family of heterotrimeric G-proteins (Wang et al., 1999), providing a surface to which PLC can dock, leading to the activation of the native protein.

All three isoforms of PLC possess type II topology C2 domain and PLCβ, γ lack the full complement of five Ca²⁺-co-ordinating acidic side chains. It was shown that PLCγ expressed in COS cells, failed to bind cellular membranes in vitro in the presence of Ca²⁺ concentrations known to cause binding of cPLA2 and PKCβ1 (Clark et al., 1995). Clearly, much remains to be known about the mechanisms of action of the PLC C2 domains.

**ras GTPase-activating protein (rasGAP)**

p120GAP is a GTPase activating protein (GAP) for the small G-protein p21 Ras (Trahey and McCormick, 1987). The role of the C2 domain of p120-rasGAP has been determined by examining the Ca²⁺-dependent membrane binding characteristics of a bacterially expressed GST fusion protein of the CaLB subregion of the rasGAP C2 domain (Gawler et al., 1995a,b). The fusion protein was capable of Ca²⁺-dependent lipid binding, showing a preference for negatively charged phospholipids such as phosphatidylserine and phosphatidylinositol in the presence of low micromolar Ca²⁺ *in vitro* (Gawler et al., 1995a,b). This region of the rasGAP C2 domain was also capable of mediating Ca²⁺-dependent membrane binding *in vivo*. Attachment of this region to a transformation-defective v-src which lacked its own N-terminal myristoylation sequence and could not translocate correctly to the membrane restored transformation ability and localization with the
membrane fraction in vivo (Gawler et al., 1995a). As previously mentioned, the CaLB of rasGAP was found to mediate an interaction with annexin VI both in vitro and in vivo (Davis et al., 1996). Interestingly, annexin VI has been implicated in tumor suppressor function (Theobald et al., 1995) raising the possibility that this activity could be related to its ability to interact with rasGAP, thereby regulating p21ras activity. While it was found that a truncated version of p120GAP, that was incapable of binding to phospholipids, was able to bind to fibroblast membranes in a Ca\(^{2+}\)- and concentration-dependent manner, it was also capable of binding to annexin VI in an amino acid sequence (AA 618-632) specific but Ca\(^{2+}\)-independent manner (Chow et al., 1999). Interestingly, this truncated p120GAP, when bound to annexin VI in the presence of Ca\(^{2+}\), regained its ability to bind phospholipid (phosphatidylserine) vesicles. It was suggested that annexin VI may simultaneously mediate an interaction with p120GAP and membrane phospholipids, underlying the importance that this interaction may have in regulating p21ras activity (Chow et al., 1999).

Other C2 domain containing proteins

There are many other examples of proteins with C2 domains that are not described here, as much less is known about their function. These include rabphilin-3A, known to bind the membrane-bound GTPase Rab3A involved in neurotransmitter release (Geppert et al., 1994), DOC2, a protein highly expressed in the brain that is enriched in synaptic vesicles and believed to play a role in neurotransmitter release (Orita et al., 1995, 1997), perforin, a protein found in the secretory granules of cytotoxic T cells that binds and inserts itself into cellular membranes to form pores that result in the lysis of target cells (Lichtenheld et al., 1988; Liu et al., 1995), UNC-13, a brain-specific protein originally cloned in C. elegans and believed to play a role in synaptic transmission (Maruyama and Brenner, 1991; Brose et al., 1995) and the dual-specificity phosphatase PTEN, a tumor supressor, whose C2 domain structure has been solved recently (Lee et al. 1999). The structure of PTEN reveals a lack of the conserved Ca\(^{2+}\) ligands (Asp) yet, displays other structural features similar to C2 domains known to interact with membranes (Lee et al., 1999) and indeed, the PTEN C2 domain does show a Ca\(^{2+}\)-independent affinity for phospholipid membranes in vitro. Mutations in the lysine rich region of the CBR3-like loop of PTEN resulted in a loss of its tumor
suppressor function, and rendered a phenotype of growth of glioblastoma cells similar to growth of glioblastoma tumor cells (Lee et al., 1999). These results suggested that the binding of PTEN to phospholipids is important for its tumor suppressor function. Contact points between the C2 domain and the phosphatase domain of PTEN were mapped and found to include several residues that have been found to be mutated in cancers, indicating the integrity of the interface between the two domains is important for the function of PTEN (Lee et al., 1999).

IX. Annexins

Annexins are a family of Ca\(^{2+}\) and phospholipid binding proteins that contain multiple repeats of a highly conserved 70 amino-acid domain and are structurally very similar (reviewed in Creutz, 1992; Moss, 1997; Gerke and Moss, 1997). Although these proteins have been implicated in numerous physiological functions all involving membranes and Ca\(^{2+}\), the exact role for annexins is not understood. There are at least ten annexins in mammals and several of these have homologues in lower eukaryotes (Smith and Moss, 1994). One distinguishing feature of annexins is that each has a unique N-terminus of varying length. These N-termini are likely responsible for the unique functions of the various family members. Indeed, the N-terminal sequence of many annexins harbors phosphorylation sites and the sites for interactions with other proteins such as annexin II interaction with the S-100 EF-hand type Ca\(^{2+}\) binding protein p11 (Zokas and Glenney, 1987) and annexin XI interaction with calcyclin (Minami et al., 1992).

In the folded molecule each 70 amino-acid annexin repeat comprises five \(\alpha\)-helices wound into a superhelix (Concha et al., 1993; Huber et al., 1990a). The helices are connected by loops which are involved in Ca\(^{2+}\) co-ordination (Huber et al., 1990b). The Ca\(^{2+}\) binding sites differ from the EF-hand type, helix-loop-helix, Ca\(^{2+}\) binding site found in proteins like parvalbumin and troponin C (Weng et al., 1993). In the annexins, the Ca\(^{2+}\) ions co-ordinate to carbonyl and carboxyl oxygens of the peptide bonds located in the loop regions. All annexins with the exception of annexin XIII bind to membranes in a Ca\(^{2+}\)-dependent fashion. The members of the annexin XIII
family are N-terminal myristoylated and therefore, constitutively associated with the membrane (Wice et al., 1992, Fiedler et al., 1995, Lafont et al., 1998).

Annexin XIII exists as two isoforms, annexin XIIIa and b. Annexin XIIIa was isolated in intestinal epithelia (Wice and Gordon, 1992) and lacks an N-terminal 40 amino-acid insert present in annexin XIIIb. In polarized MDCK cells annexin XIIIb was isolated to apical exocytic carriers or rafts (Wandinger-Ness et al., 1990; Fiedler et al., 1995) that are microdomains of sphingolipids and cholesterol which recruit apically destined proteins in the trans-Golgi and are involved in transport to the apical membrane (reviewed in Simons and Ikonen, 1997). Immunofluorescence experiments have shown that annexin XIIIb is located at the apical membrane of MDCK cells and an antibody raised against annexin XIIIb inhibited the apical delivery of vesicular carriers when introduced into streptolysin-O (SLO)-permeabilized cells (Fiedler et al., 1995). Using in vitro assays, Lafont et al. (1998) showed that only myristoylated annexin XIIIb can stimulate apical transport whereas the unmyristoylated form inhibits this route. Moreover, the formation of apical carriers from the trans-Golgi is inhibited by an anti-annexin XIIIb antibody and is recovered by introduction of myristoylated recombinant annexin XIIIb (Lafont et al., 1998). These results suggest that annexin XIIIb directly participates in apical membrane delivery and furthers the notion of the involvement of the annexins in membrane trafficking events.

X. Summary

In summary, there now exists a plethora of evidence defining the mechanisms by which short-lived proteins in eukaryotic cells are degraded by the ubiquitin system. In this pathway, proteins are targeted for degradation by covalent attachment of ubiquitin, a highly conserved 76 amino acid protein which signals proteolysis by the 26S proteasome or by endocytosis and lysosomal degradation. Ubiquitin-mediated degradation of regulatory proteins plays a vital role in the control of numerous cellular processes including cell-cycle progression, signal transduction, as well as receptor downregulation, and has been implicated in responses by the immune system and
in development. Abnormalities in the proteins comprising the ubiquitin system have been shown to lead to various pathological conditions. The selectivity of protein degradation is largely governed by the enzymes involved in ligating ubiquitin to the target molecule, the E3 enzymes or ubiquitin-protein ligases. These enzymes/enzyme complexes recognize specific signals in their substrates to allow for this specificity and different E3s respond to different signals. In particular, the Nedd4/Rsp5 family of protein ubiquitin ligases in yeast and mammalian cells have been shown to regulate the stability of many membrane proteins through ubiquitination. These enzymes, with Nedd4 as the archetype, possess an N-terminal C2 domain, 2-4 WW domains and C-terminal HECT or ubiquitin ligase domains. While the WW domains of Nedd4 have been shown to bind to proline-rich sequences (PY motifs) in the cytosolic tails of one of its substrates, ENaC and the HECT domain was shown to possess the catalytic activity required for ubiquitin ligation, no function had been ascribed to the C2 domain of Nedd4.

C2 domains have been identified in numerous signalling molecules and have been found to mediate Ca\(^{2+}\)-dependent membrane and phospholipid association both \textit{in vivo} and \textit{in vitro}. Consequently, they are believed to play a pivotal role in intracellular membrane targeting and cellular localization. In addition, many C2 domains have been found to interact with proteins, which may represent an alternative or parallel mechanism for intracellular trafficking. Much is known about the structure of the C2 motif and how this relates to its functioning and it is likely that the specificity of interactions for each C2 domain will, in part, be reflective of the differences in the amino acid composition of the calcium binding regions or loops (CBRs).

Annexins represent a family of Ca\(^{2+}\) and phospholipid binding proteins. Although their cellular role is unclear, many annexins have been implicated in membrane trafficking events. In particular, annexin XIIIb has been shown to play a role in apical membrane transport in polarized epithelial cells.
X. Rationale and Goals of Research

The goal of my research was to investigate the role of the C2 domain of Nedd4 in regulating the function of this ubiquitin-protein ligase. I hypothesized that the C2 domain of Nedd4 is vital to the intracellular targeting of the molecule. By mediating Ca\(^{2+}\)-dependent interactions with either membranes or intracellular proteins, I proposed that the C2 domain directs or influences the subcellular localization of the molecule thereby bringing it into close proximity with its potential substrates, such as ENaC.
Chapter Two:
The C2 Domain of Nedd4 Mediates Ca\(^{2+}\)-dependent Membrane Association

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My contribution to this work was to perform all experiments and to provide the intellectual drive behind the experimentation. Herman Yeger and Perry Howard were acknowledged for supplying protocols for immunofluorescence and confocal microscopy. Olivier Staub was acknowledged for providing the rNedd4 cDNA and anti-Nedd4 antibodies.
Chapter Two: The C2 Domain of Nedd4 Mediates Ca^{2+}-dependent Membrane Association

I. Summary

Nedd4 (Neuronal precursor cell-expressed developmentally down-regulated 4) is a protein-ubiquitin ligase (E3) containing a HECT domain, 3 or 4 WW domains and a C2 domain. Several C2 domains of other proteins have been shown to mediate Ca^{2+}-dependent membrane association. To test whether the C2 domain of Nedd4 fulfills a similar function we determined the distribution of Nedd4, endogenously expressed in MDCK cells, in response to elevations in cytosolic Ca^{2+}. We observed that Nedd4 was redistributed from the cytosolic to the particulate fraction in response to ionomycin plus Ca^{2+} treatment. A similar treatment of polarized MDCK cells led to primarily an apical but also lateral membrane localization of Nedd4, as determined by immunostaining and confocal microscopy. The Ca^{2+}-induced association of Nedd4 with the membrane persisted for approximately 30-45 min. To determine if the C2 domain, alone, could bind membranes, the Nedd4-C2 domain was expressed as a GST fusion protein and was used in in vitro binding experiments with purified membranes. Our results show that the GST-Nedd4-C2 fusion was sufficient to bind cellular membranes in a Ca^{2+}-dependent manner. To determine whether the C2 domain displayed a preference for charged phospholipids, as was shown for several other C2 domains, this GST-Nedd4-C2 was used in in vitro binding experiments with purified phospholipids. We found that not only could the C2 domain mediate Ca^{2+}-dependent interactions with purified phosphatidylserine, phosphatidylinositol and phosphatidylcholine liposomes in vitro, but that the interactions appeared to be of equal intensity, revealing no preference towards charge. To determine whether the C2 domain was responsible and necessary for mediating the Ca^{2+}-dependent membrane association seen in cells, an epitope-tagged Nedd4 lacking its C2 domain was stably expressed in MDCK cells and its cellular localization in response to increases in cytosolic Ca^{2+} was assessed. The epitope-tagged Nedd4 construct lacking the C2 domain failed to mediate the Ca^{2+}-induced plasma membrane localization seen with the wild-type (epitope-tagged) Nedd4. These results demonstrated that the C2 domain of Nedd4 acts
as a bona fide C2 domain which binds phospholipids and membranes in a Ca\textsuperscript{2+}-dependent manner and is involved in localizing the protein primarily to the apical region of polarized epithelial cells in response to Ca\textsuperscript{2+}.

II. Introduction

Nedd4 was previously shown to interact with the epithelial Na\textsuperscript{+} channel (ENaC), an interaction mediated by an association of its WW domains with the proline-rich PY motifs of ENaC (Staub et al., 1996). Deletion or mutations within the PY motifs of the β and γ subunits have been genetically linked to Liddle Syndrome (Shimkets et al., 1994; Hansson et al., 1995a,b; Tamura et al., 1996; Inoue et al., 1998), a hereditary form of arterial hypertension caused by an abnormal increase in ENaC activity (Schild et al., 1995; Schild et al., 1996). More recently, work from our lab and that of others (Pirozzi et al., 1997) has described interaction of Nedd4 and Nedd4-like proteins with other PY motif-containing proteins, also mediated by the WW domains. We therefore speculate that substrate specificity for the E3 activity of Nedd4 mediated by its HECT domain is conferred, at least in part, by its WW domains (Staub and Rotin, 1996). Unlike the WW or HECT domains, however, the function of the putative C2 domain of Nedd4 has not been elucidated.

The C2 domain is a module present in numerous proteins (Ponting and Parker, 1996; Nalefski and Falke, 1996) and has been shown to be a functional domain in several of them (Clark et al., 1991; Bazzi and Nelsestuen, 1987; Bazzi and Nelsestuen, 1990; Gawler et al., 1995; Nalefski et al., 1994; Brose et al., 1992). The C2 domains described to date show significant diversity in the molecules they interact with, which include Ca\textsuperscript{2+}, phospholipids, intracellular proteins and inositol polyphosphates. The C2 domain spans approximately 130 amino acids and the NMR and crystal structure reveals that the domain folds into an 8-stranded β-sandwich motif structured around a 4-stranded core known as the "C2 key" (Sutton et al., 1995; Shao et al., 1996; Essen et al., 1996) which is lined with conserved aspartate (or in some cases asparagine or glutamate) residues, the Ca\textsuperscript{2+}-binding sites (Sutton et al., 1995; Shao et al., 1996; Essen et al., 1996) (as described in Introduction section).
Although originally identified as a Ca\(^{2+}\) and membrane binding module, accumulating evidence suggests that at least some C2 domains bind proteins as well (see Chapter 3). The synaptotagmin C2A and C2B domains bind several proteins (reviewed in Sudhof and Rizo, 1996) including Ca\(^{2+}\)-dependent binding to syntaxin, a membrane protein in the presynaptic terminal, and AP-2, a heterooligomeric adaptor complex in clathrin coated pits (Li et al., 1995; Zhang et al., 1994; Sugita et al., 1996). In addition, Ca\(^{2+}\)-induced translocated PKC interacts with several proteins from the particulate fraction that only bind in the presence of PKC activators (Ron et al., 1995). These proteins, RACKs (receptors for activated C- kinase), are substrates of activated PKC (Uberall et al., 1997) and peptides that mimic the binding site of PKC to RACK (which contain the PKC-C2 domain) inhibit PKC translocation. Recombinant fragments of synaptotagmin containing the C2 region bind to RACKs and inhibit PKC translocation and association with RACKs (Mochly-Rosen et al., 1992). In addition, recent work (Davis et al., 1996) has demonstrated a direct interaction between Annexin VII and the CaLB domain of p120 GAP, thereby furthering the notion that C2/CaLB domains interact with proteins as well.

As several C2 domains of other molecules had been characterized and been shown to mediate Ca\(^{2+}\)-dependent membrane targeting, we hypothesized that the C2 domain of Nedd4 may be fulfilling a similar function. In the present study we show that Nedd4 expressed endogenously in MDCK cells associates with membranes and localizes to the plasma membrane in a Ca\(^{2+}\)-dependent fashion, with a particular preference towards the apical and lateral regions of these polarized epithelial cells. We also demonstrate that the Nedd4-C2 domain by itself is capable of mediating a Ca\(^{2+}\)-dependent cellular membrane and phospholipid binding, and that Nedd4 lacking its C2 domain (heterologously expressed in MDCK cells) fails to associate with the plasma membrane in response to Ca\(^{2+}\). These results support our hypothesis that the putative C2 domain of Nedd4 acts as a bonafide C2 domain which binds phospholipids and membranes in a Ca\(^{2+}\)-dependent fashion, and furthermore, is involved in localizing the protein primarily to the apical and lateral regions of polarized epithelial cells in response to Ca\(^{2+}\). These findings may support a role for the C2 domain
in regulating the activity of the ubiquitin ligase by mediating the localization and hence, proximity to substrates, of Nedd4.

II. Experimental Procedures

Membrane preparation

The epithelial MDCK (Madin-Darby Canine Kidney) cells, expressing endogenous Nedd4, were grown on 100 mm coated plates in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) and grown at 37°C, 5% CO2-atmosphere. For ionophore treatment, cells were serum-starved for 16 hr, trypsinized, resuspended in serum-free media and harvested. Cells were then washed twice with Ca²⁺-free medium (140 mM NaCl, 6 mM KCl, 1 mM MgCl₂, 0.1 mM EDTA, 20 mM glucose and 20 mM HEPES) and then incubated in that medium containing also 1 mM ethylene glycol-bis(b-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), pH 7.3 for 5 min at 37°C in the presence or absence of 1 μM ionomycin and the indicated amount of free Ca²⁺ (maintained with Ca²⁺/EGTA buffers). The application of ionomycin in the presence of Ca²⁺ has been shown to induce increases in cytosolic calcium in MDCK cells (Lang et al., 1990; Delles et al., 1995). The cells were then collected and washed twice with Ca²⁺-free medium (for -Ca²⁺ conditions) or +Ca²⁺ medium (same as -Ca²⁺ medium but containing 1.1 mM CaCl₂) and re-suspended in +/-Ca²⁺ medium with 10μg/ml of both leupeptin and aprotinin and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were then homogenized and nuclei and mitochondria pelleted at 1000x g (5 min, 4°C). The supernatant was then spun at 100,000x g for 30 min (4°C) and the cytosolic (supernatant, S100) and pellet (particulate, P100) fractions were collected for further analysis.

Immunoprecipitations

Particulate (P100) fractions were resuspended in lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 1% Triton-X-100, 1 mM PMSF, 10 μg/ml of both
leupeptin and aprotinin). Nedd4 was immunoprecipitated from the S100 and P100 fractions using an affinity purified polyclonal antibody raised against GST-Nedd4-WWII (Staub et al., 1996) by incubating for 4 h at 4°C. The immunocomplexes were collected with protein-A-Sepharose beads, washed five times with HNTG buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 0.1% Triton-X-100, 10% glycerol). Proteins were separated on 8% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with anti-Nedd4-WWII antibodies, followed by horseradish peroxidase-conjugated (HRP) secondary antibodies and ECL detection (Amersham Canada Ltd., Oakville, Ontario).

**Preparation of GST fusion protein constructs**

GST fusion proteins were prepared by PCR amplification of the region of rNedd4 cDNA (Staub et al., 1996) corresponding to the C2 domain (nucleotides 182-574). PCR fragments were subcloned with flanking BamHI and EcoRI sites into the corresponding sites in pGEX-2TK (Pharmacia Biotech Inc., Baie d'Urfe, Quebec). The plasmid containing the C2 insert, verified by sequencing, were used to transform the HB101 strain of Escherichia coli. Fusion protein expression was induced with 0.2 mM isopropyl β-D-thiogalactoside (IPTG) (Pharmacia) for 4-5 hrs and bacteria were collected and lysed by sonication in phosphate-buffered saline (PBS) containing aprotinin, leupeptin and PMSF (concentrations as stated above). Fusion proteins were purified from the bacterial lysate with glutathione-agarose beads and eluted with 30 mM reduced glutathione (pH 8.0).

**In vitro binding experiments**

For translocation assays, 200 µg (by protein determination) of crude membranes from MDCK cells, prepared as described above, were diluted into Ca²⁺-free medium, pH 7.5, containing 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF and 10 mg/ml of both leupeptin and aprotinin. Membranes were then resuspended by sonication (5 x 1 sec) and incubated with purified GST alone or GST-C2 (10 nM) in the presence or absence of Ca²⁺ for 30 min. at 30°C. Membrane
associated proteins were separated on 10% SDS-PAGE and immunoblotted with anti-GST antibodies as described above.

For liposome binding experiments, liposomes of varying composition (either mixtures of, or pure phosphatidylserine, phosphatidylinositol or phosphatidylcholine) were used. Approximately 1mg of the phospholipid in chloroform was dried under nitrogen, added to 1 ml of sucrose containing media (25 mM HEPES, 0.1 M NaCl, 1 mM EDTA, 1 mM DTT, 20% sucrose pH 7.5) and sonicated at 4°C 5 x 1 sec to resuspend. 100 μl of liposomes were incubated with 10 nM purified GST-C2 or GST alone in the presence of Ca²⁺ (1 mM) in a final assay volume of 1 ml. Liposomes were collected by centrifugation at 100,000 x g for 30 min at 4°C and proteins associated with them separated on 10% SDS-PAGE and immunoblotted with anti GST antibodies, as described above.

**Generation of stable MDCK cells lines expressing epitope-tagged Nedd4 or C2-deleted Nedd4.**

A C2-deleted (ΔC2) Nedd4 was created by deleting amino acids 79-191 (nucleotides 179-571) of Nedd4 using PCR. This construct, as well as full-length Nedd4 used as a control, were epitope tagged with the T7-epitope (MASMTGGQMQM) placed at the N-terminus of the molecule rather than the C-terminal (N-terminal tagging was chosen because all HECT domains, including that of Nedd4 are located at the very C-terminus of the protein, suggesting that addition of sequences at that position may interfere with its enzymatic activity). These constructs, called T-Nedd4 or T-ΔC2-Nedd4, were subcloned into the pRC-CMV vector (Invitrogen Corporation, San Diego, California). Stable cell lines were generated by transfecting MDCK cells with the T-Nedd4 or T-ΔC2-Nedd4 constructs using lipofectamine (Gibco BRL, Life Technologies, Gaithersburg, Maryland) and selection in 1 mg/ml G418 (Gibco). Positive clones were tested for protein expression using anti T7 antibodies (Novagen, Madison, Wisconsin).
**Immunostaining and confocal microscopy**

MDCK cells, either wild type or those expressing T-Nedd4 or T-ΔC2-Nedd4, were grown to confluence on permeant filters (Falcon 0.4 μm pore size, 6 well format; Becton Dickinson Labware, Lincoln Park, New Jersey) and then treated or not with Ca\(^{2+}\) plus ionomycin as described above. Following treatment, filters were washed and fixed briefly in 10% neutral buffered formalin, washed 2x in PBS and stored at 4°C. Cells on membranes were then permeabilized at room temperature in a humidity chamber for 30 min with 1% Triton-X-100 in Tris-buffered saline (30 mM Tris pH 7.5, 150 mM NaCl). The filters were cut from the inserts and incubated in 5% normal goat serum in Antibody Diluting Buffer (Dimension Labs, Mississauga, Ontario) for 30 min. followed by incubation for 2 h in the primary antibody (either 10 μg/ml affinity pure anti-Nedd4-WWII antibodies, or 1:1000 dilution of anti T7 antibodies). Filters were then washed 4x with 30 mM Tris pH 7.5, 150 mM NaCl, 1% BSA, 0.05% Triton-X-100 (TBS buffer) followed by incubation with biotinylated goat anti-rabbit or anti-mouse secondary antibody (Molecular Probes, Eugene, Oregon) for 45 min (diluted 1:250 and 1:200 for anti rabbit and anti mouse secondary antibodies, respectively). This was followed by several washes in TBS buffer and one final wash in bicarbonate saline buffer (16 mM bicarbonate, 150 mM NaCl supplemented with 2 mM HEPES, pH 8.5). The filters were then incubated for 45 min with avidin-Texas Red (Molecular Probes) (1:100 dilution in bicarbonate saline) followed by several additional bicarbonate saline washes and finally mounted in Vectashield (Vector Labs, Burlingame, California). The fixed and stained cells were viewed using a Zeiss LSM-4A inverted confocal microscope with X 63 oil objective (NA=1.3). Texas-Red (λex= 596, λem= 615) was detected by krypton-argon laser excitation (568 nm) and detection with a photomultiplier after an emission filter (>590 nm). Serial sections were acquired by line averaging the frames (n=8) at descending z levels. Starting at the apical surface (i.e. the coverslip) a minimum of 20 optical sections (each 0.5 μm thick) were obtained for the monolayer. The photomicrographs represent multiple sections in the monolayer observed on each coverslip repeated for at least three independent preparations.
III. Results

*Ca<sup>2+</sup>*-dependent association of endogenous Nedd4 with cellular membranes

Numerous proteins contain regions homologous to the C2 domain (Figure 10 in Ch. 1), most of which are thought to interact with cellular membranes. To assess whether Nedd4 displays a Ca<sup>2+</sup> dependent association with membranes in cells, we examined the changes in the subcellular distribution of endogenously expressed Nedd4 following an increase in intracellular Ca<sup>2+</sup> in MDCK cells. MDCK cells were used because they represent a well characterized epithelial cell line, forming monolayers of polarized cells when grown to confluence, and because they express high levels of endogenous Nedd4 (Staub et al., 1996). The elevation in intracellular Ca<sup>2+</sup> concentrations was achieved by incubating cells with 1 μM ionomycin in the presence of 1 mM Ca<sup>2+</sup> for 5 min. Cells were then either fractionated and the soluble (S100) or particulate (P100) fractions analyzed for the presence of associated Nedd4 by immunoblotting, or immunostained with anti Nedd4 antibodies to follow intracellular localization of the protein. Figure 2-1 (left panel) shows that in response to Ca<sup>2+</sup> plus ionomycin treatment, the majority of endogenous Nedd4 (115 kDa band) was redistributed from the cytosolic to the particulate fraction. This redistribution was not caused by ionomycin itself, because treatment of cells with ionomycin alone (1 μM) did not alter the primarily cytosolic distribution of Nedd4 (Figure 2-1, right panel).

To follow directly the Ca<sup>2+</sup>-dependent subcellular distribution of endogenous Nedd4 in living cells, particularly in cells which are polarized, MDCK cells were grown to confluence on permeant filters to allow them to become polarized. Under these conditions, they form tight junctions and a sheet of high resistance epithelial monolayer (Dho and Rotin, unpublished). Following treatment of cells without or with Ca<sup>2+</sup> plus ionomycin, cells were permeabilized and immunostained with anti-Nedd4 antibodies followed by Texas Red-conjugated secondary
Figure 2-1. **Ca\textsuperscript{2+}-induced association of Nedd4 with crude membranes of MDCK cells.**

MDCK cells serum-starved for 16 hrs. were incubated for 5 min at 37°C in the presence (+) or absence (-) of 1 μM ionomycin plus 1 mM CaCl\textsubscript{2}, homogenized and separated into particulate P100 (P) and soluble S100 (S) fractions by high speed centrifugation. Nedd4 proteins were then immunoprecipitated from each fraction with polyclonal anti-Nedd4 antibodies (anti-Nedd4-WW2), separated on 10% SDS-PAGE and immunoblotted with Nedd4-WW2 antibodies (left panel). A control depicting endogenous expression of Nedd4 (~115 kDa) in MDCK cells (MDCK lysate) is also included (left panel). MDCK cells treated with ionomycin alone exhibited the same Nedd4 distribution profile as the untreated cells (right panel).
Fraction: P S P S MDCK lysate P S
Ca^{2+}: + + - - ionomycin alone

Nedd4
antibodies, as detailed in the Experimental Procedures section. The cells were then viewed by confocal microscopy, using a LSM-410 Zeiss laser scanning microscope. Figure 2-2 (A,B) depicts two series of horizontal (XY) sections of MDCK cells stained with affinity pure anti-Nedd4 antibodies, taken at 0.5 mm intervals starting from the apical (top) surface, and representing either untreated control cells (Figure 2-2A), or Ca^{2+} plus ionomycin - treated cells (Figure 2-2B). As evident from Figure 2-2B, following Ca^{2+} plus ionomycin treatment, the majority of Nedd4 was localized to the plasma membrane, preferentially accumulating at the apical and lateral membranes; only baseline levels of Nedd4 were detected in the membrane fraction of untreated cells (Figure 2-2A). Accordingly, vertical (XZ) reconstructions (summations) of all the images depicted in Figures 2-2A,B indeed demonstrated strong staining for Nedd4 at the apical (top) region of the treated cells (Figure 2-2C; +Ca^{2+}), while the distribution of Nedd4 in the untreated cells was relatively even throughout the cell (Figure 2-2C; -Ca^{2+}). A time-course of Nedd4 association with the apical/subapical membrane revealed the interaction persisted for approximately 30-45 min (Figure 2-3). These results, therefore, support the above observation of Ca^{2+}-induced association of Nedd4 with membranes. Moreover, they demonstrate that in polarized epithelia such as MDCK cells, endogenously expressed Nedd4 is preferentially redistributed to the apical and lateral regions of the cell in response to elevated intracellular levels of Ca^{2+}.

**The Nedd4-C2 domain is sufficient to mediate Ca^{2+}-dependent membrane association in vitro**

In order to determine whether the Ca^{2+}-dependent membrane association of Nedd4 was due to the action of the C2 domain alone and not to other regions of the protein, a GST-fusion protein encompassing the C2 domain was constructed and its association with purified membranes in response to Ca^{2+} was determined. Thus, 200 µg of MDCK crude membrane fractions (P100) were incubated with soluble GST-Nedd4-C2 or GST alone (10 nM each) in the presence of increasing concentrations of Ca^{2+} (+ 1 mM EGTA). Membranes were then collected, proteins separated on SDS-PAGE and immunoblotted with anti GST antibodies, to determine the amount...
Figure 2-2. Confoal micrographs of Ca^{2+}-dependent apical and lateral membrane localization of Nedd4 in polarized MDCK cells.

MDCK cells were grown to confluency on permeable filters to allow them to become polarized. They were then either not treated (-Ca^{2+}) or treated (+Ca^{2+}) with Ca^{2+} plus ionomycin, as described in Figure 2. Cells were subsequently fixed with 10% formalin and immunostained with affinity pure anti-rabbit Nedd4 primary antibodies followed by goat anti-rabbit Texas-Red conjugated secondary antibodies. Serial cross sections (XY) of polarized MDCK cells either Ca^{2+} untreated (A) or treated (B) were taken from the top of the monolayer (the apical surface) down to the glass slide (basal surface) at 0.5 μm intervals. These XY images were then summed to generate vertical (XZ) image reconstructions (C), depicting Nedd4 vertical distribution in Ca^{2+}-treated (+Ca^{2+}) and untreated (-Ca^{2+}) polarized MDCK cells.
Figure 2-3. *Time course of Ca\(^{2+}\)-dependent apical and lateral membrane association of Nedd4 in polarized MDCK cells.*

MDCK cells were grown and treated as described in Figure 2-2 except cells were fixed with 10% formalin at certain time points (labeled on figure) following treatment with Ca\(^{2+}\) plus ionomycin. Cells were then immunostained for Nedd4 as described above. These XY sections were all taken at an interval of 3.0 μm below the apical surface.
of membrane-associated GST-Nedd4-C2. Our results show (Figure 2-4) a basal association between the P100 fraction and the GST-Nedd4-C2 in the range of 0-300 nM Ca\(^{2+}\), consistent with our initial observation of limited membrane association of Nedd4 even in the absence of elevated intracellular Ca\(^{2+}\) levels (see Figure 2-1). However, there was a clear and reproducible increase in membrane-associated GST-Nedd4-C2 when Ca\(^{2+}\) concentrations were raised from 300 to 500 nM, with no further increase in binding at concentrations up to 1000 nM (Figure 2-4). GST alone did not bind to MDCK membranes. These results, therefore, demonstrate that the C2 domain of Nedd4 alone was sufficient to bind MDCK membranes in vitro, and that this binding was Ca\(^{2+}\) dependent at Ca\(^{2+}\) concentrations > 300 nM.

In several Ca\(^{2+}\)-responsive proteins, the C2 domain displays specificity towards negatively charged phospholipids (Daveltov and Sudhof, 1993; Chapman and Jahn, 1994; Gawler et al., 1995). To determine whether the C2 domain of Nedd4 binds phospholipids and whether it shows preference towards negatively charged ones, binding experiments with purified GST-Nedd4-C2 and purified liposomes of varying composition were performed. Either phosphatidylserine (PS), phosphatidylinositol (PI) stabilized with phosphatidylcholine (PC), or pure PC, were prepared as described in the Experimental Procedures section. They were then incubated with soluble GST-Nedd4-C2 or GST alone (10 nM each) in the absence or presence of 1 mM Ca\(^{2+}\). Liposomes were subsequently sedimented and associated GST-Nedd4-C2 (or GST alone) analyzed by immunoblotting with anti-GST antibodies, as described above. Figure 2-5 shows that GST-Nedd4-C2, but not GST alone, was able to bind to pure phospholipid liposomes. This association was partially (~50%) augmented in the presence of Ca\(^{2+}\), but did not display obvious specificity for any lipid type, as no significant difference between the association of the GST-Nedd4-C2 with pure PC, pure PS or a 1:1 mixture of PC:PI was detected. Thus, the C2 domain of Nedd4 alone is capable of binding in a Ca\(^{2+}\)-responsive manner to either MDCK membranes or to pure phospholipids.
Figure 2-4. Ca\(^{2+}\)-dependent binding of GST-Nedd4-C2 to MDCK crude membranes.

200 μg of crude MDCK membranes were incubated with GST alone or with GST-Nedd4-C2 (10 nM each) in the presence of the indicated concentrations of Ca\(^{2+}\) for 30 min at 30°C. The membranes were collected, proteins separated on 10% SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-GST antibodies. Arrows indicate the GST-C2 fusion protein.
Figure 2-5. Ca$^{2+}$-dependent association of GST-Nedd4-C2 with phospholipids.

Liposomes of the indicated composition were incubated with 10 nM purified GST or GST-Nedd4-C2 in the presence of 1 mM Ca$^{2+}$. Liposomes were then sedimented and the associated protein separated on 10% SDS-PAGE and immunoblotted with anti-GST antibodies (as described in Figure 2-3 above). The lower panel represents the total amount of protein used in the binding experiments.
LIPOSOME COMPOSITION:

Ca^{2+}: + + - -

100% Phosphatidylcholine

100% Phosphatidylserine

50% Phosphatidylcholine/50% Phosphatidylinositol

TOTAL PROTEIN:

GST-C2

GST

GST GST
-C2

-C2
Deletion of the C2 domain abrogates Ca\(^{2+}\)-dependent membrane association of Nedd4 in vivo

To test whether the Nedd4-C2 domain was responsible for the observed Ca\(^{2+}\)-dependent membrane association of Nedd4 in living cells, stable MDCK cell lines expressing either T7-epitope tagged full length Nedd4 (T-Nedd4), or T7-epitope tagged C2-deleted Nedd4 (T-ΔC2-Nedd4), were generated (Figure 2-6A). Several clones of transfected MDCK were tested for protein production and the two clones expressing approximately equal amounts of protein, T-Nedd4 (2-11) and T-ΔC2-Nedd4 (2-27) (Figure 2-6B), were then tested for Ca\(^{2+}\)-dependent subcellular localization using immunostaining with anti-T7 antibodies and confocal microscopy. To ensure that the addition of the T7 epitope onto Nedd4 by itself did not affected membrane localization in response to calcium, T-Nedd4 (2-11) monolayers grown on filters were treated with Ca\(^{2+}\)/ionomycin as above, fixed, stained with anti-T7 antibodies and examined with the confocal microscope. Our results show that the T-Nedd4 heterologously expressed in MDCK cells displayed a similar Ca\(^{2+}\)-response as the endogenous Nedd4 (shown in Figure 2-2) in that it was mainly cytosolic in untreated cells, but was localized to the plasma membrane in response to Ca\(^{2+}\) plus ionomycin treatment (Figure 2-6C). We, therefore, proceeded to test membrane localization of the C2-deleted (T-ΔC2-Nedd4) protein in MDCK cells following Ca\(^{2+}\)/ionomycin treatment using fluorescence microscopy. In contrast to the Ca\(^{2+}\)-induced membrane association seen with T-Nedd4 construct (Figure 2-7, top right panel), the T-ΔC2-Nedd4 did not associate with the plasma membrane following Ca\(^{2+}\) plus ionomycin treatment (Figure 2-7, middle right panel). As before, no significant plasma membrane association of Nedd4 was observed in untreated control cells and a similar distribution was seen for T-ΔC2-Nedd4 expressing cells (Figure 2-7, top and middle left panels). Although the anti-T7 antibody displays a non-specific cross-reactive nuclear staining, this was unrelated to the expression of transfected Nedd4 because there was no cytosolic or membrane staining in untransfected MDCK cells (Figure 2-7, bottom panel). Taken together, these results indicate that the C2 domain of Nedd4 is necessary for the Ca\(^{2+}\)-induced redistribution of the protein from the cytosol to the plasma membrane.
Figure 2-6. Epitope-tagged Nedd4 in transfected MDCK displays Ca²⁺-dependent plasma membrane association.

(A) Schematic representation of the T7-epitope tagged Nedd4 (T-Nedd4) and the T7-epitope tagged C2-deleted Nedd4 (T-ΔC2-Nedd4) constructs used for stable transfection into MDCK cells. (B) Expression of T-Nedd4 (clone 2-11) and T-ΔC2-Nedd4 (clone 2-27) in MDCK cells stably transfected with the constructs depicted in panel A. Proteins from the lysate of the transfected cells were separated on 10% SDS-PAGE, transferred to nitrocellulose and immunoblotted with monoclonal anti-T7 antibodies, to determine levels of protein expression. Lysate of untransfected MDCK cells (MDCK lysate) immunoblotted with affinity-pure anti-Nedd4-WWII antibodies used as a control is depicted in the right lane. (C) Intracellular localization of T-Nedd4 in untreated (a) or Ca²⁺ plus ionomycin-treated (b) transfected MDCK cells as viewed with the confocal microscope. Cells were fixed and permeabilized as described in Figure 3 above, and immunostained with monoclonal anti-T7 antibodies followed by goat anti-mouse secondary antibodies conjugated to Texas-Red. XY images shown represent slices through the subapical region of the membrane taken 3.0 μm from the apical surface.
Figure 2-7. Lack of $\text{Ca}^{2+}$-induced membrane localization of C2-deleted Nedd4 stably expressed in MDCK cells.

Fluorescent micrographs of intracellular localization of T-Nedd4 (top panels) and T-$\Delta$C2-Nedd4 (middle panels) in untreated (left panels), in $\text{Ca}^{2+}$ plus ionomycin-treated (right panels) transfected MDCK cells, or in untransfected cells (bottom panel). Cells were fixed and permeabilized as described in Figure 3 above, and immunostained with monoclonal anti-T7 antibodies followed by goat anti-mouse secondary antibodies conjugated to Texas-Red. Staining with anti-T7 antibodies gave a background nuclear stain which was unrelated to transfection (bottom panel). Photographs represent total fluorescence as viewed with rhodamine optics on a standard fluorescent microscope. Arrows point to areas of plasma membrane localization.
IV. Discussion

In this report, we show that the ubiquitin protein ligase Nedd4 possesses a functional C2 domain: upon increases in cytosolic calcium, endogenous Nedd4 associates with membranes and, interestingly, displays polarized distribution in that it localizes to the apical and lateral membranes of polarized MDCK cells. This membrane localization is abolished in the absence of the C2 domain. Moreover, the C2 domain alone is sufficient to mediate Ca\(^{2+}\)-dependent membrane and phospholipid association.

Several mechanisms could explain the localization of Nedd4 to the apical region of MDCK polarized epithelia. The distribution of various lipid classes in the membrane regions of cultured MDCK cells has been extensively studied (Simons and van Meer 1988). These reports demonstrate that the apical membrane has a high glycosphingolipid content. This high glycosphingolipid content at the apical membrane may serve to increase the phospholipid packing density at this region, thereby providing a favorable environment for Nedd4-C2 association. Clearly, a greater understanding of the dynamics of C2/lipid interactions is necessary to fully understand the translocation mechanism. Alternatively, other factors may be involved in binding of Nedd4 to the apical site. Although in this report we show that a Nedd4-C2 fusion protein is sufficient to bind purified membrane in a Ca\(^{2+}\)-dependent manner, this does not exclude the involvement of other proteins that are either translocated to the apical membrane upon increases in cellular calcium or that are already present there and retain Nedd4 upon its Ca\(^{2+}\)-dependent (C2-mediated) arrival. Indeed, it has been shown for several proteins that their C2 domain engages in interactions that may influence their cellular targeting (see introduction) therefore, it is possible that C2-protein interactions may be involved in Nedd4 cellular translocation as well.

The Ca\(^{2+}\)-dependent polarized membrane localization of Nedd4 in epithelial cells is reminiscent of the specialized translocation of synaptotagmin to nerve terminals in response to calcium. Synaptotagmin is an essential component of the Ca\(^{2+}\) sensor for the final step in nerve terminal fusion leading to neurotransmitter release (Elferink et al., 1993; reviewed in Sudhof and
Rizo, 1997). The function of synaptotagmin in exocytosis is mediated by its association with syntaxins (Li et al., 1995), which are plasma membrane proteins that form complexes with other vesicular proteins (Sollner et al., 1993; McMahon et al., 1993). This interaction serves to localize synaptic vesicles to the nerve terminal for proper transmitter release. Although the function of the Nedd4 apical/lateral membrane translocation is not known, it is possible that its calcium responsive targeting may increase the specificity of interactions of this ubiquitin protein ligase with its substrate(s); in this model, the apically located substrate would only become ubiquitinated following an increase in intracellular levels of Ca\(^{2+}\).

The interaction between the C2 domain and its calcium ligand is thought to occur via 5 conserved aspartates that line the opening of the C2 key motif, corresponding to Asp 172, 178, 230, 232, 238 of synaptotagmin (Sutton et al., 1995; Shao et al., 1996; Essen et al., 1996). Many of the C2 domains identified thus far including that of Nedd4, however, do not possess the full complement of conserved aspartates (Ponting and Parker, 1996; Nalefski and Falke, 1996). While structural data suggests that all five aspartates are necessary for calcium binding (Sutton et al., 1995; Shao et al., 1996) the C2 domains of molecules such as rasGAP and cPLA2, which have been shown to mediate calcium-dependent membrane localization, do not possess all these conserved aspartates (Gawler et al., 1995; Clark et al., 1991); in fact, the 40 amino acid CaLB region of rasGAP is sufficient to mediate calcium-dependent phospholipid-binding (Gawler et al., 1995a; 1995b) despite the fact that it does not possess any of the conserved aspartates. In these molecules the aspartate residues are often replaced with other oxygen-containing sidechains (Asn, Thr) that may be able to co-ordinate Ca\(^{2+}\) binding (J. Falke; personal communication). Thus, the observation of C2-dependent association of Nedd4 with the plasma membrane, despite lacking one of its conserved aspartates, is consistent with findings in other C2 domains. Whether the differences in conservation of co-ordinating residues reflect the different affinities for calcium is presently unclear.

The Ca\(^{2+}\)-dependent membrane and phospholipid binding properties of Nedd4 C2 are similar to what has been reported for other C2 domains. There was a visible increase in binding of
purified GST-C2 Nedd4 to cellular membranes between 300 and 500 nM Ca\(^{2+}\). This finding is consistent with similar binding studies done with rasGAP (Gawler et al., 1995b) where GST-GAP binding was exponential between 10\(^{-7}\) and 10\(^{-6}\) M free Ca\(^{2+}\). In addition, previous studies with the C2 domains of synaptotagmin and rasGAP (Davletov and Sudhof, 1993; Chapman and Jahn, 1994; Gawler et al., 1995a) show a preference of the C2 domain for negatively charged phospholipids upon Ca\(^{2+}\)-binding. Contrary to this, our results show lack of preference of Nedd4-C2 domain toward negatively charged phospholipids. This finding, however, is in agreement with that of the cPLA2-C2 domain, which was also shown not to have such preference (Nalefski et al., 1994); in fact, that domain shows approximately ten-fold higher affinity for zwitterionic (PC) phospholipids than anionic phospholipids (J. Falke, personal communication).

Our lab has recently demonstrated association of Nedd4 with ENaC (Staub et al., 1996), and that ENaC is ubiquitinated in vivo (Staub et al., 1997). Although we do not know yet whether Nedd4 is directly involved in ubiquitinating ENaC, this is a likely possibility, as it is capable of suppressing its function by controlling its stability at the cell surface (Goulet et al., 1998; Abriel et al., 1999). ENaC is located at the apical membrane of epithelial cells such as those in the distal nephron, distal colon and lung epithelia, and one possible scenario is that elevation of intracellular Ca\(^{2+}\) may target Nedd4 to the apical membrane, where ENaC is located. This would then allow for the Nedd4-WW domains to associate with the channel and for the Nedd4-HECT domain to ubiquitinate it. This model therefore predicts that elevation of intracellular Ca\(^{2+}\) would cause inhibition of ENaC activity resulting from enhanced channel ubiquitination and degradation. Indeed, earlier studies have documented inhibition of amiloride-sensitive Na\(^{+}\) channel activity by elevated intracellular Ca\(^{2+}\) levels (Palmer and Frindt, 1987). Moreover, studies from our own lab with ENaC expressed in MDCK cells revealed a biphasic inhibition of ENaC activity following dialysis with 1 \(\mu\)M free Ca\(^{2+}\): an initial rapid (within 5 min) inhibition likely caused by changes in channel gating, followed by a secondary slow inhibition (5-20 min) which we suspect may originate from a decrease in channel numbers at the cell surface (Ishikawa et al., 1998). We speculate that this slow phase may involve Nedd4 activity. This model does not preclude the

124
possibility that the Nedd4-C2 domain is also involved in ENaC endocytosis/degradation, perhaps in an analogy to the second C2 domain of synaptotagmin, which mediates interactions with the clathrin-associated protein AP2 (Zhang et al., 1994).

In summary, the presence of a functional C2 domain in Nedd4 may help in determining the array of substrates (ENaC and others) which are targeted for ubiquitination by this ubiquitin protein ligase, by directing subcellular distribution of Nedd4 to the location of these proteins in a Ca\(^{2+}\)-dependent fashion. Specificity of binding to these putative target protein(s) may be then mediated by the WW domains, and subsequent ubiquitination by the HECT domain.
Chapter Three:

Apical Membrane Targeting of Nedd4 is Mediated by an Association of its C2 Domain with Annexin XIIIb

* This work is submitted for publication in J. Cell Biology:


My contribution to this work was to identify the interaction between Nedd4 and annexin XIII and to biochemically characterize the interaction. I also performed preliminary experiments using confocal microscopy which showed co-localization of the two proteins in cells and provided the intellectual drive behind the experimentation. Frank Lafont (in the lab of Kai Simons) was acknowledged for performing the confocal microscopy of the annexin XIIIb inducible MDCK cell lines (made by Sandra Lecat) as well as the raft isolation experiments. Paul Verdake was acknowledged for his work on the immunogold labelling experiments.
Chapter Three: Apical Membrane Targeting of Nedd4 is Mediated by an Association of its C2 Domain with Annexin XIIIb

I. Summary
Nedd4 is a ubiquitin protein ligase composed of a C2 domain, 3 or 4 WW domains and an E3 HECT domain. We have previously shown a Ca\textsuperscript{2+}-dependent plasma membrane localization of Nedd4 mediated by the Nedd4-C2 domain, with the majority of the protein targeted to the apical membrane of polarized MDCK cells. To study the apical membrane preference of Nedd4, we searched for Nedd4-C2 domain-interacting proteins that might be involved in targeting it to the apical surface. Using immobilized Nedd4-C2 domain to capture interacting proteins from MDCK cell lysate in the presence of Ca\textsuperscript{2+}, we isolated a 35-40 kDa protein from a silver stained SDS-PAGE, which was then identified by MALDI-TOF mass spectrometry as annexin XIII. Annexin XIII is involved in apical membrane targeting by virtue of its association with apically destined membrane rafts. It has two known isoforms, a and b, that are apically localized, although XIIIa is also found in the basolateral membrane. To confirm these results, \textit{in vitro} binding experiments between immobilized GST-Nedd4-C2 and either endogenous annexin XIII, transfected epitope tagged annexin XIII, or Ni\textsuperscript{2+}-purified His-tagged annexin XIII were performed. All experiments showed that Nedd4-C2 domain interacted with both annexin XIIIa and b in presence of Ca\textsuperscript{2+} and experiments with purified annexin XIII showed that this interaction was direct. Co-precipitation experiments showed that the interaction between the two proteins was taking place in cells in response to elevations in intracellular Ca\textsuperscript{2+}. Immunofluorescence and immuno-gold electron microscopy revealed that Nedd4 and annexin XIIIb co-localized in apical membrane rafts, as well as at the trans-Golgi, apical membrane microvilli and endosomes. Moreover, we demonstrated that Nedd4 associated with raft lipid microdomains in a Ca\textsuperscript{2+}-dependent manner, as determined by detergent extraction and flotation assays. These results suggest that the apical membrane localization of Nedd4 was mediated by an association of its C2 domain with the apically-targeted annexin XIIIb.
II. Introduction

Nedd4 is a ubiquitin protein ligase comprised of a C2 domain, 3 or 4 WW domains and a ubiquitin protein ligase (E3) HECT domain (Kumar et al., 1992). The C2 domain is a protein-lipid, protein-protein interaction module originally identified as the second of four conserved regions of Ca"-responsive isoforms of protein kinase C (PKCaβγ) (Coussens et al., 1986; Knopf et al., 1986) and later found in other molecules such as cytoplasmic phospholipase A₂ (cPLA₂), synaptotagmin, p120 ras GTPase activating protein (rasGAP), and phosphoinositide specific phospholipase C (PLC-βγδ) (reviewed in Ponting and Parker 1996; Nalefski and Falke 1996). The list of ligands to which the C2 domain is capable of binding extends from Ca", phospholipids, inositol polyphosphates to intracellular proteins (Ponting and Parker 1996; Nalefski and Falke 1996; Rizo and Sudhof 1998).

We have recently demonstrated a Ca"-dependent association of the C2 domain of Nedd4 with membranes and phospholipids, and that this domain is necessary for translocating Nedd4 to the plasma membrane, particularly the apical region of polarized MDCK epithelial cells, in response to elevation of intracellular Ca" (Plant et al., 1997). However, this Ca"-dependent apical membrane preference of the Nedd4-C2 domain was puzzling, as it was not clear how the C2 domain could distinguish between the apical vs. basolateral membranes, especially as the inner leaflet of these membranes in MDCK cells is homogeneous with respect to its lipid composition (van Meer and Simons 1986). Moreover, our work has shown a lack of preference of the Nedd4-C2 domain towards charged phospholipids (Plant et al., 1997), clearly dissimilar to several other C2 domains (Gawler et al., 1995; Davelov and Sudhof, 1993; Chapman and Jahn, 1994; Yamaguchi et al., 1993). Thus, the purpose of the current study was to investigate how the Nedd4-C2 domain is preferentially mobilized to the apical region of polarized MDCK cells, and to determine whether a C2 domain-interacting protein(s) may facilitate this Ca"-dependent apical targeting.

Using pull down experiments with immobilized GST-Nedd4-C2 domain and MDCK II cell lysate followed by mass spectrometry (MALDI-TOF) analysis, we have identified annexin XIII as a binding partner of the Nedd4-C2 domain. We further demonstrate a Ca"-dependent association of
the C2 domain of Nedd4 with annexin XIIIa and b, which are epithelial specific isoforms of the annexin family (Wice and Gordon 1992; Fiedler et al., 1995). Annexin XIIIb is associated with apical rafts (Lafont et al., 1998). Rafts are 50-70 nm diameter lipid microdomains enriched in glycosphingolipids and cholesterol (Simons and Ikonen, 1997; Brown and London, 1999), and play an important role in cholesterol metabolism, sorting mechanisms and cell signaling. They have also been implicated in the pathogenesis of several diseases (Simons and Ikonen, 1997). Of particular interest, regarding this study, is the proposed function for rafts in the delivery of proteins destined for the apical membrane of polarized MDCK cells (Simons and Ikonen, 1997). We demonstrate, using a inducible MDCK cell line expressing annexin XIIIb that, upon a rise in intracellular Ca\(^{2+}\) concentration and expression of annexin XIIIb, Nedd4 is preferentially associates with the apical surface. Moreover, we show co-localization of annexin XIIIb and Nedd4 in apical carriers and at the apical plasma membrane. We further demonstrate that Nedd4 is recruited into rafts by annexin XIIIb in presence of Ca\(^{2+}\). We propose, therefore, that the apical membrane targeting of Nedd4 may be mediated or facilitated by a Ca\(^{2+}\)-dependent association of its C2 domain with annexin XIIIb.

II. Experimental Procedures

Constructs

Bacterially-expressed GST fusion proteins: GST fusion proteins of the rat Nedd4-C2 domain were prepared by PCR amplification of the region of rNedd4 cDNA (amino acid residues 77-219, Staub et al., 1996) corresponding to the boundaries defined in Nalefski and Falke (1995) for a type II topology C2 domain. PCR products were subcloned with flanking Bam HI and Eco RI sites into the corresponding sites in pGEX-2TK (Pharmacia Biotech Inc.). The plasmid containing the C2 insert, verified by sequencing, was used to transform the HB101 strain of Escherichia coli. Fusion protein production was as previously described (Plant et al., 1997).
**Bacterially-expressed Histidine-tagged constructs:** HA-tagged annexin XIIa and b were generated by introducing the HA tag sequence YPYDVPDYAG at the C termini of annexin XIIa or b (at amino acid residues 316 and 357, respectively, (Fiedler et al., 1995)) by PCR. Full-length HA-tagged annexin XIIa and b were subcloned with flanking NdeI and XhoI sites into the pET-30b(+) bacterial expression vector, in frame with a C-terminal 6x His tag (Novagen). The plasmid sequences were verified by sequencing and were used to transform the HB101 strain of *Escherichia coli* and proteins were produced and purified as described previously (Kanelis et al., 1998). His-HA-tagged truncated annexin XIIa/b was created by PCR of HA-annexin XIIa with primers encompassing the endonexin folds region defined previously (amino acids 6-316 of annexin XIIa; Fiedler et al., 1995) and subsequent subcloning of the PCR product into the pET-30b(+) bacterial expression vector as described above.

**Mammalian expression vectors:** Full-length HA-tagged annexin XIIa and b were subcloned into the pRc-CMV vector (Invitrogen Corp., San Diego, CA). The Nedd4-C2 domain (amino acid residues 77-219, Staub et al., 1996) was subcloned into pEBG mammalian expression vector in frame with GST, as previously described (Wallace et al., 1998). Myc-tagged annexin XIIb was generated with the myc tag introduced at the C terminus of annexin XIIb and the construct was expressed in a lac switchable MDCK II cell line, according to Lecat et al. (in preparation).

**Identification of interacting proteins with MALDI-TOF mass spectrometry.**

Epithelial MDCK II cells were grown to confluency on 100 mm diameter coated plates in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37°C, 5% CO₂ atmosphere. Cells were then lysed with lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 1% Triton X-100) with a protease inhibitor cocktail containing 1 mM phenylmethylsulfonfylfluoride (PMSF) and 10 μg/ml of each leupeptin, aprotinin and pepstatin. The lysate was spun at 14,000xg (5 min, 4°C) to remove mitochondria and nuclei. The supernatant was incubated with purified GST-C2 fusion
protein or GST alone immobilized on glutathione agarose beads in the presence or absence of Ca\(^{2+}\) (1 \(\mu\)M final concentration), 10 mM EGTA and 1 mM MgCl\(_2\) at 4°C for 1 hr. The beads were sedimented at 10,000xg for 15 sec and were washed five times with HNTG (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100 and 10% glycerol). Proteins were separated on a 10% SDS-PAGE and following electrophoresis the gel was silver stained using a modified protocol from Current Protocols in Protein Science (John Wiley and Sons Inc.). The gel was fixed in 50% MeOH and 10% acetic acid for 20 min, rinsed in 20% ethanol followed by water for 10 min each. It was then reduced by incubation in sodium thiosulphate (0.2 g/L) for 1 min, rinsed twice with water and incubated in a silver nitrate solution (2.0 g/L) for 30 min. The gel was then rinsed with water, developed by several incubations in developing solution (30 g/L sodium carbonate, 1.4 ml of formaldehyde solution (37% solution/L) and 10 mg/L sodium thiosulphate) and then stopped by incubation in a 1% acetic acid solution for at least 20 min. Bands present only in the +Ca\(^{2+}\) pulldown with the GST-Nedd4-C2 domain were excised and the proteins were trypsin-digested in the gel and extracted using a protocol described previously (Shevchenko et al., 1996). Extracted peptides were purified using a micro reverse phase cartridge (Michrom BioResources, Auburn, CA.) and were identified using MALDI-TOF mass spectrometry with the following parameters: linear mode, 92% grid voltage, 0.150% guide wire voltage, delayed extraction 200 ns, low mass gate 800 Da and laser intensity 1650. Samples were loaded in a matrix solution containing 20 mg/ml a-cyano-4-hydroxy-trans cinnamic acid (SIGMA) in 50% acetone/50% isopropanol. Masses obtained using MALDI-TOF were analyzed using the ProFound database (http://prowl.rockefeller.edu/cgi-bin/ProFound). The masses used for the search derived from highly resolved peaks in the 900 to 3000 Da range and on average 10-15 masses were used in the search.

**In vitro binding experiments**

Pulldown experiments, as described above, were repeated by incubating GST-Nedd4-C2 or GST alone with lysate from MDCK II cells or from 293T cells (grown in the conditions described for
MDCK II cells) transfected with HA-annexin XIIIa or b. 293T cells were transiently transfected using the Ca\textsubscript{2}PO\textsubscript{4} method (described in Chen and Okayama 1987), harvested and lysed as described above for MDCK II cells. Following electrophoresis on 10% SDS-PAGE, the proteins were transferred onto nitrocellulose and blotted with anti-annexin XIIIb antibodies (described previously; Fiedler et al., 1995) to detect endogenous annexin XIIIb, or with anti-HA antibodies to detect transfected HA-annexin XIIIa/b, followed by anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies (Boehringer Mannheim Canada) and ECL detection (Amersham Corp.). Ca\textsuperscript{2+}-dependent pulldown experiments were done in an identical fashion as described above but for Ca\textsuperscript{2+}-free (-Ca\textsuperscript{2+}) conditions, only 10 mM EGTA and 1 mM MgCl\textsubscript{2} were added to the lysate. Binding experiments involving purified His-HA-annexin XIIIa/b or His-HA truncated annexin XIII and GST-C2 or GST alone were done as described above, in the presence of 1 µM (final concentration) Ca\textsuperscript{2+} with 10 mM EGTA and 1 mM MgCl\textsubscript{2}. GST and GST-C2 on beads were collected by centrifugation and following 5 washes with HNTG, proteins were eluted from the beads and separated by electrophoresis on 10% SDS-PAGE. The proteins were then transferred onto nitrocellulose and blotted with anti-HA antibodies to detect bound annexin XIII proteins.

Co-precipitation experiments
The GST-Nedd4-C2 domain (expressed in the mammalian expression vector pEGB) was transiently co-transfected into 293T cells alone or together with full-length HA-tagged annexin XIIIa or b. Prior to lysis, the cells were either treated or not with Ca\textsuperscript{2+}-medium (140 mM NaCl, 6 mM KCl, 1.1 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 0.1 mM EGTA 20 mM glucose, 20 mM HEPES) together with 1 µM ionomycin to increase intracellular Ca\textsuperscript{2+}. The application of ionomycin in the presence of Ca\textsuperscript{2+} has been shown to induce increases in cytosolic [Ca\textsuperscript{2+}] in MDCK cells (Lang et al., 1990; Delles et al., 1995). Cells were then lysed in lysis buffer plus protease inhibitors (as above), spun at 14,000 xg for 5 min and the supernatant was incubated at 4°C for approximately 30 min with glutathione agarose beads to capture the transfected GST-Nedd4-C2 domain. The beads were then
washed five times with HNTG, proteins separated on 10% SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-GST antibodies to detect Nedd4-C2, or with anti-HA antibodies to detect tagged annexin XIIIb or b.

**Immunofluorescence staining and confocal analysis**

Annexin XIIIb tagged on the C-terminus with the myc epitope was overexpressed in Lac repressor-producing MDCK(lac) cells for the use of the Lac switchable system (McCarthy et al., 1996; Lecat et al., in preparation). Polarized annexin XIIIb-myc MDCK(lac) (clone 55, Lecat et al., in preparation) grown on polycarbonate filters (0.45 μm pore size, Costar, Cambridge, USA) were induced overnight with 1 mM SodiumButyrate and 5 mM IPTG. For Ca²⁺-treatment, cells were washed twice with Ca²⁺-free media (140 mM NaCl, 6 mM KCl, 1 mM MgCl₂, 0.1 mM EGTA, 20 mM glucose, 20 mM HEPES) and then incubated in media with or without Ca²⁺ (described above) and 1 μM ionomycin for 5 min at 37°C. Filters were then washed and fixed in methanol at -20°C for 6 min, and washed with phosphate-buffered saline. The filters were cut from the inserts and the cells incubated in 0.2% fish skin gelatin (FSG, Sigma, Deisenhofen, Germany) in PBS for 30 min followed by incubation overnight at 4°C with the primary antibodies diluted in PBS with 0.2% FSG (10 μg/ml of either affinity pure anti-Nedd4 or anti-Anx XIIIb antibodies). DNA was stained with propidium iodide (0.05 μg/ml) for 5 min in PBS as described (Lafont et al., 1994). Filters were then washed four times with PBS followed by incubation with goat anti-rabbit fluorescein conjugated antibodies (Dianova, Hamburg, Germany) in PBS with 0.2% FSG for 1 hr at 37°C. This was followed by four washes in PBS. Cells were placed in mounting medium in PBS-glycerol (Merck, Darmstadt, Germany) 1:1 with 0.1% NaN₃ and 100 mg/ml DABCO [1,4-diazabicyclo-2.2.2-octane]. Coverslips were positioned on thin bridges cut from cellophane and sealed with nail polish. The fixed and stained cells were viewed using a Leica NTS confocal.
**Detergent extraction, cyclodextrin treatment and flotation assays**

MDCK(lac) cells expressing the annexin XIIIb-myc construct were used. After induction, as detailed above, cells were scraped and processed with Triton X-100 as described (Lafont et al., 1998). Methyl-β-cyclodextrin 10 mM was added directly onto living cells for 1 hr at 37°C. Buffers used were: TNE buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EGTA) and TNCa buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM CaCl2). In both cases, the buffers were supplemented with DTT (5 mM) and a cocktail of protease inhibitors (CLAP: chymostatin, leupeptin, antipain, pepstatin A; final concentration 10 μg/ml each). Samples were placed in 40% OptiPrep and overlaid with 25% and 0% OptiPrep either in TNE or TNCa. Samples were centrifuged 4 hrs at 40,000 rpm in a SW 60 rotor (Beckman) at 4°C. Fractions were collected from the top and protein were methanol/chloroform precipitated before being analysed by SDS-PAGE and Western blotting with anti-Nedd4 antibodies (Staub et al., 1996), anti-annexin XIIIb antibodies (Fiedler et al., 1995), and anti-caveolin-1 antibody (Santa Cruz, Santa Cruz, CA.). For quantitation filters were incubated with 35S labelled secondary antibodies (Amersham) before being air dried. Each band density was measured using phosphoimager (Fuji, Tokyo, Japan) and analyzed with the AIDA 2D software (Raytest, Straubenhardt, Germany).

**Electron Microscopy**

Double or triple immuno-gold labeling, on ultrathin cryo-sections, was carried out as detailed in Lafont et al. (1998) using polarized MDCK cells either expressing or not expressing the VSV G-epitope tagged sialyltransferase (Scheiffele et al., 1998). TGN-derived carriers vesicles were isolated from influenza infected MDCK cells. Cells were perforated by mechanically ripping off the apical plasma membrane as previously described (Lafont et al., 1998). The buffer used to isolate apical exocytic vesicles contained 500 nM free Ca2+. Protein A-coupled gold particles were purchased from the Department of Cell Biology, Faculty of Medicine, Utrecht, Netherlands. Samples were examined under a Zeiss transmission 10 C electron microscope (Zeiss, Germany).
III. Results

**Identification of annexin XIII as a binding partner for Nedd4-C2 domain**

In order to identify proteins that may be involved in C2-mediated targeting of Nedd4 to the apical region of polarized cells, we performed pulldown experiments with a fusion protein of the Nedd4-C2 domain. Lysates from MDCK II cells were prepared as described in Experimental Procedures section, and incubated in the presence or absence of 1 μM Ca\(^{2+}\) with immobilized GST-Nedd4-C2 domain. After separation of bound proteins by SDS-PAGE, silver-staining of the gel revealed the presence of several unique bands precipitating with the C2 domain fusion protein in the presence of Ca\(^{2+}\). The most prominent of these bands, migrating at ~35-40 kDa (Fig. 3-1A), was excised from the gel, purified and analyzed by MALDI-TOF mass spectrometry. Using the ProFound program (http://prowl.rockefeller.edu/cgi-bin/ProFound), this band was identified as annexin XIII/XIIIa (human intestine-specific annexin) (Fig.3-1B,C) originally described by Wice and Gordon (1992). The canine annexin XIIIa homologue, and a 40 kDa spliced variant which contains a 41 amino acid insert at its N terminus, annexin XIIIb, were subsequently cloned by Fiedler et al., (1995) and shown to be expressed in dog intestine and kidney epithelial (MDCK II) cells. Annexin XIIIb is 90% identical and 96% similar to human annexin XIIIa (Fiedler et al., 1995), and was found to be involved in vesicle trafficking to the apical plasma membrane in polarized MDCK cells. The annexin XIII subfamily of the annexins is the only one known to be myristoylated (Moss 1997) (Fig. 3-1C) and hence constitutively associated with membranes. Myristoylated annexin XIIIb (but not an unmyristoylated form) was shown to associate with and function in the formation of apical carrier vesicles from the trans-golgi network (TGN) that serve in apical delivery (Lafont et al., 1998).
Figure 3-1. Annexin XIIIb is identified as an interacting protein of Nedd4-C2 domain. (A) Proteins, from a pulldown of MDCK lysate with GST-C2, were separated by SDS-PAGE followed by silver-staining of the gel which revealed the presence of a prominent band migrating at \(-35-40\) kDa (marked *), which precipitated with the C2 domain fusion protein in the presence of \(\text{Ca}^{2+}\). This band was excised from the gel, purified and analyzed by MALDI-TOF mass spectrometry. Using the ProFound program, this band was identified as annexin XIII/XIIIa (human intestine-specific annexin). (B) The sequence of the canine isoform of annexin XIII, annexin XIIIb, was put into the PAWS program to calculate the masses of tryptic fragments from a theoretical cleavage (amino acids corresponding to tryptic fragments in brackets), and these masses were compared to the masses from MALDI-TOF analysis. 12 out of the 15 masses obtained through the MALDI-TOF analysis corresponded to the theoretical values for annexin XIIIb cleavage and represented 45% of the proteins fragments (peptides in bold print). As only fragments \(>900\) Da were used in the search, a comparison with the masses from a theoretical trypsin cleavage of annexin XIIIb \(>900\) Da showed that the masses obtained through MALDI-TOF analysis covered 76% of the protein sequence. (C) Domain structure of annexin XIII. Annexin XIII exists as two isoforms annexin XIIIa and b. The annexin XIII subfamily of the annexins is the only one known to be myristoylated (Moss 1997) and hence constitutively associated with membranes. Annexin XIIIb contains a 41 amino acid insert as a result of alternative splicing. Both isoforms possess the hallmark of annexin family members; four \(\text{Ca}^{2+}\)-binding endonexin repeats at the C-terminus.
B.

PEPTIDE MASSES IDENTIFIED BY MALDI-TOF MASS SPECTROMETRY AND CORRESPONDING AMINOACIDS OF ANNEXIN XIII (trypsin cleavage):

<table>
<thead>
<tr>
<th>Mass</th>
<th>Peptide Fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1775.21 (5-20)</td>
<td>1703.0 (142-157) 1755.78 (301-316)</td>
</tr>
<tr>
<td>2783.9 (21-48)</td>
<td>1198.5 (191-201) 983.80 (342-350)</td>
</tr>
<tr>
<td>1365.4 (51-62)</td>
<td>982.04 (218-226)</td>
</tr>
<tr>
<td>1662.2 (70-85)</td>
<td>1591.9 (227-240)</td>
</tr>
<tr>
<td>1691.9 (120-134)</td>
<td>1748.2 (260-275)</td>
</tr>
</tbody>
</table>

ANNEXIN 13B SEQUENCE COVERAGE

MGNR HSQSYLSQGQOLPK DIOPSAAVQPLSHPSGSGEPEAOQP AKA SHHGFVDHDAK KLNKACK GMGTDEAAITEILSSR TSDERQQIKQKYKATYGKDLEEVFKSDLGNGFEK TALALDRPASYDARQLQKAMKGLGTDEAVLIEILCTRTTNKEIMAIKEAYQRLFDRS LESDVKADTSGNLK AILVSLLOANR DEGDDVDKDLAGQDAK DLYDAGDGR WGTDELAFNVLAK RSHKQLRATFQAYQILIDK DICEAIEAETSQDLOK AYLTLVRCAR DOEYFADR LYKSMK GTGTDEETLIHHIVTR AEVDLQGIKAKFQEKYQKSLSDMR SDTSGDFQK LLVALLH

TOTAL PERCENTAGE COVERAGE: 45%
PERCENT COVERAGE OF PEPTIDE FRAGMENTS > 900 Da: 76%
C.

Annexin XIII

13a

myristol

13b

40 a.a.
unique region

Ca Ca Ca

endonexin repeats
**Annexin XIIIa/b and Nedd4-C2 domain interact in vitro and in living cells**

To verify the interaction of annexin XIII with the Nedd4-C2 domain, we repeated the pulldown experiments (used for the initial identification of annexin XIII) by incubating immobilized GST-Nedd4-C2 fusion protein with lysate from MDCK II cells expressing endogenous annexin XIII. We also performed the same experiment with lysate from 293T cells transfected with HA-tagged annexin XIIIa or b, in the presence of Ca^{2+}. Following precipitation, SDS-PAGE and transfer to nitrocellulose, the proteins were detected by immunoblotting with either anti-annexin XIIIb antibodies to detect endogenous annexin XIIIb, or with anti-HA antibodies to detect transfected annexin XIIIa and b. Our results show that immobilized GST-Nedd4-C2 domain (but not GST alone) was able to precipitate annexin XIIIb from MDCK II cells as well as annexin XIIIa and b (35 and 40 kDa bands, respectively) from transfected 293T cells (Fig. 3-2A), suggesting the Nedd4-C2 domain can bind annexin XIII. These interactions were dependent on the presence of Ca^{2+}, as chelating the free Ca^{2+} in the binding buffer with EGTA (-Ca^{2+}), abrogated binding of annexin XIIIa or b to the Nedd4-C2 domain (Fig. 3-2B).

To determine if the interaction between annexin XIII and the Nedd4-C2 domain could take place in cells, a mammalian GST-Nedd4-C2 expression construct was transfected into 293T cells together with HA-annexin XIIIa or b. Before harvesting, the cells were either treated or not with the Ca^{2+} ionophore ionomycin, plus Ca^{2+}, to increase cytosolic Ca^{2+} concentrations. Cells were then lysed and the C2 domain was precipitated from cellular lysate by incubation with glutathione agarose beads. As shown in Figure 3-3, annexin XIIIa and b co-precipitated with the Nedd4-C2 domain only when the cells had been Ca^{2+}-treated. The annexin XIIIa and b constructs expressed alone did not precipitate with the beads (Fig. 3-3). Collectively, these results show that the C2 domain of Nedd4 binds annexin XIIIa and b in a Ca^{2+}-dependent manner both in vitro and in living cells.

To test whether the interaction between the C2 domain and the annexin isoforms was direct, and not requiring another cellular component, purified GST-C2 or GST alone was
Figure 3-2. Ca$^{2+}$-dependent co-precipitation of Nedd4-C2 domain and annexin XIIIa and b in vitro. (A) The C2 domain of Nedd4, expressed as a GST fusion protein (GST-C2), or GST alone, was incubated with lysate from MDCK II cells (left; MDCK cells) or with lysate from 293T cells transiently transfected with HA-tagged annexin XIIIa or b (right; transfected (Tx) 293T cells, 13a or 13b) in the presence of Ca$^{2+}$. GST or GST-C2 were precipitated by incubation with glutathione agarose beads and co-precipitated proteins were separated on 10% SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-annexin XIIIb (13b) antibodies (left) to detect endogenous annexin XIIIb, or with anti-HA antibodies (right) to detect transfected HA-tagged annexin XIIIa and b. Aliquots of the lysate were also analyzed for levels of expression of either endogenous annexin XIIIb or transfected annexin XIIIa and b (13a, 13b) together with untransfected lysate (-). Total GST or GST-C2 used in the pulldown experiments are shown (Ponceau S). (B) Identical experiments to those described in (A) were performed with 293T cells transiently transfected with HA-annexin XIIIa and b (13a, 13b) except in the presence (+) or absence (-) of Ca$^{2+}$. Western blots were performed as described for (A) using an anti-HA antibody.
A

MDCK cells

Transfected 293T cells

**Pulldown**

<table>
<thead>
<tr>
<th>GST-C2</th>
<th>GST</th>
<th>lysate</th>
<th>GST-C2</th>
<th>GST</th>
</tr>
</thead>
</table>

**Ponceau-S**

<table>
<thead>
<tr>
<th>Anx 13b</th>
</tr>
</thead>
</table>

**Blot: anti-annexin 13b**

**Pulldown**

<table>
<thead>
<tr>
<th>GST-C2</th>
<th>GST</th>
<th>lysate</th>
</tr>
</thead>
</table>

| 13a | 13b | -- |

| 13a | 13b | -- |

| 13a | 13b | -- |

**Blot: anti-HA**

<table>
<thead>
<tr>
<th>HA-Anx</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>13b</th>
</tr>
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<table>
<thead>
<tr>
<th>13a</th>
</tr>
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</table>
**Figure 3.3. Ca$^{2+}$-dependent co-precipitation of Nedd4-C2 domain and annexin XIIIa and b from mammalian cells.** The Nedd4-C2 domain (C2) expressed as a GST fusion protein (in the mammalian expression vector pEBG) was transiently cotransfected with HA-tagged annexin XIIIa or b (13a, 13b) in pRC-CMV into 293T cells. Transfected cells were either treated (+Ca$^{2+}$) or not (-Ca$^{2+}$) with Ca$^{2+}$/ionomycin to raise intracellular Ca$^{2+}$ levels. Cells were then lysed and the lysate was incubated with glutathione agarose beads to precipitate GST-Nedd4-C2 and associated proteins. Proteins were then separated on 10% SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-HA antibodies to detect co-precipitated HA-tagged annexin XIIIa and b, or with anti-GST antibodies to detect GST-Nedd4-C2. Precipitation and immunoblotting of annexin XIIIa (13a) or annexin XIIIb (13b), or GST-C2 expressed alone (GST-C2), used as controls, are shown as well. Bottom panels show aliquots of the lysate that were analyzed for protein expression of either the HA-tagged annexin XIIIa (bottom left) or GST-Nedd4-C2 (bottom right). Tx, transfected; untx, untransfected.
incubated with purified His-HA-tagged annexin XIIIa or b (described above) in the presence of Ca\textsuperscript{2+}. As shown in Fig. 3-4A, the interaction proved to be a direct one, as the full-length annexins bound to the GST-C2 on beads but not to the GST alone. To narrow down the region of annexin XIIIa/b that was mediating the binding to Nedd4-C2, a His-HA-tagged truncated version of annexin XIIIa/b (E), encompassing only the endonexin repeats (found in all annexin family members) was purified and incubated with GST-Nedd4-C2 and GST, as described above. This construct failed to bind to the same degree as the full length proteins (Fig. 3-4A). This is not due to protein misfolding since the endonexin repeats have been shown to fold independently (reviewed in Liemann and Huber, 1997). Three experiments identical to those described in (A) were performed and the Western blots were quantitated using Fuji ImageQuant. The intensities of binding of annexin XIIIa/b and the endonexin folds to the GST-Nedd4-C2 were normalized to the intensity of binding to GST alone, and were averaged over the three experiments and plotted (Fig. 3-4B). Again, the truncated version of annexin XIII failed to bind to the same degree as the full length proteins.

**Ca\textsuperscript{2+}-dependent re-distribution of Nedd4 in MDCK cells expressing annexin XIIIb**

Stable MDCK II cell lines expressing myc-tagged annexin XIIIb in an inducible manner were used to determine whether the protein co-distributes with endogenous Nedd4 in response to Ca\textsuperscript{2+}. The characterization of the cell line, MDCK(lac)/55, is described elsewhere (Lecat et al., in preparation). Briefly, the synthesis of the myc-tagged annexin XIIIb is repressed due to the stable expression of the lac repressor that can be inhibited upon IPTG treatment. It should be noted that these cells differ from those used in the Chapter Two localization studies in that in these MDCK(lac)/55 cells, the expression of endogenous annexin XIIIb is down-regulated prior to induction by IPTG and the efficiency of apical delivery is reduced (Cheong et al., 1999). Immunofluorescence analysis using anti-annexin XIIIb and anti-Nedd4 antibodies revealed that the distribution of Nedd4 was not dramatically modified by the induction of myc-tagged annexin XIIIb expression (Fig. 3-5). However, following Ca\textsuperscript{2+}/ionomycin treatment, Nedd4 was redistributed mainly to the apical compartment. In cells that did not receive Ca\textsuperscript{2+}/ionomycin pre-treatment and in which annexin XIIIb
Figure 3-4. Annexin XIIIa and b but not isolated Endonexin Folds Bind GST-Nedd4-C2 Directly In Vitro. (A) Purified GST-C2 or GST alone (~5 μg each) were incubated in the presence of annexin XIIIa (A), b (B) or truncated annexin XIII expressing only the endonexin folds region (E) (5 μg each, purified as described above) with Ca$^{2+}$ in the binding solution (described above). Western blots were performed, as described, using an anti-HA antibody. (B) Three experiments identical to those described in (A) were quantitated using Fuji ImageQuant. Briefly, Western blots were developed with ECL but the signal was collected by a CCD camera. The intensity of the bands was analysed, quantitated and expressed as intensity in arbitrary units (AU)/mm$^2$. The intensities of binding of annexin XIIIa/b and the endonexin folds to the GST-Nedd4-C2 were normalized to the intensity of binding to GST alone, and were averaged over the three experiments and plotted.
A.

Pulldown:  
<table>
<thead>
<tr>
<th></th>
<th>GST-C2</th>
<th>GST</th>
<th>total protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-Anx XIII:</td>
<td>A</td>
<td>B</td>
<td>E</td>
</tr>
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</table>

40 kDa

Blot: anti-HA

B.

<table>
<thead>
<tr>
<th>intensity (AU/mm²)</th>
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<tbody>
<tr>
<td>3500000</td>
</tr>
<tr>
<td>3000000</td>
</tr>
<tr>
<td>2500000</td>
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<tr>
<td>1500000</td>
</tr>
<tr>
<td>1000000</td>
</tr>
<tr>
<td>500000</td>
</tr>
<tr>
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</tbody>
</table>

annexin XIIIa  
annexin XIIIb  
endonexin folds
Figure 3-5. $Ca^{2+}$-dependent distribution of Nedd4 and annexin XIIIb in MDCK cells. Filter-grown polarized MDCK(lac) switchable cells expressing annexin XIIIb were either induced or not induced overnight with IPTG and SodiumButyrate. The internal concentration of $Ca^{2+}$ was increased (or not) following incubation of the cells with a $Ca^{2+}$/ionomycin-containing buffer as detailed in the Material and Methods. Cells were processed for immunofluorescence labeling using either anti-annexin XIIIb or anti-Nedd4 antibodies and FITC-conjugated secondary antibodies (green). Nuclei were stained with propidium iodide (red) in all panels. X-Z confocal sections are shown. Notice the basolateral staining of Nedd4 in $Ca^{2+}$/ionomycin-treated cells and the apical staining after induction of annexin XIIIb expression (arrowheads). Bar, 15 $\mu$m.
<table>
<thead>
<tr>
<th>Not-induced</th>
<th>Induced</th>
</tr>
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<tbody>
<tr>
<td>Anti-XIIIb</td>
<td></td>
</tr>
<tr>
<td>Nedd4</td>
<td></td>
</tr>
<tr>
<td>Anti-XIIb Ca²⁺-dependent</td>
<td></td>
</tr>
<tr>
<td>Nedd1 Ca²⁺-independent</td>
<td></td>
</tr>
</tbody>
</table>
was not overexpressed, Nedd4 was detected in both apical and basolateral compartments. These results are compatible with the previously reported Ca$^{2+}$-dependent membrane-association of Nedd4 (Plant et al., 1997) (Chapter Two).

**Subcellular localization of Nedd4 in the apical pathway in MDCK cells**

We investigated in greater detail, the distribution of both Nedd4 and annexin XIIIb using immunogold labeling of ultrathin cryo-sections and processing the samples for analysis in electron microscopy. The number of gold particles associated with either annexin XIIIb or Nedd4 on surfaces located within 500 nm from the apical plasma membrane were counted and then expressed as associated gold particles/μm$^2$. We found that following annexin XIII overexpression, the number of annexin XIIIb associated gold particles/μm$^2$ increased (from 7.2±1.3 to 11.1±1.1, n=10) significantly (t-test, p<0.05) as did the number of Nedd4 associated gold particles/μm$^2$(1.3±0.3 to 2.8±0.3, n=10). We observed co-localization of Nedd4 and annexin XIIIb at the apical plasma membrane and in tubulo-vesicular structures underneath the apical surface (Fig. 3-6A), suggesting they were apical carriers en route to the surface. Structures of large size (>200 nm), some of them containing internal membranes, were also immunodecorated for both proteins, suggesting they were endosomal structures and multivesicular bodies which contain Nedd4 and annexin XIIIb (Fig. 3-6B). We also examined whether Nedd4 was associated with TGN membranes by testing its co-localization with the trans-Golgi resident enzyme sialyltransferase (Fig. 3-6C). We did not find co-localization of Nedd4 and sialyltransferase, while we could observe clear co-localization of annexin XIIIb with this TGN marker, as previously reported (Lafont et al, 1998). Interestingly, the ratios of annexin XIIIb and Nedd4 associated with the apical membrane before and after annexin XIIIb overexpression, were of similar magnitude (i.e. annexin XIIIb was elevated 1.7 fold and Nedd4 1.6 fold after overexpression of annexin XIIIb). These results suggest that more Nedd4 is recruited to apical membranes as more annexin XIIIb is present there.

In order to directly visualize the localization of Nedd4 in exocytic apical carriers, we studied its distribution in trans-golgi network (TGN)-derived vesicles isolated from influenza infected
Figure 3-6. Co-localization of annexin XIIIb and Nedd4 in MDCK cells. Double immunolabelling on ultrathin cryo-sections of MDCK cells showing co-localization of Nedd4 (15 nm gold) and annexin XIIIb (10 nm gold) at the apical surface (A) and in internal membranes structures of large size (B). Note in B the presence of membranes in the immunodecorated structures. (C) Triple labelling showing localization of sialyltransferase (5 nm gold), Nedd4 (10 nm gold) and annexin XIIIb (15 nm gold). Arrowheads, co-localization of Nedd4 and annexin XIIIb; small arrows, co-localization of annexin XIIIb and sialyltransferase. Bars, 100 nm.
MDCK cells using immuno-gold electron microscopy. As shown in Figure 3-7, Nedd4 (10 nm gold particles) was present in the isolated vesicles identified by the presence of the apical marker hemagglutinin (15 nm). Annexin XIIIb (5 nm) was also found associated with the apical carriers, as previously reported (Lafont et al., 1998). Collectively, these results suggest that the binding of Nedd4 to annexin XIIIb occurs at an early step prior to the arrival at the apical plasma membrane, but after the carriers have budded off the TGN.

**Annexin XIIIb expression enhances the association of Nedd4 with rafts**

Since annexin XIIIb was described as being associated with rafts in apical carriers (Lafont et al., 1998), we analyzed whether Nedd4 was associated with rafts as well. We first examined whether Nedd4 could be found floating in association with detergent insoluble material. To this end, we made use of the stably transfected switchable MDCK (lac) cell line expressing annexin XIIIb (Lecat et al., in preparation) and quantitated the amount of Nedd4, annexin XIIIb or caveolin associated with the raft fraction. In these cells, in which the endogenous expression of annexin XIIIb is down-regulated prior to induction, Nedd4 was hardly detectable in the top fraction of the OptiPrep gradient (2% was recovered in the top 3 fractions) indicating that it was not associated with light membranes resistant to Triton extraction (Fig. 3-8, top left). The low level of Nedd4 found floating was probably due to association with endogenous annexin XIIIb. We then investigated whether the inducible expression of annexin XIIIb could have an effect on the association of Nedd4 with the detergent resistant membranes. After induction, Nedd4 was clearly recovered at the top of the gradient (23% recovered in the top three fractions), suggesting that annexin XIIIb could recruit to or facilitate the association of Nedd4 with detergent insoluble membranes (Fig. 3-8, +Anx XIIIb). Annexin XIIIb overexpression did not alter the level of Nedd4 expression. To determine whether Nedd4 was raft-associated, rafts were disorganized using the cholesterol sequestering drug methyl-β-cyclodextrin (+MeβCD). The floatation patterns of both Nedd4 and annexin XIIIb were dramatically perturbed following MeβCD treatment, with no proteins left in the top fraction (Fig. 3-8, +Anx XIIIb + MeβCD). Finally, the raft-association of
Figure 3-7. Nedd4 localization in apical exocytic carriers. Co-localization of HA (15 nm gold), Nedd4 (10 nm gold), and annexin XIIIb (5 nm gold) in isolated apical carriers. Bar, 60 nm.
Figure 3-8. Ca^{2+}- and annexin XIIIb-dependent raft-association of Nedd4. Control MDCK(lac) cells (top left) or cells induced overnight to express annexin XIIIb (+Anx XIIIb, top right) were scraped, Triton X-100 extracted on ice, placed in 40% OptiPrep and overlaid with 25% and 0% OptiPrep in Ca^{2+}-containing (TNCa) buffer. Fractions were collected from the top, proteins precipitated and analyzed by SDS-PAGE and Western blotting with either anti-caveolin (Cav-1), anti-annexin XIIIb (Anx XIIIb) or anti-Nedd4 antibodies. Induced cells were treated with MeβCD before being scraped and processed as described elsewhere (+Anx XIIIb +MeβCD, bottom left panel). (+Anx XIIIb -Ca^{2+}, bottom right panel), induced cells were scraped, detergent extracted and the floatation was performed in Ca^{2+}-free (TNE) buffer.
Nedd4 was examined in the absence of Ca$^{2+}$ (Fig. 3-8, + Anx XIIIb -Ca$^{2+}$). Under such conditions, as previously reported (Lafont et al., 1998), annexin XIIIb was still associated with apical rafts (27% recovered in the top three fractions) which is to be expected as annexin XIIIb association with apical rafts is constitutive (due to its myristoylation) and not dependent on calcium. Nedd4, however, was virtually undetectable in the light fraction (6% in the top three fractions), demonstrating that the association of Nedd4 with rafts is Ca$^{2+}$-dependent, consistent with our previous membrane binding results (Plant et al., 1997). Caveolin-1, which is a protein constitutive of and tightly bound to apical rafts (Scheiffele et al., 1998), was used as a positive control for raft association in these experiments. The association of caveolin with rafts is strong and resistant to cholesterol sequestering drugs and to removal of calcium. Similar results were obtained when annexin XIIIb was overexpressed using an adenovirus expression system (Lecat et al., unpublished data).

IV. Discussion

One of the distinguishing features of polarized epithelial cells is the compartmentalization of their plasma membrane into distinct apical and basolateral regions. These specialized domains are maintained by tight junctions and are characterized by differences in lipid and protein composition, the foundations of the functional specificity of each region (Simons and Fuller 1985; van Meer and Simons 1986, 1988; Simons and Wandinger-Ness 1990; Simons 1993-94). The differences in composition of the domains is generally believed to originate in the TGN where classes of proteins and lipids are included or excluded from microdomains destined for a specific membrane (Griffiths and Simons 1986; Rodriguez-Boulan and Nelson 1989; Simons and Ikonen 1997). For example, sphingolipids and cholesterol are preferentially packaged into platforms or rafts within the TGN, and are targeted to the apical membrane (Simons and Ikonen 1997; Brown and London 1998). These transportable segments of membrane associate with specific classes of proteins that are either destined for the apical region of polarized epithelia or play an important role in raft formation.
We have shown previously that increases in intracellular Ca\textsuperscript{2+} prompted a redistribution of Nedd4 from the cytoplasm to the plasma membrane region of polarized epithelia, in particular the apical region of polarized MDCK cells (Plant et al., 1997). In this study we have established a relationship between the ubiquitin protein ligase Nedd4 and the apical raft associated annexin XIIIb. Annexin XIIIb, which is associated with the membrane in the absence of Ca\textsuperscript{2+}, likely due to its myristoylation, has been shown to associate with, and proposed to be involved in, the formation of apical rafts in the TGN (Lafont et al., 1998). As such, its distribution within the cell is predominantly TGN, along the apical route in tubulovesicular structures and at the apical membrane (Lafont et al., 1998). It is likely that the Ca\textsuperscript{2+}-dependent association of Nedd4 with annexin XIIIb forms the basis for the partitioning of Nedd4 to the apical region of polarized MDCK cells. The finding that Nedd4, in response to increases in intracellular Ca\textsuperscript{2+}, binds to and localizes to the same membrane compartments as annexin XIIIb (Fig. 3-4) is in agreement with the observed distribution of Nedd4 in apical carriers, at the apical plasma membrane and in endosomal compartments, as demonstrated through immuno-gold electron microscopy (Figures 3-5 and 3-6). Furthermore, overexpression of annexin XIIIb caused an increased association of Nedd4 with apical rafts, an interaction that was ostensibly Ca\textsuperscript{2+}-dependent, because it was abrogated by the addition of the Ca\textsuperscript{2+} chelator EGTA.

The exact mechanisms involved in mediating this Ca\textsuperscript{2+}-dependent interaction between annexin XIIIb and the Nedd4-C2 domain are unknown. Structural studies of the C2 domain have shown that the co-ordination of the Ca\textsuperscript{2+} ions is provided, in part, by the side chains of the conserved aspartates in the loop regions of the C2 key motif (corresponding to Asp 172, 178, 230, 232 and 238 of synaptotagmin; Sutton et al., 1995; Shao et al., 1996) and in synaptotagmin and PKC\ensuremath{\alpha}, mutation of all or several of these residues to asparagine affect the Ca\textsuperscript{2+}-dependent membrane binding properties of the C2 domain (Sutton et al. 1995; Medkova and Cho 1998). In the Nedd4-C2 domain, four of the five aspartate residues (Asp 95, 101, 153 and 161) are conserved. This finding, in addition to the fact that not all C2 domains possess the full complement of
aspartates yet still display Ca\textsuperscript{2+}-dependent membrane association (Nalefski \textit{et al.} 1994; Gawler \textit{et al.} 1995), may further reflect differences in functional specificity of the different C2 domains, as well as variability in the mechanisms of Ca\textsuperscript{2+} co-ordination and Ca\textsuperscript{2+} affinity.

The C2 domain of Nedd4 also binds to annexin XIIIa, which is targeted to the apical membrane of MDCK cells but is also found in basolateral rafts (Lecat \textit{et al.}, in preparation), where it is engaged in a pathway that remains to be precisely characterized. The higher content of annexin XIII at the apical compartment due to the presence of both annexin XIIIa and b could explain the preferential apical distribution of Nedd4. Based on the observations of Nedd4-C2 domain binding to annexin XIII (this study), and of rasGAP-CaLB domain binding to annexin VI (Davis \textit{et al.}, 1996), we hypothesize that other C2 domain-containing proteins may interact with other annexins.

In addition to our demonstration of the role of the Nedd4-C2 domain in Ca\textsuperscript{2+}-dependent plasma membrane targeting in polarized epithelia (Plant \textit{et al.}, 1997 and this study), recent reports have proposed other functions for this domain, not necessarily mutually exclusive with the role in targeting we have demonstrated. For example, it has been shown that following caspase activation during the onset of apoptosis, Nedd4 is cleaved at the N-terminus, clipping off its C2 domain (Harvey \textit{et al.}, 1998). This cleavage may result in reduced stability of Nedd4 or affect the ability of Nedd4 to localize properly within the cell in order to bind its physiological targets (Harvey \textit{et al.}, 1998). Interestingly, the C2 domain of the \textit{Saccharomyces cerevisiae} homologue of Nedd4, Npi1/RSP5, was shown recently to be important for internalization of the Gap1 permease (Springael \textit{et al.}, 1998). Although the C2 domain of RSP5 is dispensable for survival in yeast (unlike the HECT domain), and is not required for ubiquitination of Gap1 \textit{per se}, this finding extends the function of the C2 domain of E3 enzymes to include endocytosis of ubiquitinated substrates. In fact, the C2B domain of synaptotagmin is implicated in the regulation of endocytosis by virtue of its association with the adaptor AP-2 complex. Whether the C2 domain of Nedd4 functions in a similar manner is yet to be determined. Furthermore, an interaction between the C2 domain of Nedd4 and the adaptor molecule Grb10, primarily via a phosphotyrosine-independent association with the Grb10-SH2 domain, has recently been described and was suggested to serve as
an alternative mechanism for targeting Nedd4 to potential substrates (Morrione et al., 1999; Appendix I). Finally, the finding that Nedd4 is associated with apical rafts raises the possibility that ubiquitination may play a role in regulating the dynamics of rafts, as already demonstrated for phosphorylation (Brown and London, 1998).
Chapter Four:

Summarizing Discussion and Future Directions
Chapter Four: Summarizing Discussion and Future Directions

In the previous chapters, a role for the C2 domain of the protein ubiquitin ligase Nedd4 has been described. We have found that its C2 domain is capable of Ca\(^{2+}\)-dependent membrane/lipid binding when expressed as a GST fusion protein, and that it is, alone, responsible for mediating Ca\(^{2+}\)-dependent membrane association of Nedd4. I have also reported that the C2 domain does not display any obvious preference for charged lipids, yet, seems to display a preference for the apical region of polarized epithelia (MDCK cells) in response to Ca\(^{2+}\)-mediated targeting. The association of Nedd4 with the membrane was shown to persist for approximately 30-45 min, following its Ca\(^{2+}\)-mediated delivery. This preference for the apical region may be mediated by an association of the C2 domain with the apically targeted protein annexin XIIIb. The C2 domain interacts with annexin XIIIb in a Ca\(^{2+}\)-dependent manner and co-localizes with it in living cells and in isolated apical rafts, in a Ca\(^{2+}\)-dependent fashion. In fact, when annexin XIIIb expression is stimulated using an inducible system, it targets Nedd4 to the apical membrane in response to Ca\(^{2+}\), as Ca\(^{2+}\) allows its association with Nedd4. In the absence of annexin XIIIb overexpression, Nedd4 does not display this re-distribution from the cytosol. The interaction between Nedd4-C2 and annexin XIII was shown to be direct, not requiring additional cellular components, and the interaction may be taking place through the unique N-terminal sequences of annexin XIII, as mutants expressing only the endonexin folds fail to bind to the C2 domain.

Protein-protein interactions are commonplace in the cell, especially in the context of creating scaffolds of signalling molecules at the plasma membrane for transduction of signals from extracellular to intracellular regions (Pawson and Scott, 1997). Through interactions between various conserved modular domains, proteins are able to localize, both spatially and temporally, to their cellular targets in response to external cues, in order to transduce a specific signal. The C2 domain is certainly an example of this. Aside from engaging in a Ca\(^{2+}\)-dependent protein-protein interactions in order to localize Nedd4 to the membrane, the area of its potential substrates, the C2 domain also can interact with the modules of adaptor molecules, that are part of membrane
complexes, potentially serving as signaling scaffolds. It is tempting to speculate that the protein interactions which the C2 domain engages in are integral to the mechanisms facilitating Nedd4 interactions and actions upon potential substrates in cells.

The ultimate consequence of Nedd4 targeting to the plasma membrane, and particularly to the apical region as a result of its interactions with annexin XIIIb, is proximity to specific substrates such as ENaC. Such targeting would facilitate binding of Nedd4 to these substrates via its WW domains, subsequent substrate ubiquitination, via the HECT domain, and likely endocytosis and degradation of the protein (Fig. 4-1). If intracellular Ca^{2+} was to affect delivery of Nedd4 to its cellular targets, Ca^{2+} would be expected to affect the stability or activity of the Nedd4 targets. Indeed, whole-cell patch clamp studies of MDCK cells heterologously expressing ENaC, a known target of Nedd4 (Staub et al., 1996; Goulet et al., 1998; Abriel et al., 1999), have revealed an inhibition of amiloride sensitive Na^{+} current through ENaC in response to elevation of intracellular Ca^{2+} (Ishikawa et al., 1998), although we do not know yet whether Nedd4 is involved in this inhibition.

In the remainder of this chapter I will discuss a number of future directions, stemming from this work, that may be undertaken to gain a deeper understanding of the role of the C2 domain in Nedd4 functioning.
Figure 4-1. Proposed model of Nedd4 targeting and regulation of ENaC activity. In response to elevations in intracellular Ca$^{2+}$, Nedd4 associates with Annexin XIIIb which serves to localize Nedd4 to the apical region of polarized epithelia. Here, it can interact with its substrates, such as ENaC, via an interaction between its WW domains and the PY motifs present in the ENaC subunits. It is possible that such an interaction may induce conformational changes in Nedd4, orienting the HECT domain in a favorable conformation for ubiquitination of ENaC. This targets the channel for degradation via the endosomal/lysosomal system.
Regulation of ENaC by Nedd4

[Diagram showing the regulation process of ENaC by Nedd4, involving steps like Annexin 13b, Ca²⁺, and Ubiquitination and lysosomal degradation.]
A. A role for the C2 domain of Nedd4 in endocytosis

Several recent findings support the hypothesis that the C2 domain of Nedd4 may be in the internalization of ubiquitinated substrates. Primarily, our studies with the interaction of Nedd4 and annexin XIIIb clearly demonstrated the presence of Nedd4 and annexin XIIIb in endosomal compartments. This was seen through immuno-electron microscopy of polarized MDCK cells in the presence of elevated intracellular Ca\(^{2+}\) (to induce the re-distribution of Nedd4 to the apical region of these cells). The physiological significance of this targeting is unknown, but it is tempting to speculate that following Ca\(^{2+}\)-mediated delivery of Nedd4 to the apical region of polarized cells (via an interaction of its C2 domain with the apically targeted annexin XIIIb), Nedd4 is brought in to close proximity with its potential substrates where it ubiquitinates them and is then internalized with them following the ubiquitination event.

The role of the C2 domain in this process can be further speculated given the finding that the N-terminal region (specifically the C2 domain) of the Nedd4 homologue, Rsp5, is required for proper endocytosis of its substrate, Gap1p. Although the ubiquitination of Gap1p is unaffected by the C2 deletion, these mutants are unable to internalize, suggesting that the C2 domain is mediating this process (Springael et al., 1999). A relationship between Rsp5 and the endocytotic machinery has been established; two hybrid interactions have revealed an interaction between Rsp5 and Pan1 (a relative of Eps15), which is involved in endocytosis (Zolladek et al., 1997). Pan1 interacts with many proteins, including yAP180A, a yeast homologue of a class of clathrin assembly proteins (AP180) (Wendland and Emr, 1998). Furthermore, it is well documented that the C2B domain of synaptotagmin is involved in internalization processes by virtue of its association with the clathrin adaptor complex AP-2 (Zhang et al. 1994).

Recent studies have shown that the recruitment of the synaptotagmin C2B domain to the AP-2 complex is enhanced in the presence of tyrosine based endocytic motifs Yxx\(\phi\) (Haucke and De Camilli, 1999 and Chapter One). Extending these findings to the scenario of Nedd4-C2 and ENaC, it is possible that the tyrosine based motifs found in the C-termini of all three subunits of the channel (\(\alpha\): YATL; \(\beta\): YDSL; \(\gamma\): YNTL) (Canessa et al., 1994) are interacting with the clathrin
complex, an activity which prompts the interaction of Nedd4-C2 and AP-2 (analogous to synaptotagmin C2B). Clearly, the precise role of Nedd4, particularly the C2 domain, in the endocytic process needs to be determined. It would be necessary to, primarily, establish a relationship between Nedd4 and components of the clathrin complex by co-precipitation and in vitro binding studies with adaptins since these have been shown to bind other C2 domains. In addition, mutational analysis can be performed on the tyrosine based motifs found in ENaC to determine whether these regions of the channel are necessary for its internalization. The internalization of ENaC can be assessed by immunogold-EM (as was done with Nedd4 and Anx XIIIb) or by immunofluorescence using antibodies directed towards the channel subunits.

B. The role of Nedd4 C2 in regulating epithelial Na⁺-channel activity.

Although the work described in this thesis has demonstrated the targeting of Nedd4 to the apical region of polarized MDCK cells, neither the direct ubiquitination of ENaC by Nedd4 nor the requirement of the C2 domain in this process has been shown. To determine the effect of the C2 domain on the channel it is necessary to explore the nature of the negative regulation of ENaC by Ca²⁺ as described earlier (Ishikawa et al., 1998). Our hypothesis is that this effect is due to the Ca²⁺-dependent apical localization of Nedd4, in the proximity of ENaC, and subsequent downregulation of the channel by decreasing its cell surface expression by ubiquitination and degradation. In order to test this hypothesis, the stability of the channel on the cell surface must be assessed using surface labeling in the presence and absence of Ca²⁺. This would clarify whether the affect of Ca²⁺ on channel activity is due to effects on channel stability or to an affect of Ca²⁺ on the conductive properties of the channel (i.e. gating, conductance). It would also be informative to determine the Ca²⁺-dependent effects of a dominant-negative form of Nedd4 (bearing the C860S mutation in its HECT domain) on the activity of the channel, expressed in MDCK cells, to assess affect of Nedd4, and the C2 domain in particular, on the channel activity. In order to do this, a construct of Nedd4 bearing the C860S mutation could be transfected and overexpressed with an
inducible system in ENaC expressing MDCK cells that are subsequently treated with or without Ca\textsuperscript{2+}/ionomycin, and electrophysiology be carried out to assess the channel properties. In addition, antisense-mRNA to Nedd4 or inhibitory antibodies to Nedd4 WW domains could be introduced in these ENaC expressing cells to determine the affects on channel function.

The relationships of ENaC with rafts remains to be analyzed. It is not known at what point Nedd4 and ENaC interact, that is, if the interaction takes place in apical rafts destined for the apical membrane or at the apical membrane itself. The presence of ENaC in these apical rafts must be tested. Recent data suggests that ENaC may be associated with these microdomains; when co-expressed all ENaC subunits are recovered in a Triton-insoluble fraction (Prince and Welsh 1999). To determine the presence of the channel in these apical rafts, the rafts must be isolated from MDCK cells expressing the ENaC subunits and then the fraction containing the rafts can be blotted with antibodies against the channel subunits. For \textit{in vivo} studies, immuno-EM can be performed on apical rafts isolated from ENaC expressing MDCK cells using anti-ENaC antibodies.

C. A Role for the C2 domain in mediating targeting of Nedd4 to potential substrates.

In this thesis, the interactions that take place between the C2 domain and annexin XIIIa/b have been described, although the nature of these interactions remains elusive. Preliminary \textit{in vitro} binding studies with the Nedd4-C2 and the two isoforms of annexin XIII have suggested the interactions occurring through the N-terminal region of annexin. A truncated version of these proteins, encompassing only the endonexin repeats (found in all annexin family members) failed to bind to the same degree as the full length proteins (Chapter Three). This is not due to protein misfolding since the endonexin repeats have been shown to fold independently (reviewed in Liemann and Huber, 1997). A region of Anx XIII likely to be mediating this interaction is the extreme N-terminal region of annexin XIII, not including the 40 amino acid insert found in Anx XIIIb. Mutations can be made in these 6 amino acids to determine which are essential for binding to the C2 domain. The region of the Nedd4 C2 engaged in these interactions is unknown and needs
clarification. A series of deletion mutations/point mutations of the C2 domain can be used in \textit{in vitro} binding experiments to determine a general region of interaction. This can be followed by more detailed analysis using point mutations of this region to determine specific contact points within the C2 domain.

We have been part of a collaboration that described the interaction between the C2 domain and the SH2 domain of the adaptor molecule, Grb10 (Appendix I). This interaction is non-phosphotyrosine-dependent and interestingly, non-Ca\textsuperscript{2+}-dependent as well. As Grb10 has been shown to interact with the IGF-I receptor, the interaction between the Nedd4-C2 and Grb10 SH2 domains may serve to target Nedd4 to the IGF-I receptor within the cell; indeed, we have shown that the IGF-I receptor is ubiquitinated in cells (as previously reported; Sepp-Lorenzino \textit{et al.}, 1995). Nothing, however, is known about the nature of the interaction between the C2 domain of Nedd4 and the SH2 domain of Grb10. This interaction represents a novel relationship for the SH2 of Grb10. Currently, we are part of a collaboration with Dr. Julie Forman-Kay to solve the solution structure of the Nedd4 C2 complexed with the Grb10 SH2 domain. The structure will give greater insight to the region of the C2 domain involved in the interaction.

The interaction between Nedd4-C2 and Grb10 may be another example of the involvement of the Nedd4 C2 domain in protein complexes that serve to localize the ubiquitin-protein ligase to its potential targets. By partaking in the interaction with the SH2 domain of Grb10, the C2 domain may act to bring the enzyme in close proximity to its potential substrates (IGF-IR receptor), akin to its interaction between annexin X\textsubscript{III}b which serves to deliver Nedd4 to the apical region of polarized MDCK cells (the location of its substrate, ENaC). Although we were able to show IGF-I receptor ubiquitination, we currently do not know whether this is due to the action of Nedd4 on the receptor directly. To determine whether this interaction serves to ubiquitinate the IGF-IR, Grb10 constructs expressing mutations in the SH2 domain that disrupt the interaction with the C2 domain can be co-expressed with the IGF-IR in cells that express Nedd4. The ubiquitination of the receptor under these conditions can be assessed through ubiquitination assays, as described previously. An attempt
to reconstitute the receptor ubiquitination in vitro with Nedd4 would determine whether Nedd4 was responsible for the ubiquitination seen in vivo.

Summary

In conclusion, in the previous chapters, I have described a role for the C2 domain of the ubiquitin-protein ligase Nedd4 in mediating the Ca\(^{2+}\)-dependent targeting of this E3 to the membrane via protein-protein and protein-lipid interactions. In this chapter, I have outlined a number of possible directions for future work, which involve more detailed studies on the interactions in which the Nedd4-C2 is engaged. Specifically, the nature of the interaction between Nedd4-C2 and annexin X\textsubscript{III}b needs to be characterized more thoroughly, as does the mechanism of Ca\(^{2+}\) inhibition of ENaC. Studies in these areas will further validate the role for the C2 domain in targeting of Nedd4 to its substrates within the cell.
APPENDIX I:

The C2 Domain of Ned4 Mediates a Phosphotyrosine-independent Interaction with the SH2 Domain of Grb10

* This work was published in the Journal of Biological Chemistry.


My contribution to this collaboration was to perform the ubiquitination assays on Grb10 and the IGF-IR.
APPENDIX I: The C2 Domain of Nedd4 Mediates a Phosphotyrosine-independent Interaction with the SH2 Domain of Grb10

I. Summary

A yeast two-hybrid screen was used to identify proteins interacting with mouse Grb10, an adaptor protein known to interact with both the insulin and insulin-like growth factor-I receptors. A mouse cDNA clone containing the C2 domain of mouse Nedd4 was isolated in the screen. The interaction with Grb10 in the two-hybrid system was confirmed using the full-length Nedd4, and it was abolished by deleting the last 148 amino acids of Grb10, a region that includes the SH2 domain and the newly identified BPS domain. In the two-hybrid system, the SH2 domain and the BPS domain of Grb10 together were capable of binding to the C2 domain of Nedd4, yet, a stronger binding was mediated by the SH2 domain alone. The interaction between Grb10 and Nedd4 was also reproduced in vivo in mouse embryo fibroblasts, where endogenous Nedd4 co-immunoprecipitated with both the endogenous and a transfected tagged-Grb10 in a Ca²⁺-independent manner. In addition, this interaction was shown to be phosphotyrosine-independent, an observation that supported the two-hybrid binding results. The interaction between Grb10-SH2 and Nedd4-C2 likely does not serve to ubiquitinate Grb10, as Grb10 failed to become ubiquitinated in vivo. The IGF-IR, however, was shown to be ubiquitinated in cells which raised the possibility that this interaction may be used to target other proteins, like tyrosine kinase receptors, for ubiquitination by Nedd4.

II. Introduction

Grb10 was originally isolated using the CORT technique with the EGF receptor (Ooi et al., 1995), and is a member of a family of adapter proteins that includes at least seven isoforms in human and mouse. Grb10 was recently identified as an interacting partner with either the IGF-I receptor (IGF-1R) (Morrione et al., 1996; Dey et al., 1996; O’Neill et al., 1996), the insulin receptor (Liu et al., 1995; Hansen et al., 1996; Laviola et al., 1997; Frantz et al., 1997; Dong et al.,

174
1997a), or both (O’Neill et al., 1996). All Grb10 isoforms contain a highly conserved SH2 domain at the C-terminus, a PH domain in the central region and a less conserved N-Terminus, containing proline-rich sequences, considered to be possible binding sites for SH3 domain-containing proteins (Frantz et al., 1997; Dong et al., 1997b). Recently, another functional domain of the Grb10 protein has been identified and called BPS (Between the Pleckstrin Homology and SH2 domains) domain (He et al., 1998). The function of the different Grb10 isoforms is not fully clear and the data available are also partially discordant. An inhibitory effect of mGrb10 (Ooi et al., 1995) on IGF-I-mediated mitogenesis has been shown (Morrione et al., 1997) in addition to an inhibitory effect on IR signaling with a human isoform of Grb10 (Liu et al., 1995), but O’Neill et al., (1996) reported opposite results on IGF-IR and IR signaling with another human variant. The most likely explanation of these data is that different isoforms may have different functions and may compete for common substrates. Recently, Grb10 has been identified as a maternally expressed imprinted gene (Meg) on mouse chromosome 11 (Miyoshi et al., 1998), and it has been suggested that it may be a candidate gene for the Silver-Russell syndrome in humans. More recently, the hGrb10 SH2 domain has been shown to interact with both the Raf1 and MEK1 kinases, and these interactions were phosphotyrosine-independent (Nantel et al., 1998). Grb10 has been also reported to interact with the Growth hormone receptor (Moutussamy et al., 1998), the ELK receptor (Stein et al., 1996) and with BCR-ABL tyrosine kinase (Bai et al., 1998). To identify other proteins that interact with Grb10, a yeast two-hybrid screen was performed with the full-length Grb10 as a bait. A cDNA clone of mouse Nedd4 was isolated and this interaction was confirmed in vivo and in vitro and, furthermore, was shown to be between the C2 domain of mNedd4 and the SH2 domain of mGrb10. The interaction may not serve to ubiquitinate Grb10 as it failed to become ubiquitinated in cells. Interestingly, the IGF-IR was ubiquitinated, as previously reported (Sepp-Lorenzino et al., 1995) raising the possibility that by interacting with Grb10, Nedd4 may come into contact with other proteins such as growth factor receptors and ubiquitinate them. The finding of the interaction between the Grb10-SH2 and Nedd4-C2 extended the range of protein substrates of the C2 domain, but also marked the first description of an interaction between a C2 domain and an SH2 domain.
Moreover, the finding illuminated a novel mechanism whereby Nedd4 is targeted to potential substrates, such as IGF-IR, for ubiquitination and regulation of receptor function, utilizing adaptor proteins.

II. Experimental Procedures

Yeast Two-Hybrid System

pRK5-Grb10 plasmid (a kind gift from Dr. Ben Margolis), was cut with Hind III, treated with Klenow and then cut with Sac I. The purified fragment, containing the full-length coding sequence for mGrb10, was cloned in the GAL4 DNA-binding domain of the pAS2-1 yeast cloning and expression vector, digested with Sma I (Clontech Lab, Inc.). The Y190 yeast strain (Harper et al., 1993) was first transformed with pAS2-1/Grb10 plasmid, tested for the expression of the hybrid protein by western blotting using antibodies against the Gal 4 DNA binding domain (Clontech), and then transformed with a mouse embryo cDNA library, cloned in pVP16 vector (a kind gift of Drs. Stanley Hollenberg and Ann Vojtek (Vojtek et al., 1993)). Co-transformants were plated onto -trp-leu-his selective medium, supplemented with 25 mM 3-aminotriazole. His+ colonies were then assayed for β galactosidase activity, by a filter assay as described (Vojtek et al., 1993). Segregation of the bait plasmid was performed by cyclohexamide selection (as described in Clontech instructions) and confirmed by replica plating on plates lacking leu only or lacking both trp and leu, and β-galactosidase filter assay. Trp- leu+ colonies were then mated with Y187 yeast strain, transformed with the bait plasmid as the positive control, and with the pLAM plasmid (Clontech) as the negative control and further analyzed for β-galactosidase activity by filter assay (Vojtek et al., 1993).

Plasmid Construction

Grb10 deletion mutants bait plasmids. The mGrb10 1-473 (ΔSH2*BPS) was constructed by cloning an EcoR I/filled/Sca I fragment in the Sma I site of pAS2-1. The mGrb10-SH2*BPS (aa
was created inserting the EcoRI/EcoRI filled fragment from pSA2-1-Grb10 into the NcoI/filled site of the pAS2-1 vector. The mGrb10-BPS (aa 475-518) was cloned inserting the NcoI/NcoI/blunt fragment from pAS2-1-Grb10 into the SmaI site of the pAS2-1 vector. The mGrb10-SH2 (aa 518-621) was created inserting the NcoI/BamHI filled fragment from pAS2-1-Grb10 into the NcoI/BamHI filled site of the pAS2-1 vector.

**Other yeast plasmids.** The full-length Nedd4 was created by cutting the SpeI/ApaI fragment from pBS-Nedd4 and cloning into the SpeI/ApaI sites of pGAD-GH AD yeast vector. The interactions were then analyzed transforming the baits alone or co-transforming baits and preys plasmid into Y187 yeast strain and testing the colonies for β-galactosidase activity by filter assay.

**Cell Lines**

R-/IR, R-/IR/Grb10 and p6/Grb10 cells were previously described (Morrione *et al.*, 1997): they are all mouse embryo fibroblasts, over-expressing Grb10 (R-/IR/Grb10 and p6/Grb10); R-/IR/Grb10 and p6/Grb10 cells over-express also the same number of the insulin (R-/IR/Grb10) or the IGF-I receptor (p6/Grb10), respectively (Morrione *et al.*, 1997).

**Immunoprecipitation and Immunoblotting**

Cells lysates of exponentially growing cells (1 mg of protein) were immunoprecipitated in HNTG buffer [20mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 0.2 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, 2mg/ml of Aprotinin] with the following antibodies: For Grb10 with a monoclonal antibody against the Myc tag (Oncogene Science), for Nedd4 with polyclonal antibodies as described (Kumar *et al.*, 1997). The immunoprecipitates were resolved by SDS-PAGE and transferred to a nitrocellulose filter. The membranes were then probed with an anti-Grb10 polyclonal antibody (#309; a kind gift of Dr. Ben Margolis), anti-Nedd4 polyclonal antibody (Kumar *et al.*, 1992), or anti-phosphotyrosine antibodies (Transduction Laboratories), followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (Oncogene Science), or protein A, horseradish peroxidase linked
(Amersham Corp. instructions). Blots were then developed with the ECL system according to the manufacturer's instruction (Amersham Corp).

**Ca**

2+-dependent co-immunoprecipitation experiments

These experiments were performed as described by Plant *et al.*, (1997) with some modifications. Briefly R-/IR cells over-expressing mGrb10 were starved for 24 hrs in Ca**2+**-free serum-free medium (Gibco), washed twice with washing buffer (250 mM sucrose, 10 mM HEPES pH 6.8, 1 mM EDTA) and then incubated in Ca**2+**-free medium (140 mM NaCl, 6 mM KCl, 1 mM MgCl₂, 0.1 mM EDTA, 20 mM glucose and 20 mM HEPES) in the presence or absence of 1.1 mM CaCl₂ and 1 μM ionomycin (Calbiochem) for 5 minutes at 37°C. Ionomycin in the presence of calcium has been shown previously to increase levels of cytosolic calcium (Plant *et al.*, 1997; Lang *et al.*, 1990; Delles *et al.*, 1995). The cells were then washed twice with Ca**2+**-free medium (-Ca**2+** conditions) or +Ca**2+** medium (+Ca**2+** conditions) and then lysed in lysis buffer with or without 1.1 mM CaCl₂ and processed for co-immunoprecipitation as described above.

**Growth factor stimulation**

R-/IR/Grb10 and p6/Grb10 cells were serum-starved for 72 hrs and then stimulated with 100 ng/ml of insulin (Sigma) (R-/IR/Grb10) or 50 ng/ml of IGF-I (Gibco) (p6/Grb10) for 10 minutes at 37°C. Cells were then lysed and immunoprecipitate as described above.

**Detection of ubiquitination in vivo**

This method has been described in detail by Staub *et al.*, (1997). Briefly R-/IR/Grb10 or p6/Grb10 cells over-expressing Grb10 were transiently transfected with a plasmid encoding His-tagged ubiquitin (His-Ub), expressing eight His-tagged ubiquitin molecules under the control of a CMV promoter (Treier *et al.*, 1994). 48 hrs after Ca**2+** phosphate transfection cells were lysed in lysis buffer containing 50 μM N-acetyl-L-leucinyl-L-leucinyl-L-norleucynal (LLnL) and incubated with Ni**2+** -NTA agarose beads (Qiagen) on a rotating wheel for 4 hrs at 4°C. The beads containing the
histidinated (hence ubiquitinated) bound proteins were washed twice with HNTG plus imidazole and three times with lysis buffer and bound proteins were then separated on 8% SDS-PAGE, transferred to a nitrocellulose filter and probed with anti-Grb10 antibodies as previously described. Alternatively, an 8 HA-tagged ubiquitin molecule-expressing construct was transfected into cells over-expressing Grb10 and IGF-1R (p6/Grb10) and following immunoprecipitation with anti-IGF-IR antibodies proteins were immunoblotted with anti-HA antibodies to detect ubiquitinated proteins.

III. Results

Grb10 interacts with Nedd4 in the two-hybrid system

We used mGrb10 (Ooi et al., 1995) as a bait to screen a mouse embryo library (Vojtek et al., 1993) in the yeast two-hybrid system (Chien et al., 1991). One of the cDNA clones isolated (see Experimental Procedures) encoded the N-terminal portion of mouse Nedd4 (Kumar et al., 1992), spanning the entire C2/CaLB (Ca Lipid binding) domain (Clark et al., 1991; Rizo et al., 1998), from amino acid residue 65 to residue 237. A full-length Nedd4 was then used to confirm this interaction: as shown in figure A-1, Grb10 strongly interacts with the full-length Nedd4 (amino acids 1-887), confirming the specificity of the interaction. In addition, no β-galactosidase activation was detected with the bait alone, or after co-transformation of the bait with the AD vector alone, or between an unrelated bait (laminin) and Nedd4 (data not shown). To localize the domain of Grb10 responsible for the interaction, we constructed a mGrb10 bait lacking the C-terminal 148 amino acids, a region that includes the newly identified BPS domain (He et al., 1998) and the SH2 domain (Ooi et al., 1995). This deletion (Fig. A-1) completely abolished the interaction with the C2 domain of Nedd4, while with the full length Nedd4 the interaction was barely above a detectable level. This lack of binding was not due to lack of expression of the truncated bait, because immunoblotting with antibodies against the GAL4 binding domain revealed stronger
Figure A-1. mGrb10 interacts with mNedd4 in the two-hybrid system. Baits and preys were cotransformed into Y187 yeast strain and β-galactosidase activity was determined by filter assay (see Experimental Procedures). The level of interaction is defined as: +++ very strong; ++ strong; + good; + weak; -/+ barely detectable – undetectable. Three colonies from β-galactosidase filter assay are presented as examples. The numbers reported define amino acids position.
expression of the truncated than of the full length Grb10 (data not shown). To further narrow down the domain of Grb10 responsible for the interaction with Nedd4, we constructed three additional mGrb10 deletion mutants: one expressing the BPS domain alone (aa 475-518), one expressing the SH2 domain alone (aa 518-621) and one expressing both the BPS + SH2 domains (aa 475-621).

As shown in figure A-1, both the BPS (aa 475-518) and the SH2 (aa 518-621) domains can independently bind Nedd4, but the SH2 domain seems to bind more strongly. The BPS+SH2 Grb10 mutant (aa 475-621) is interacting with Nedd4 with an affinity comparable with the one of the SH2 alone, confirming that the SH2 domain is likely the domain mostly responsible for the interaction.

As a control, we tested the level of expression of the deletion mutant fusion proteins by western blot using antibodies against the GAL4 binding domain; our results show that the mutant proteins were all expressed at comparable levels to the full-length mGrb10 (data not shown). In addition, no β-galactosidase activation was detected with any of the mutant baits alone, nor following co-transformation of the baits with the AD vector alone (data not shown).

**mGrb10 and Nedd4 interact in mammalian cells**

To confirm these data in mammalian cells, we performed a series of co-immunoprecipitation experiments in mouse embryo fibroblasts (R-/IR) or in mouse embryo fibroblasts over-expressing mGrb10 fused to a myc-tag (R-/IR/Grb10) (Morrione et al., 1997). In addition to Grb10, these cells express high levels of endogenous Nedd4 (Fig. A-2, lane 3). Thus, exponentially growing cells were lysed and immunoprecipitated with an anti-myc antibody. As shown in figure A-2, panel A, Grb10 was immunoprecipitated by Myc antibodies in R-/IR/Grb10 cells (lane1), while no Grb10 protein was detectable in R-/IR cells used as a control (lane 2). Grb10 was clearly able to co-immunoprecipitate the endogenous Nedd4 (lane 1) expressed in these cells. No Nedd4 protein was detectable in immunoprecipitated R-/IR cells (lane 2), confirming the specificity of Myc antibodies for the tagged Grb10. The same experiment was then repeated using Nedd4 antibodies: the endogenous Nedd4 (Fig. A-2, panel B, lanes 1 and 2, over-exposed to visualize the co-immunoprecipitated proteins), is co-immunoprecipitating both the over-expressed Myc-tagged
Figure A-2. Grb10 co-immunoprecipitates \textit{in vivo} with Nedd4. Lysates from R-/IR cells, in lane 2 or R-/IR-Grb10 cells expressing Grb10 fused to a Myc tag (see Experimental Procedures (lane 1) were immunoprecipitated with either antibodies for the Myc tag (panel A) or anti Nedd4 antibodies (panel B) and probed with Grb10 and Nedd4 antibodies. Lane 3 of panels A and B are whole cell lysates from sample of R-/IR/Grb10 cells.
### A

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Grb 10

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Nedd4

**IP**  
anti-myc  
whole lysate

### B

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Nedd4

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Grb 10

**IP**  
anti-Nedd4  
whole lysate
Grb10 in R-/IR/Grb10 cells (lane 1) and the endogenous Grb10 (lane 2) present in the R-/IR cells (lane 3). This experiment demonstrates that endogenous Nedd4 forms a complex with endogenous Grb10 (or with heterologously expressed Grb10) in living cells, suggesting the two proteins likely interact with each other under physiological conditions.

**Nedd4 co-immunoprecipitates with Grb10 in a Ca\(^{2+}\) and phosphorylation-independent manner**

Because the C2 domain of Nedd4 has been demonstrated to target Nedd4 to the plasma membrane in response to Ca\(^{2+}\) (Plant *et al.*, 1997), we investigated whether Ca\(^{2+}\) also plays a role in the interaction between Grb10 and the Nedd4-C2 domain. To this end, we performed a series of co-immunoprecipitation experiments in R-/IR cells over-expressing Grb10, starved in Ca\(^{2+}\)-free-serum-free medium and then stimulated with Ca\(^{2+}\) in the presence of ionomycin (see Experimental Procedures). Our results show that there was no detectable difference in the level of Grb10 and Nedd4 co-immunoprecipitating in the presence or absence of Ca\(^{2+}\) plus ionomycin treatment (Fig.A-3 panel C, D, E). Moreover, tyrosyl-phosphorylation of Nedd4 did not seem to be affected by increasing Ca\(^{2+}\) concentrations (Fig. A-3C).

To further characterize the interaction between Grb10 and the C2 domain of Nedd4, we tested whether Nedd4 co-immunoprecipitating with Grb10 is tyrosyl-phosphorylated. Exponentially growing R-/IR cells overexpressing Grb10 were immunoprecipitated with an anti-Myc antibody to precipitate Grb10, and the blot was stained with anti phosphotyrosine antibodies. As shown in figure A-3 (panel A), there is a tyrosyl-phosphorylated band migrating at expected size of Nedd4 (lanes 1, 2). To confirm that this band is indeed Nedd4, the blot was stripped and reprobed with anti Nedd4 antibodies (panel B); the Nedd4 protein (lane 1), clearly detectable in the co-immunoprecipitation, is perfectly overlapping with the tyrosyl-phosphorylated band shown in panel A (lane 1), suggesting that Nedd4 was tyrosyl-phosphorylated. As a very small fraction of the total phosphorylated Nedd4 co-immunoprecipitates with Nedd4, it is likely that Grb10 preferentially associates with unphosphorylated Nedd4.
Figure A-3. **Nedd4 co-immunoprecipitating in vivo with Grb10 is weakly tyrosyl-phosphorylated and the interaction is Ca\(^{2+}\)-independent.** Exponentially growing R-/IR-Grb10 (1 mg) cells were immunoprecipitated with anti Myc antibodies (panels A and B, lane 1) and probed with anti phosphotyrosine antibodies (panel A) or anti Nedd4 antibodies (panel B). Ca\(^{2+}\)- and serum-starved R-/IR-Grb10 cells (panels C, D and E, lanes 1, 3 and 5) were stimulated with Ca\(^{2+}\) + ionomycin (panels C, D, E, lanes 2, 4 and 6) and immunoprecipitated with anti Nedd4 antibodies (lanes 1 and 2) or anti Myc antibodies (lanes 3 and 4), and probed with anti phosphotyrosine (panel C), Nedd4 (panel D) or Grb10 (panel E) antibodies. Lane 2 in panels A and B, lanes 5 and 6 in panels C, D and E are total cell lysates loaded as a control.
### IP: anti-myc

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#### A

- **P-tyr blot**
  - 200 -
  - 97 -

#### B

- **Nedd4 blot**
  - 200 -
  - 97 -

#### C

- **P-tyr blot**
  - 97 -

#### D

- **Nedd4 blot**
  - 97 -

#### E

- **Grb10 blot**
  - 68 -
The interaction between Grb10 and Nedd4 is constitutive

To investigate if the binding between Grb10 and Nedd4 is regulated by growth factors, we used two different cell lines over-expressing myc-tagged mGrb10 in combination with either the insulin receptor (R-/IR/Grb10) or the IGF-IR (p6/Grb10) (Morrione et al., 1997). The cells were serum-starved, stimulated with insulin (R-/IR/Grb10) or IGF-I (p6/Grb10) (see experimental procedures) and then immunoprecipitated with either Nedd4 or Myc antibodies. As seen in figure A-4, in both cell lines, we detected co-immunoprecipitation of Grb10 with Nedd4 in both stimulated and unstimulated cells. These results therefore demonstrate that the interaction is constitutive and not modulated by growth factors. The identity of the additional proteins immunoprecipitated by Nedd4 antibodies or co-precipitated with Nedd4 in either the insulin or the IGF-I-stimulated cells is currently unknown. Only a small fraction of the total Nedd4 protein appears to be tyrosine-phosphorylated either by insulin (in R-/IR/Grb10 cells), or by IGF-I (in the p6/Grb10 cells), and a tyrosyl-phosphorylated Nedd4 is detectable in the coimmunoprecipitation by myc antibodies only after a longer exposure of the film; it is likely therefore that the majority of the Nedd4 protein is co-immunoprecipitating with Grb10 in a phosphotyrosine-independent manner, in agreement with the original identification of the interaction in the yeast two hybrid system.

Grb10 is not ubiquitinated in vivo but the IGF-IR is ubiquitinated

Because Nedd4 is a ubiquitin protein ligase (E3) (Kumar et al., 1992; Kumar et al., 1997; Bork et al., 1994; Staub et al., 1996) we wanted to investigate if Grb10, which interacts with it, is a target for ubiquitination by Nedd4. We therefore tested whether Grb10 is ubiquitinated in vivo, using a previously described methodology (Staub et al., 1997). Thus, we transiently transfected R-/IR cells over-expressing Grb10 with a plasmid encoding His-tagged multiubiquitin (Treier et al., 1994), precipitated the histidinated (hence ubiquitinated) cellular proteins with Ni²⁺ agarose beads and immunoblotted the precipitated proteins with anti Grb10 antibodies. As can be seen in figure 4-5A, we did not detect any ubiquitination of Grb10, despite relatively strong ubiquitination of the α
Figure A-4. Effect of IGF-I and insulin on Grb10/Nedd4 binding. R-/IR/Grb10 cells and p6/Grb10 cells were serum-starved for 72 hrs, stimulated (see Experimental Procedures) with insulin (R-/IR/Grb10) or IGF-I (p6/Grb10), immunoprecipitated with either anti-Nedd4 or anti-myc antibodies and then probed with anti-phosphotyrosine, anti-Nedd4 and anti-Grb10 antibodies.
Figure A-5. **Lack of in vivo ubiquitination of Grb10 protein.** Lack of *in vivo* ubiquitination of Grb10 and ubiquitination of the IGF-I receptor expressed in R-/IR/Grb10 cells. R-/IR/Grb10 cells stably expressing Grb10 were transiently transfected (+) or not (-) with HA-tagged arENaC (ENaC Tx) and/or His-ubiquitin (His-Ub Tx). The cells were lysed and lysates were incubated with Ni²⁺-NTA agarose beads (Ni²⁺) to precipitate histidinated (ubiquitinated) proteins. These proteins were separated on 8% SDS-PAGE, transferred to nitrocellulose and blotted with (A) anti-HA antibodies (to detect ubiquitinated arENaC) or anti-Grb10 antibodies. Ubiquitinated species appear as a high molecular weight smear. Lysates represent expression of the protein. (B) 3T3 cells stably expressing IGF-IR and myc tagged Grb10 were transiently transfected (+) or not (-) with an 8x HA-tagged ubiquitin construct (HA-Ub Tx). Cell lysates were immunoprecipitated with anti-IGF-IRb or anti-Grb10 antibodies and blotted with anti-HA antibodies to detect ubiquitinated species. The lysate from HA-Ub transfected (+) cells serves as a positive control for HA-Ub transfection. Arrows indicate the molecular weight of the corresponding proteins.
subunit of the epithelial Na⁺ channel (αENaC) used as a positive control (Staub et al., 1997). These results show that Grb10 is not ubiquitinated in vivo in exponentially growing R-IR cells over-expressing Grb10. We repeated this experiment in p6/Grb10 cells and once again did not detect any ubiquitination of Grb10 (data not shown). Because Grb10 is known to interact with the IGF-I receptor, we wanted to determine whether the receptor, itself, was ubiquitinated. We performed a similar assay for in vivo ubiquitination of the IGF-IR, using R-IR cells over-expressing Grb10 that were transfected with HA-tagged ubiquitin. Following immunoprecipitation of the receptor and immunoblotting with anti-HA antibodies, we demonstrated that the receptor was ubiquitinated (Fig. A-5B).

IV. Discussion

We and others have recently isolated mGrb10 (Ooi et al., 1995) as an interacting protein with the IGF-I receptor (Morrione et al., 1996; Dey et al., 1996; O’Neill et al., 1996) and we showed an inhibitory effect of mGrb10 on IGF-I-mediated mitogenesis (Morrione et al., 1997). To identify new interacting partners for Grb10, we performed a yeast two-hybrid screen of a mouse embryo library (Vojtek et al., 1993) using mGrb10 as a bait, and identified Nedd4 as a Grb10 interacting protein. We further showed that: (i) The C2 domain of Nedd4 is sufficient for the interaction; (ii) A deletion of the mGrb10 C-terminal 148 amino acids, a region that includes the newly identified BPS domain and the SH2 domain, abolishes the interaction with Nedd4; (iii) The SH2 domain alone of Grb10 shows the strongest interaction with Nedd4 but the BPS domain alone is also able to bind Nedd4; (iv) Grb10 and Nedd4 interact in vivo, as assessed by co-immunoprecipitation experiments; (vi) Nedd4 co-immunoprecipitating in vivo with Grb10 is tyrosyl-phosphorylated and this interaction is Ca²⁺-independent; (vii) the interaction is constitutive; and (viii) Grb10 is not ubiquitinated in vivo in mouse embryo fibroblasts over-expressing Grb10.

Mouse Nedd4, originally identified by a subtractive cloning in neural precursor mouse cells (Kumar et al., 1992), has been shown to be expressed in a variety of embryonic tissues. Nedd4 is a
ubiquitin protein ligase (E3) containing 3 WW domains (4 in the mouse), and a HECT domain. The WW domains of rat Nedd 4 have been shown to interact with the epithelial sodium channel (ENaC), recognizing proline-rich PY motifs (Staub et al., 1996) and regulating channel activity and ubiquitination (Goulet et al. 1998; Abriel et al., 1999), while the C2 domain is mediating Ca\(^{2+}\)-dependent translocation to the plasma membrane (Plant et al., 1997).

C2 domains encompass approximately 130 residues (including the CaLB region) (Clark et al., 1991), and contains five conserved aspartates which provide Ca\(^{2+}\)-binding sites (Sutton et al., 1995; Shao et al., 1996; Essen et al., 1996). The domain has been shown to mediate Ca\(^{2+}\)-stimulated phospholipid and membrane binding (Plant et al., 1997; reviewed in Nalefski and Falke, 1996). Evidence is accumulating showing that some C2 domains can bind proteins as well: the C2A and C2B domains of synaptotagmin bind different proteins (reviewed in Sudhof and Rizo, 1996), the PKC\(\beta\)-C2 protein binds RACKs (receptor for activated C-kinase) upon activation (Ron et al., 1995), and it has been recently shown that p120\(^{GAP}\) is interacting with Annexin VI by its CaLB domain (Davis et al., 1996), confirming the role of C2/CaLB domains in protein-protein interaction. The fact that Grb10 is interacting with Nedd4 in a Ca\(^{2+}\)-independent manner is in agreement with increasing lines of evidences showing that some C2 domains which are Ca\(^{2+}\)-regulated can bind other molecules in a Ca\(^{2+}\)-independent manner (reviewed in Rizo and Sudhof, 1998).

The interaction between Grb10 and Nedd4 \textit{in vivo} is phosphotyrosine-independent, and is not influenced by mitogenic agents. Our finding that an SH2 domain can bind a C2 domain and that this is a tyrosine phosphorylation-independent interaction is novel and interesting, as it suggests an association which does not involve the binding pocket in the SH2 domain reserved for phosphotyrosine. Other phosphotyrosine-independent interactions with SH2 domains have already been reported in the literature (Pendergast et al., 1991; Muller et al., 1992; Cleghon and Morrison, 1994; Raffel et al., 1996). Moreover, it has been recently shown that the Grb10 SH2 domain can bind Raf1 and MEK1 kinases in a phosphotyrosine-independent manner, and that the interaction with Raf1 is constitutive and not modulated by growth factors (Nantel et al., 1998). How the SH2
domain of Grb10 binds the C2 domain of Nedd4 is unknown, and we can not currently preclude
the possibility of phospholipids involvement in this interactions, since the latter have been
demonstrated to bind both SH2 and C2 domains (Rizo and Sudhof, 1998; Rameh et al., 1995).

Ubiquitination of proteins usually tag them for rapid degradation (reviewed in Ciechanover,
1994) and many lines of evidence are now accumulating on the role of the ubiquitin-proteosome
system in regulating and degrading a number of cytosolic proteins, including cell cycle proteins
(reviewed in Deshaies 1995). Some transmembrane proteins are ubiquitinated as well, including
several tyrosine kinase receptors like the EGF receptor and the PDGF receptor (Galcheva-Garbova
et al., 1995; Mori et al., 1992), and ubiquitination seems to be involved in their subsequent
degradation by the endosomal/lysosomal pathway (Hochstrasser, 1996). We could not detect any
ubiquitination in vivo of Grb10: however we can propose that the interaction between Grb10 and
Nedd4 may be used to target other proteins, such as the IGF-1 receptor or the insulin receptor, for
ubiquitination. Indeed, our results and those of others (Sepp-Lorenzino et al., 1995) have
demonstrated ubiquitination of the IGF-1 receptor in vivo, prompting the need to further examine
the possibility that by interacting with the IGF-IR and Nedd4, Grb10 may promote receptor
ubiquitination by Nedd4.
References


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228


230


APPENDIX II:

Alternative Mechanisms of Vacuolar Acidification in H+-ATPase-Deficient Yeast

Pamela J. Plant¹,², Morris F. Manolson¹,², Sergio Grinstein¹,², and Nicolas Demaurex³

¹Division of Cell Biology, Hospital for Sick Children, Toronto, Ont., M5G 1X8, Canada, ²Department of Biochemistry, University of Toronto, Toronto, Ont., M5S 2Z9, Canada, and ³Department of Physiology, University of Geneva Medical Center, 1211 Geneva 4, Switzerland

* This work was published in the Journal of Biological Chemistry (1999) 274 (52): 37270-37279
SUMMARY

Acidification of the endosomal/lysosomal pathway by the vacuolar-type proton translocating ATPase (V-ATPase)\(^1\) is necessary for a variety of essential eukaryotic cellular functions. Nevertheless, yeast lacking V-ATPase activity (\(\Delta vma\)) are viable when grown at low pH, suggesting alternative methods of organellar acidification. This was confirmed by directly measuring the vacuolar pH by ratio fluorescence imaging. When \(\Delta vma\) yeast were cultured and tested in the acidic conditions required for growth of V-ATPase-deficient mutants, the vacuolar pH was 5.9. Fluid-phase pinocytosis of acidic extracellular medium cannot account for these observations, because the V-ATPase-independent vacuolar acidification was unaffected in mutants deficient in endocytosis. Similarly, internalization of the plasmalemmal H\(^+\)-ATPase (Pma1p) was ruled out, because overexpression of Pma1p failed to complement the \(\Delta vma\) phenotype and did not potentiate the vacuolar acidification. To test whether weak electrolytes present in the culture medium could ferry acid equivalents to the vacuole, wild-type and the \(\Delta vma\) yeast were subjected to sudden changes in extracellular pH. In both cell types, the vacuoles rapidly alkalinized when external pH was raised from 5.5 (the approx. pH of the culture medium) to 7.5, and re-acidified when the yeast were returned to medium of pH 5.5. Importantly, these rapid pH changes were only observed when NH\(_4^+\), routinely added as a nitrogen source, was present. The NH\(_4^+\)-dependent acidification was not due to efflux of NH\(_3\) from the vacuole, as cells equilibrated to pH 7.5 in the absence of weak electrolytes rapidly acidified when challenged with an acidic medium containing NH\(_4^+\). These findings suggest that while NH\(_3\) can act as a cell-permeant proton scavenger, NH\(_4^+\) may function as a protonophore, facilitating equilibration of the pH across the plasma and vacuolar membranes of yeast. The high concentration of NH\(_4^+\) frequently added as a nitrogen source to yeast culture media, together with effective NH\(_4^+\) transporters, thereby facilitate vacuolar acidification when cells are suspended in acidic solutions.
INTRODUCTION

Acidification of defined endomembrane compartments along the endocytic and secretory pathways is essential for cellular function. Intraorganellar acidification appears to control vesicular traffic (1) as well as receptor-ligand dissociation within endosomes (1). Similarly, acidification is thought to be essential for protein degradation in lysosomes (2) and for microbial elimination in phagosomes (3). In yeast and plants acidification of the vacuole provides the driving force for secondary transport of a variety of ions and metabolites (4-7).

Acid equivalents are concentrated in the lumen of these organelles by active H⁺ pumping mediated by the vacuolar-type ATPase (V-ATPase), an evolutionarily conserved multimeric enzyme (see (8-11) for review). Considering the array of critical cellular functions that depend on the acidification generated by the V-ATPase, this enzyme would be predicted to be essential for cell survival. Accordingly, attempts to isolate Neurospora mutants with defective V-ATPase have failed repeatedly (12). By contrast, a variety of yeast mutants devoid of detectable V-ATPase activity (Δvma) have been isolated (see (10) for review). Interestingly, such mutants are viable only when grown in rich media buffered to acidic pH. These V-ATPase deficient mutants are unable to grow in media buffered to neutral pH (13-15), in media containing high concentrations of Ca²⁺ (16) or Zn²⁺, or when glycerol is used as the sole carbon source (reviewed in (10)). These observations suggest that acidification might not be essential for growth, but required only under stress conditions. Alternatively, when grown under rich acidic conditions, yeast may possess additional mechanisms independent of the V-ATPase to acidify their endomembrane compartments. In this regard, it is unclear whether Δvma mutants can in fact acidify their vacuole. Flow cytometric determinations of vacuolar pH in Δvphl (17) and Δvma2 mutants (13) yielded values that are somewhat lower than those reported for the cytosolic pH of yeast (18,19). While the significance of this pH difference has not been ascertained, the existence of V-ATPase-independent methods of vacuolar acidification must be considered.

Several alternative pathways for vacuolar acidification can be envisaged. Nelson and Nelson (15) proposed that yeast might acidify their endocytic compartments, and ultimately their vacuole,
by fluid-phase uptake of acid equivalents from the external medium. In support of this hypothesis, Munn and Reizmann (20) found that Δvma mutants were unable to survive when they were also defective in fluid-phase and receptor-mediated endocytosis. Alternatively, vacuolar acidification in Δvma mutants could result from internalization of the plasma membrane H⁺-ATPase (Pma1p), a monomeric protein that normally extrudes protons from the cytosol to the surrounding environment (21,22). If internalized in its active state, Pma1p might contribute to the acidification of intracellular organelles. Finally, when yeast are grown in rich media at low pH, acidification of the vacuole may result simply from the passive leakage of extracellular weak acids, which could reach and dissociate in the endosomal or vacuolar lumen.

In this report, we developed a sensitive microspectroscopic method to determine the pH within vacuoles of intact S. cerevisiae. Using this approach, we proceeded to compare the pH of vacuoles from wild type and Δvma mutant yeast and analyzed the mechanisms underlying the partial acidification observed in V-ATPase-deficient mutants.
EXPERIMENTAL PROCEDURES

Materials and media. 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein -acetoxyxethyl ester (BCECF-AM) was obtained from Molecular Probes Inc. (Eugene, Oregon USA). Bafilomycin-A1, concanamycin A and concanavalin A were from Sigma. YPD medium contained 1% yeast extract, 2% Bacto-peptone (both from Difco; Detroit, Michigan USA) and 2% dextrose.

Strains and plasmids. The following yeast strains were used: BJ926: MATa/α trp1/+/his1 prc1-126/prc1-126 pep4-3/pep4-3 prb1-1122/prb1-1122 can1/can1 gal2/gal2. SF838-5A (Δvma4) MATα leu2-3,112 ade6 ura3-52 vma4Δ::URA3 (kindly supplied by Dr. T. Stevens, University of Oregon). RH268-1C: MATa, end4, ura3, leu2 his4, bar1-1 (kindly supplied by Dr. H. Riezman, University of Basel). The plasmid YCp2HSE-PMAL (kindly provided to us by Dr. C. Slayman, Yale University) contains the full-length PMA1 gene preceded by two tandem copies of the heat shock element (23).

Growth and labeling of yeast. Strains were grown in rich medium (YPD) or minimal medium for transformed yeast as described before in Ausubel et al. (24). Buffered medium was prepared by the addition of 50 mM 3-(N-morpholino)ethanesulfonic acid (MES) to either rich or minimal media, and the pH adjusted to the indicated value with NaOH. YPD plates supplemented with 100 mM CaCl2, 4 mM ZnCl2, or buffered to pH 7.5 with 50 mM MES-Tris were prepared as described in Manolson et al. (25). Growth phenotypes of Δvma and Δvma-YCp2HSE-PMA1 were assessed as described before (25), after incubating the plates at 30°C or 37°C for 3 days for -leu (pH 5.5), 100 mM CaCl2, and pH 7.5 buffered plates, and for 5 days for plates containing 4 mM ZnCl2.

For vacuolar pH determinations, yeast were harvested at 10⁷ cells/ml and resuspended in rich medium containing 50 μM BCECF-AM and incubated at the specified temperature for 15-30 min. For measurements of cytosolic pH, the cells were loaded in rich medium buffered to pH 7.5. Yeast were then sedimented, washed three times in rich medium, resuspended at 2x10⁷ cells/mL in the indicated synthetic growth media and used immediately for imaging.

243
Video microscopy and pH imaging. Preparation of cells. For imaging, 100 μl of the BCECF-loaded yeast suspension was plated onto glass coverslips that had been pre-coated with concanavalin A (Sigma) as described (26). The coverslips were inserted into a temperature-controlled perfusion chamber (Medical Systems Corp., Greenvale, NY) and placed on the stage of an inverted microscope (Axiovert 100 TV, Zeiss, Germany). Image acquisition proceeded immediately after the addition of 1 ml of the appropriate recording solution to the chamber. Yeast pH was recorded in the synthetic growth media described above, as normal growth media or YPD produced background autofluorescence.

Fluorescence microscopy. Ratio fluorescence imaging was performed as described (27), using a 63x/1.25 NeoFluar objective (Zeiss), a 75 W Xenon epifluorescence lamp, and a shutter/filter-wheel assembly for wavelength selection (Sutter Instruments, Novato, CA). Images were acquired on a 1317x1035 pixels cooled digital CCD camera running at 1 MHz (Princeton Instruments, Trenton, NJ). Image acquisition and excitation filter selection was controlled by the Metafluor software (Universal Imaging, West Chester, PA). Red illumination allowed concomitant visualization of the cells by differential interference contrast (DIC), using a separate video camera (Dage-MTI, Michigan City, IN).

The bright fluorescence of BCECF at both excitation wavelengths (440 and 490 nm) and the use of high transmission objectives allowed to image the fluorescence of yeast vacuoles at the full resolution of the CCD array (i.e. no binning, pixel size 0.1 x 0.1 μm; e.g. Fig. 1). For statistical analysis, however, lower resolution images were used (4x4 binning; pixel size = 0.4 x 0.4 μm) in order to decrease exposure time and minimize photobleaching and possible phototoxicity. The 490 nm and 440 nm fluorescence images were corrected for shading to compensate for uneven illumination, the background was subtracted and a threshold of 5 times the value of the background noise (RMS) was applied before obtaining a pixel-by-pixel ratio of the two images. Sub-threshold pixels were neither displayed nor used for subsequent analysis to prevent artifacts caused by ratioing near-zero values.
Calibration. At the end of each experiment, a calibration curve of fluorescence ratio vs. pH was obtained in situ by sequentially perfusing the cells with media containing 50 mM 2-(N-morpholino)ethanesulfonic acid (MES), 50 mM Hepes, 50 mM KCl, 50 mM NaCl, 0.2 M ammonium acetate, 10 mM NaN₃, 10 mM 2-deoxyglucose, 50 mM carbonylcyanide m-chlorophenylhydrazone (CCCP), buffered at 4 different pH values ranging from 5.0-7.0 with NaOH as previously described (17).

Image processing. An automated procedure was implemented to produce pH histograms or time graphs from the image data, using the pH independent (440 nm) image to define individual cells. A small percentage of the yeast (<5%) had sustained cellular damage and displayed a bright fluorescence (>100 fold the normal intensity). These cells were excluded from the analysis by applying a high intensity threshold, whereas a low intensity threshold was used to define the edges of the vacuole. The image was then binarized, and the fluorescent objects outlined by the computer (sketchionization). A size criterion was applied to retain only objects within ± 2 standard deviations of the average size of yeast vacuoles. The average fluorescence ratio of individual yeast vacuoles was then calculated and converted to pH. This procedure allowed to analyze an average of 20 yeast per image, thus producing statistically significant information from images acquired within short (<10 min) intervals.

Immunoblotting Yeast lysates from SF838-5A (vma4Δ) transformed with YCp2HSE-PMA1 were prepared by growing cells at 30°C to mid-log phase and then shifting to 37°C for 1 h. Cells were harvested, washed and resuspended in 63 mM Tris-HCl, pH 6.8, 1% SDS, 0.6 mM β-mercaptoethanol, 5% glycerol, 0.1 mM phenylmethylsulphonyl fluoride and 1μg/mL pepstatin A together with an equal volume of acid-washed glass beads. Samples were vortexed 3 x 30 sec at 4°C. Extracts were heated at 55°C for 10 min and cellular debris were removed by centrifugation for 5 min at 15,000xg, at 4°C. Equal amounts (50 μg) of protein were analyzed by SDS-PAGE, transferred to nitrocellulose and probed with a monoclonal antibody against Pma1p (kindly supplied by Dr. J. Teem, Florida State University). Primary antibodies were detected with
horseradish peroxidase -conjugated secondary antibody and visualized by chemiluminescence (ECL, Amersham).
RESULTS

**BCECF accumulates in the yeast vacuole.** The fluorescein derivative BCECF has been used extensively to measure the cytosolic pH in a variety of cell systems. When added to animal cells in its esterified precursor form, BCECF accumulates in the cytosol where esterases release its polyanionic, membrane impermeant form (see (28) for review). In yeast loaded in normal growth medium, we found that cytosolic accumulation of BCECF was marginal (Fig. 1). Instead, fluorescence was considerably brighter in an intracellular compartment that could be readily identified as the vacuole by simultaneous differential interference contrast (DIC) microscopy (cf. left and right panels in Fig. 1). BCECF and other heavily esterified dyes have been reported to accumulate in endomembrane compartments of fibroblasts and of **Neurospora crassa**, a close relative of **S. cerevisiae** (29). The atypical distribution of BCECF can be attributed to the abundance of hydrolases in the vacuole, which effectively cleave the acetoxyethyl ester moiety from the precursor form. Regardless of the underlying mechanism, this observation provided a convenient and reproducible method for the noninvasive measurement of vacuolar pH. Using a high intensity threshold to exclude unwanted signals, we could readily measure the vacuolar pH by ratio fluorescence imaging (Fig. 1, central panel). BCECF was found to localize to the vacuole not only in wild-type yeast, but also in V-ATPase deficient mutants (Fig. 1). In both types of cells the fluorescent probe was retained in the vacuole for extended periods: loss of BCECF was insignificant for approximately 1 h at room temperature.

**Calibration of fluorescence ratio vs. pH.** The procedure of Preston et al. (17) was used to manipulate and calibrate the vacuolar pH. The method uses solutions containing a metabolic inhibitor (deoxyglucose) to preclude regulation by endogenous transporters, a protonophore (CCCP) to increase H⁺ permeability across membranes and very high concentrations of permeant weak electrolytes (0.2 M ammonium acetate), expected to mobilize and equilibrate acid equivalents across membranes. To validate the effectiveness of this pH-clamping procedure, the behavior of BCECF within the vacuole was compared to that observed *in vitro*, solubilizing the
Figure 1. The pH-sensitive dye BCECF accumulates in yeast vacuoles. The yeast strains BJ926 (wild-type: WT) and SF838-5A (V-ATPase-deficient: Δvma4) were grown to a density of 1x10^7 cells/ml and resuspended in rich medium containing the pH sensitive dye BCECF-AM for 30 min at 30°C. Yeast were then harvested, washed, adhered to concanavalin A-coated glass coverslips, and fluorescence was imaged as described in Materials and Methods. Shown are images of the fluorescence at the pH-independent excitation wavelength of 440 nm (left), fluorescence ratio in pseudocolor (middle) and cell morphology seen by Nomarski optics (right). The pH scale was derived from the excitation ratio of BCECF fluorescence, as described in Fig. 2. Bar, 5 μm.
free acid of BCECF in calibration solutions of varying pH in the absence of yeast. Typical results comparing the pH dependence of the fluorescence ratio are illustrated in Fig. 2. As shown in Fig 2A, although the dynamic range of the signal was reduced by 1.5 fold in situ, both titration curves had comparable sigmoidal shape and yielded similar values for the apparent pK₂ of BCECF, namely 6.50 in vitro and 6.65 in situ. A reduced dynamic range in situ is invariably observed in measurements with fluorescent dyes (30), and in this case probably reflects the interaction of BCECF with vacuolar constituents. We also found that the fluorescence ratio of the population distributed normally around the mean, that all the cells in the population responded to the calibration procedure (Fig. 2B) and that the pH calibration curves were identical for wild-type and Δvma4 yeast (not shown). Based on these cumulative data, BCECF was deemed to be an adequate probe for quantitative measurements of vacuolar pH and the in situ calibration procedure was used hereafter to convert the measurements of fluorescence ratio to luminal pH.

*V-ATPase deficient yeast have acidic vacuoles when grown in acidic media.* Δvma yeast cannot grow in alkaline buffered media (pH 7.5), but grow optimally in media buffered to pH 5.5. If vacuolar acidification is essential for survival, this observation might suggest that yeast are able to acidify their vacuoles in acidic media, but not in media buffered at pH 7.5. Both wild-type and Δvma yeast were grown overnight at pH 5.5, loaded with BCECF under the same conditions and transferred to synthetic growth medium for measurement of fluorescence. Synthetic medium, as opposed to the conventional rich growth medium (yeast extract, peptone, dextrose) was used for pH determinations because the latter solution displayed a high degree of autofluorescence. Fig. 3A shows a typical distribution of vacuolar pH (pHVac) values of wild-type and Δvma yeast when recorded in medium of pH 5.5; although more alkaline than the wild-type yeast vacuoles (average pHVac = 5.45), the mutant yeast vacuoles are nevertheless considerably acidic (average pHVac = 5.9). When transferred to a medium of comparable composition buffered to pH 7.5, vacuoles of both wild-type and mutant cells became more alkaline (Fig. 3B). However, the wild-type cells underwent a comparatively minor pH shift, reaching
Figure 2. pH calibration of the vacuolar fluorescence. (A) Calibration curves of BCECF excitation ratio (440/490 nm) versus medium pH. Left panel: Calibration obtained in vitro by imaging 1 μM BCECF in the same solutions used for the in situ recordings. Right panel: in situ calibration. The vacuolar fluorescence of BJ926 yeast labeled with BCECF-AM was analyzed by ratio imaging. The vacuoles were equilibrated with recording solutions buffered to different pH values, as described in Materials and Methods. Data are mean ±SEM of >200 measurements for each condition. (B) Distribution histogram of the fluorescence ratio (abscissa) of vacuoles equilibrated at different pH (indicated over the curves).
Figure 3. Vacuoles of Δcyc1 yeast are acidic, but only when grown in pH 5.5 buffered media. Wild-type yeast (BJ926) and V-ATPase deficient mutants (SF838-5A) were labeled with BCECF-AM, and vacuolar pH was measured by fluorescence ratio imaging. (A) Yeast were imaged immediately after growth and labeling, in synthetic medium buffered to pH 5.5. (B) After the measurements at pH 5.5, the cells were equilibrated for 1 h with a similar medium buffered to pH 7.5 and analyzed again by imaging. In situ calibration curves were used to convert measurements of fluorescence ratios into vacuolar pH values.
A

Number of cells

WT  vma 4

pH 5.5

B

Number of cells

WT  vma 4

pH 5.5 to 7.5

Vacuolar pH
steady state at a level ($pH_{\text{vac}} = 5.9$) that is considerably more acidic than that of the medium. By contrast, the vacuolar alkalization was much more pronounced in V-ATPase-deficient cells (average $pH_{\text{vac}} = 7.05$). This differential behavior is likely due to the activity of the V-ATPase, a notion that was validated using bafilomycin. Wild-type cells incubated at pH 5.5 in the presence of this V-ATPase antagonist equilibrated at a $pH_{\text{vac}} = 6.06$, which was indistinguishable from that observed in the $\Delta vma$ mutants. At pH 7.5, bafilomycin increased the steady state pH of wild-type cells by >1.3 pH units. Jointly, these observations suggest that $\Delta vma$ mutants or cells in which the V-ATPase has been pharmacologically ablated are able to acidify their vacuoles only when grown at pH 5.5. In growth media buffered to pH 7.5 the vacuoles of $\Delta vma$ yeast are no longer acidic, which may account for their inability to grow under such conditions.

**The plasma membrane ATPase does not contribute to vacuolar acidity.** Yeast possess two distinct types of $H^+$-pumping ATPases, the endomembrane V-type pump and the plasma membrane P-type pump. The latter is present in the plasma membrane, where it extrudes $H^+$ from the cytosol, regulating intracellular pH and generating a transmembrane proton-motive force that is utilized by the yeast to translocate substrates by coupled transport (reviewed in (21,22,31)). In yeast, the plasma membrane $H^+$-ATPase is encoded by two $PMA$ (Plasma membrane $H^+$-ATPase) genes, $PMA1$ and $PMA2$ (32,33). Only the $PMA1$ gene is constitutively expressed and essential to growth (32). Because the lumen of the endocytic pathway is topologically equivalent to the extracellular space, endocytosis of the plasma membrane $H^+$-ATPase, possibly during the normal degradative cycle of the pump, could in principle contribute to vacuolar acidification. Alternatively, Pma1p could be mistargeted to the vacuole during its synthesis in the $\Delta vma$ mutant, due to alterations in the pH of the trans-Golgi. Accelerated internalization or synthesis of these pumps, or the increased availability of substrate, could account for the ability of the $\Delta vma$ mutants to partially acidify their vacuole at acidic extracellular pH.

Gene disruption cannot be used to ascertain the role of Pma1p in vacuolar acidification, since the gene encoding this pump, $PMA1$, is essential. Instead, it was more practical to overexpress
PMU to assess whether this would accentuate vacuolar acidification and possibly complement the Δvma phenotype, enabling the cells to grow at alkaline pH. To this end, PMAI was placed under the control of two heat shock elements (YCp2HSE-PMAI) expected to increase the level of expression of Pma1p when transformed yeast are grown at 37°C (23). To ensure that growth at 37°C effectively induced overexpression of Pma1p, lysates from transformed yeast grown at 30°C and 37°C were prepared and equal amounts of protein were analyzed by SDS-PAGE. Following transfer to nitrocellulose, immunoblots were probed with monoclonal antibodies to Pma1p. As shown in Fig. 4, yeast grown at 37°C were found to overexpress Pma1p.

Complementation of Δvma phenotypes by Pma1p overexpression was tested by growth in media supplemented with Ca²⁺ or Zn²⁺, or buffered to alkaline pH (Table 1). As reported earlier Δvma strains were unable to grow at high pH or in the presence of 100 mM Ca²⁺ or 4 mM Zn²⁺, presumably because removal of excess cations from the cytosol requires transport into the vacuole in exchange for luminal H⁺ (5). Acidification of the vacuolar lumen by the overexpressed Pma1p could result in significant growth under these conditions, despite the absence of functional V-ATPases. However, as documented in Table 1, YCp2HSE-PMAI transformed yeast were unable to grow under these conditions at either 30°C or 37°C. This was not due to toxicity associated with overexpression of Pma1p, since the cells grew normally at pH 5.5. Instead, these observations suggest that Pma1p does not contribute to vacuolar acidification.

This was confirmed by direct fluorimetric measurement of pHvac (Fig. 5). Overexpression of Pma1p in a Δvma mutant (vma4 + PMA1, grown at 37°C) did not affect the vacuolar pH under any of the conditions tested. When measured in media of pH 5.5, pHvac was 5.9 in the untransformed Δvma4 cells, as well as in the transformants, whether they were grown at or below the inductive temperature. Similarly, when the cells were bathed in media of pH 7.5, the pH of the vacuoles averaged 7.0 in all cases (Fig. 5). Although we cannot exclude that Δvma mutants might also express PMA2 in addition to PMA1, the two isoforms are expected to be functionally equivalent. Because overexpression of Pma1p had no effect on the pH of the vacuole, nor was it
Table I. Overexpression of Pma1p does not complement Δvma growth phenotypes. BJ926 (WT), SF838-5A (Δvma4) yeast and SF838-5A strains transfected with the plasmid YCp2HSE-PMA1 (Δvma4-PMA1) were grown at 30°C and 37°C on minimal media plates supplemented with 100 mM CaCl2, 4 mM ZnCl2, or buffered to pH 7.5 with 50 mM MES-Tris. Three plus signs indicate growth levels equivalent to wild-type strains; minus signs indicate no visible growth.
Figure 4. Inducible Overexpression of the plasma membrane H+-ATPase (Pma1p). The strain SF838-5A (Δvma4) transformed with the plasmid YCp2HSE-PMA1 was grown at 30°C in selective medium to mid-log phase and then either shifted to 37°C or maintained at 30°C for an additional 1 h. Lysates of the yeast were prepared as described in Materials and Methods and aliquots containing 50 μg of protein were analyzed by SDS-PAGE, transferred to nitrocellulose and probed with a monoclonal antibody against Pma1p (kindly supplied by Dr. J. Teem, Florida State University). Primary antibodies were detected with horseradish peroxidase-conjugated secondary antibody and visualized using ECL. The region immediately surrounding the 100 kDa band is illustrated; no other bands were observed on the blot. The position of the 97.4 kDa standard is indicated.
growth temperature:
30°  37°

blot: α PMA1
Figure 5. Overexpression of Pma1p does not affect vacuolar acidification. V-ATPase deficient yeast (SF838-5A) native (top panel) or containing the plasmid YCp2HSE-PMA1 were grown and labeled in pH 5.5 minimal medium at either 30°C (middle panel) or 37°C (bottom panel). Cells were harvested and resuspended in synthetic medium buffered to pH 7.5 and imaged immediately (open bars). The recording medium was then changed to pH 5.5 and the vacuolar pH measured again after a period of approx. 10 min (shaded bars).
Vacuolar pH

Number of cells

Vacuolar pH

- vma4
- vma4 + PMA1 (30°)
- vma4 + PMA1 (37°)
able to complement even partially the Δvma phenotypes, we concluded that plasmalemmal pumps are unlikely to contribute significantly to vacuolar acidification.

*Endocytosis does not contribute to vacuolar acidification.* Munn and Riezman (20) showed that mutations that disable the endocytic pathway are synthetically lethal with Δvma mutations. This finding supports the notion, originally proposed by Nelson and Nelson (15), that internalization of acidic extracellular medium by fluid-phase endocytosis may contribute to vacuolar acidification. To assess the contribution of endocytosis to vacuolar acidification in V-ATPase-deficient yeast, strains of yeast bearing a temperature sensitive mutation in endocytosis (*end4-1*) were subjected to fluorescence imaging. When such yeast are grown at 37°C for 30 min, both fluid-phase and receptor-mediated endocytosis are blocked (34). Following growth at the permissive temperature, *end4-1* were treated at 30°C or 37°C for approximately 30 min. During this period the yeast were also labeled with BCECF and then immediately used for determination of pH vac. To evaluate the role of pinocytosis of acidic medium, without the confounding effects of the V-ATPase which is present and active in the *end4-1* mutants, the cells were suspended in media containing bafilomycin. As shown in Fig. 6, cells suspended at pH 7.5 in the presence of the V-ATPase antagonist had pH vac values that were similar to those of Δvma4 mutants, confirming the effectiveness of bafilomycin (compare the top and bottom panels). More importantly, the vacuolar acidification that occurs when Δvma4 cells are transferred to pH 5.5 medium was also observed in the *end4-1* mutants incubated at the restrictive temperature (i.e. 37°C). In fact, no difference in pH vac was noted between *end4-1* cells grown at 30°C or 37°C and tested in acidic media, regardless of the presence of bafilomycin (Fig. 6). In the absence of the inhibitor, however, the *end4-1* cells became acidic even when maintained at pH 7.5 (Fig. 6), due to proton pumping by the V-ATPase. Parallel experiments demonstrated that the endocytosis defect characteristic of *end4* mutants persisted at low pH. Fluid phase endocytosis, monitored as the uptake of Lucifer yellow was markedly decreased in *end4* cells grown at the restrictive temperature, regardless of the external pH (89% inhibition at pH 7.5 vs. 91% inhibition at pH 5.5). Because nearly complete
**Figure 6. Blocking endocytosis does not affect vacuolar acidification.** Yeast with a temperature-sensitive endocytosis defect (*end4-1*) and V-ATPase deficient yeast (SF838-5A; top panel) were equilibrated for 1 h in synthetic medium buffered to pH 7.5, and vacuolar pH was measured by ratio imaging (open bars). The recording solution was then changed to synthetic medium buffered to pH 5.5 and the yeast imaged again after approx. 5 min (shaded bars). When indicated (+baf), 5 μM bafilomycin-A₁ was added to the recording solution to block endogenous V-ATPase activity in the *end4-1* mutant.
inhibition of endocytosis had little effect on pH\textsubscript{vac} at pH 5.5, we concluded that delivery of acid equivalents to the vacuole does not occur by fluid-phase endocytosis of acidic medium.

**Role of weak electrolytes on vacuolar pH.** During the course of the experiments described above, we noted that while wild-type yeast maintained an acidic vacuolar pH in the steady state when grown in alkaline media, they underwent a transient alkalinization when transferred from the medium at pH 5.5 to medium at pH 7.5. This phenomenon is illustrated in Fig. 7, where the course of re-acidification is also shown. Restoration of the acidic pH is ostensibly due to the activity of the V-ATPase, since it is not observed in the Δvma yeast (Fig. 7) and is inhibited by bafilomycin (not illustrated).

We sought an explanation for the transient alkalinization noted when changing the pH of the medium. The rapid nature of the changes suggested permeation of base equivalents into the vacuole. In this context, it is noteworthy that both rich and synthetic growth media contain 38 mM ammonium (as (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}) as a nitrogen source. We considered the possibility that ammonia (NH\textsubscript{3}), which is in equilibrium with ammonium (NH\textsubscript{4}\textsuperscript{+}), could permeate the plasma- and vacuolar membranes and act as a base equivalent as it becomes protonated in the acidic vacuolar lumen (see the scheme in Fig. 9). Because at identical [NH\textsubscript{4}\textsuperscript{+}] the concentration of NH\textsubscript{3} is ~100 fold higher at pH 7.5 than at pH 5.5, increased entry of the base is expected during the transition from acidic to alkaline medium, with net consumption of vacuolar H\textsuperscript{+}. To test this hypothesis, cells were grown in rich, (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}-containing medium at pH 5.5, loaded with BCECF and then transferred acutely to a solution with identical pH and comparable osmolarity, but devoid of NH\textsubscript{4}\textsuperscript{+} (substituted by Na\textsuperscript{+}). To circumvent any confounding effects due to the V-ATPase, Δvma cells were used. In the absence of NH\textsubscript{4}\textsuperscript{+}, replacing the pH 5.5 medium with medium at pH 7.5 failed to alkalinize the vacuole, which contrasts with the behavior noted in the presence of the weak base (Fig. 7B). Note that the starting pH\textsubscript{vac} was lower in the NH\textsubscript{4}\textsuperscript{+}-free medium than in the complete synthetic medium of comparable pH. This probably reflects efflux of the vacuolar ammonium
Figure 7. Weak acids in the growth medium can contribute to vacuolar acidification. (A) Wild type (BJ926, solid squares) and V-ATPase deficient yeast (SF838-5A, open circles) were suspended in synthetic medium of pH 5.5 and images were acquired every 30 sec. When indicated (top bar) the extracellular pH (pHo) was changed to pH 7.5. (B) Vacuolar pH was measured in V-ATPase deficient Δvma4 yeast suspended in synthetic medium of pH 5.5 containing either (NH₄)₂SO₄ (open circles) or Na₂SO₄ (solid circles). When indicated, the medium pH was increased to 7.5 and eventually returned to 5.5. Traces are representative of 6 to 9 independent experiments.
accumulated during growth, the dissociation of NH₄⁺ leaving residual H⁺ within the vacuole during the transition to the NH₄⁺-free solution.

Because weak electrolytes can seemingly alkalinize the vacuole when the pH of the medium is increased, we considered the possibility that a similar mechanism may underlie the partial acidification of the vacuole in cells suspended at pH 5.5. As shown in Fig. 7B, cells that became alkaline upon transfer from medium pH 5.5 to 7.5 rapidly acidified to their initial pH_vac when the acidic medium was restored. Importantly, this acidification was observed only when NH₄⁺ was present in the solution.

In the experiment of Fig. 7B, acidification of the vacuole during shift from pH 7.5 to 5.5 could be explained by an efflux of NH₃, caused by the sudden imposition of an outward NH₃ gradient as the weak base concentration decreases at lower pH. Therefore, it is unclear whether exit of the base or entry of the conjugated acid is responsible for the net H⁺ (equivalent) flux. To define unambiguously whether NH₄⁺ can ferry H⁺ into the vacuole, cells were initially equilibrated in alkaline medium devoid of NH₄⁺, in the presence of bafilomycin to preclude V-ATPase activity. The cells were then rapidly switched to media of pH 5.5, with or without NH₄⁺. As illustrated in Fig. 8, in the presence of the conjugated acid vacuolar pH dropped rapidly and equilibrated near pH 6.5. By contrast, pH changed very little in the absence of NH₄⁺, despite the large inward H⁺ gradient. The data of several hundred determinations are collected in part B of Fig. 8. When maintained at external pH 7.5 in the absence of NH₄⁺ (with bafilomycin), pH_vac averaged 7.35 and only a small decrease was seen after 2 h of incubation at pH 5.5 in the absence of NH₄⁺. In contrast, pH_vac reached 6.53 when NH₄⁺ was present. The rapid acidification shown in Fig. 8 cannot be accounted for by net NH₃ efflux, since the base was absent from the cell interior at the time of the pH change. Therefore, the most parsimonious explanation of these data is that ammonium (NH₄⁺) acts as a weak conjugated acid, mediating the inward delivery of H⁺ (equivalents) across the plasma and vacuolar membranes.
Fig. 8. Effect of ammonium on vacuolar acidification. Wild-type (BJ926) yeast were pre-incubated in medium of pH 7.5 without NH$_4^+$ in the presence of bafilomycin, until pH$_{\text{vac}}$ equilibrated. The pH$_{\text{vac}}$ was monitored by ratio imaging, and the medium was then switched to pH 5.5 with (solid squares) or without NH$_4^+$ (open circles). A: representative time courses of the pH changes. Data are means ± SE of 4 to 6 individual measurements. B: Steady-state vacuolar pH of cells treated as above, measured 2 h after switching to media of pH 7.5 or 5.5 with or without NH$_4^+$, as specified. The number of individual cells measured is shown in parentheses. Bars are ± one SEM.
NH₄⁺ permeates the plasma membrane and acidifies the cytosol. In order for NH₄⁺ to deliver acid equivalents to the vacuole, it must first permeate the yeast plasma membrane. Such permeation of the protonated species is expected to induce a cytosolic acidification. To assess this possibility, we implemented a strategy to measure the cytosolic pH of yeast. Earlier studies indicated that pH-sensitive fluorescent dyes accumulated in the cytosol when yeast were loaded with the precursor esters under alkaline, rather than acidic conditions (18,35). This pH-specific targeting probably reflects the different pH optima of cytosolic and vacuolar esterases, and was more pronounced with the neutral dye carboxy-SNARF-1 than with the more acidic BCECF. Consistent with this observation, we observed that, when cells were loaded under alkaline (pH 7.5) media, SNARF-1 readily loaded the yeast cytosol. Similarly, BCECF, which accumulated into the vacuole under our standard loading conditions (Fig. 1), yielded intense cytosolic staining in ~30% of cells loaded in alkaline medium (Fig. 9A, top panels). The cytosolic staining persisted when cells were subsequently challenged with acidic extracellular media (Fig. 9B), confirming that, once cleaved by cytosolic esterases, the dye was not transported into the vacuole but remained localized in the cytosol. The bright cytosolic labeling produced by BCECF could be easily separated from the vacuolar signal by applying an intensity threshold, combined with a size criterion to reject contaminating vacuoles (Fig. 9A, bottom panels). This enabled us to measure the pH of the yeast cytosol by ratio imaging, employing the same dye and calibration procedure used for the vacuolar measurements. As shown in Fig. 9C, wild-type and Δvma yeast had similar cytosolic pH when maintained in alkaline (pH 7.5) medium containing NH₄⁺. More importantly, the cytosol rapidly acidified in both cell types when challenged with acidic media containing NH₄⁺ (Fig. 9D). This effect was more rapid than the NH₄⁺-mediated acidification of the vacuole (e.g. Fig. 8), suggesting that NH₄⁺ sequentially permeates the plasma and then the vacuolar membrane of yeast.
Fig. 9. Effect of ammonium on the cytosolic pH of yeast. Wild-type (BJ926) yeast and V-ATPase deficient mutants (SF838-5A) were labeled with BCECF-AM under conditions favoring accumulation of the dye in the cytosol, as described in (18,35). A: High magnification images showing cell morphology (top left) and the typical fluorescence staining observed with the alkaline loading protocol (top right). Intense cytosolic staining is apparent in 3 cells, with weak vacuolar staining present in the remaining cells. Cells with cytosolic staining were selected by setting an intensity threshold, and contaminating vacuoles rejected by a size criterion >150 pixels (bottom left). The regions corresponding to the cytosol were used for ratio measurements (bottom right). B: Images taken before and 90 minutes after exposure of cells to acidic medium containing NH4+. Bars, 5 μm. C: Steady-state cytosolic pH of wild type (top) and Δvma yeast (bottom), measured in media of pH 7.5 or 5.5 containing NH4+. An in situ calibration, obtained as in Fig. 2, was used to convert the fluorescence ratio into cytosolic pH values. D: Time-course of the cytosolic pH changes upon exposure to acidic medium containing NH4+. Cells were pre-incubated in pH 7.5 medium devoid of NH4+. The medium was then switched to pH 5.5 (white bar) and the cells subsequently challenged with Na+ (solid squares) or NH4+ (open circles). Traces are representative of 3 separate experiments.
DISCUSSION

Video imaging of BCECF is an accurate and convenient method for measuring vacuolar and cytosolic pH. $^{31}$P-NMR and dual-excitation flow cytometry of 6-carboxyfluorescein diacetate had been used earlier to measure the pH of yeast vacuoles (13,17,19). Values of 6.1 (13) and 6.2 (17) were obtained using 6-carboxyfluorescein and a pH in the range of 5.5 to 6.0 was estimated using $^{31}$P-NMR (19). Though useful, these methods have limitations. Acquisition of NMR data is slow and it is difficult to make acute changes in the composition of the medium. Moreover, compartments other than the vacuole contribute to the resonance signal measured. A similar problem plagues the flow cytometric method since, as shown here, while esters of fluorescein derivatives accumulate in the vacuole, they are not restricted to this compartment. Indeed, they were designed to accumulate in the cytosol (36). In the case of video imaging, definition of the regions of interest, together with thresholding of unwanted signals ensure that the vacuolar or cytosolic pH is selectively measured. Moreover, by adhering the yeast to a coverslip mounted in a perfusion chamber, rapid solution changes are possible.

Vacuolar acidification is indispensable in S. cerevisiae. In eukaryotic cells, V-ATPases are responsible for generating transmembrane electrochemical gradients within several organelles. These gradients are thought to be critical for essential cellular functions such as sorting and processing of proteins, receptor recycling, and the control of vesicular traffic. In Neurospora crassa, the gene encoding the 70 kDa catalytic ATP-binding subunit, \textit{vma-1}, was shown to be essential for survival, demonstrating that V-ATPase activity is indispensable in this ascomycete fungus (37). In contrast, the filamentous fungi \textit{Ashbya gossypii} has been shown to be viable in the absence of a functional V-ATPase (38), and disruption of yeast genes encoding V-ATPase subunits resulted in only conditional lethality. Yeast devoid of all V-ATPase activity are viable when grown in unbuffered media, grow optimally in media buffered to pH 5.5 (15), but fail to grow in alkaline buffered media (pH $> 7.5$). Here we show that those growth conditions that favor viability of V-ATPase-deficient yeast also result in acidification of their vacuoles. When carried out in medium buffered to pH 5.5, measurements of vacuolar pH in \textit{Δvma} mutants yielded an average value of 5.9
This suggests that acidification of the endosomal system, but not V-ATPase activity per se, is also indispensable in yeast.

**Endocytosis does not contribute to vacuolar acidification.** Assuming that vacuolar acidification is indispensable for survival, Nelson and Nelson (15) rationalized that V-ATPase deficient yeast must have alternative means of acidifying their vacuoles. Based on the observation that *vma* yeast are only viable in acidic medium, they hypothesized that acid equivalents from the medium were internalized through fluid-phase endocytosis. A role for endocytosis in acidification was further supported by the observation that blocking endocytosis in a V-ATPase deficient strain resulted in cell death (20). Endocytosis could also contribute to acidification by delivering the plasma membrane proton-pump ATPase, Pma1p, to the vacuole. The predicted orientation of the ATPase is such that Pma1p could assist in endosomal and/or vacuolar acidification. We believe that this possibility is unlikely, as overexpression of Pma1p (which was confirmed by immunoblotting, Fig. 4) did not complement the *Δvma* phenotype (Table 1), nor could any contribution to vacuolar acidification be detected by video microscopy (Fig. 5). Moreover, blocking endocytosis by introducing the temperature sensitive mutation *end4-1* did not prevent vacuolar acidification when the cells were suspended in acidic media. This was not due to a loss of the end phenotype at acidic pH, as uptake of Lucifer yellow remained strongly inhibited under these conditions. This observation not only rules out a role for endocytic delivery of Pma1p, but also implies that vacuolar acidification does not result from uptake of the acidic fluid phase by pinocytosis, contrary to the original proposal of Nelson and Nelson (15).

**Weak acids in the growth media can contribute to vacuolar acidification.** Our measurements of *Δvma* mutants bathed in minimal medium buffered to pH 5.5, yielded an average vacuolar pH of 5.9 (Fig. 3). This is seemingly contrary to previous reports which used quinacrine to demonstrate that *Δvma* mutant vacuoles were neutral (15,25). However, this discrepancy can be readily explained by differences in the composition of the media used for the determinations. Quinacrine labeling was carried out in ammonium-containing rich media buffered to pH 7.5 (39).
Imaging determinations performed in media of similar composition and pH also resulted in a near-neutral pH value of 7.1 (Fig. 3).

Experiments such as those described above revealed that the vacuolar pH varies greatly and in some instances abruptly when the medium is replaced. What is the mechanism responsible for such changes? Alkalization of the vacuole when substituting an acidic medium for a more alkaline one can be readily understood, considering that the growth media invariably contain high concentrations of ammonium (e.g. 38 mM). This cation is in equilibrium with the unprotonated form, ammonia, which readily permeates most biological membranes. After reaching the cytosol or the acidic vacuole, ammonia becomes protonated, thereby elevating the cytosolic or vacuolar pH (Fig. 10). That ammonia is responsible for the alkalization was demonstrated by omitting ammonium from the medium. In this instance, raising the extracellular pH had little effect on either vacuolar pH or cytosolic pH over the period of time studied (Fig. 7 and 9).

The explanation for the abrupt acidification observed upon restoration of the acidic pH is less obvious. The rapidity of the effect suggests the entry and dissociation of a weak acid. On the other hand, perusal of the composition of the medium indicates that the major anions are all derived from strong acids (*i.e.* sulfate and chloride). Importantly, the acidification was observed only in media containing high concentrations of ammonium. At acidic pH, ammonium is predominantly in the protonated form, NH₄⁺, because of its high pKₐ (9.26). The experiments of Fig. 8, where the cells were pre-incubated in the absence of NH₃/NH₄⁺ ruled out the possibility that acidification is due simply to net efflux of the free base, NH₃. We therefore propose that NH₄⁺, a conjugated weak acid, mediates the delivery of acid equivalents into the vacuole (Fig. 10). Such an assumption implies that the plasma membrane possesses pathways for the permeation of the cationic species, NH₄⁺, which unlike lipophilic NH₃ cannot permeate the lipid bilayer. In this regard, yeast have been shown to express at least two separate ammonium transport systems, Mep1p and Mep2p (reviewed in (40)), on their plasmalemma. These transporters would deliver the conjugated weak acid to the cytoplasm where it deprotonates, thereby acidifying the cytosol (Fig. 10). Indeed, a rapid cytosolic acidification was observed upon exposure of cells to acidic
Figure 10. Model of vacuolar acidification in V-ATPase-deficient yeast. When the cells are grown at pH 5.5, NH₃/NH₄⁺ present in the rich or synthetic media used in most laboratories will be predominantly in the protonated form (pKₗ=9.26). NH₄⁺ can be transported into the yeast by plasmalemmal transporters, including Mep1p and Mep2p. The membrane potential generated by the plasma membrane H⁺-ATPase (Pma1p) contributes to the force driving NH₄⁺ into the cells. After reaching the cytoplasm, NH₄⁺ can deprotonate, thereby acidifying the cytosol. An acidic, NH₄⁺-rich cytosol can then promote vacuolar acidification, either by entry of NH₄⁺ itself through undefined pathways, or by entry of other weak acids present in the cytosol. Conversely, suspension of cells with acidic vacuoles in alkaline media will induce vacuolar alkalosis due to permeation and protonation of NH₃.
medium containing NH₄⁺ (Fig. 9), suggesting that NH₄⁺ readily permeates the plasma membrane of yeast. Early 31P NMR studies indicated that yeast intracellular pH was not affected by the external pH conditions or the presence of NH₄⁺ (41). However, several subsequent studies found that the intracellular pH of yeast followed the external pH when the extracellular medium was either strongly buffered (18,42-44) or contained weak organic acids such as acetate, butyrate, or succinate (19,45-47). This suggests that the plasma membrane of yeast is permeable not only to NH₄⁺, but also to other weak acids.

NH₄⁺ permeation likely reflects the activity of Mep1p and Mep2p, but might also be due to other transporters. In amphibian and mammalian cells NH₄⁺ can traverse the membrane via K⁺-selective or non-selective cation channels, through the Na⁺/K⁺ pump and via cation exchangers such as the Na⁺/H⁺ antiporter (48-51), and in some cases the permeability to NH₄⁺ is in fact greater than that to NH₃. As indicated in Fig. 10, it is not clear whether comparable ammonium transporters exist also in the vacuolar membrane. However, transport of acid equivalents across the vacuolar membrane would not necessarily occur via this pathway, since a variety of other organic weak acids exist in the cytosol, which could deliver H⁺ to the vacuolar lumen. The absence of NH₄⁺ in the medium used by Preston et al. (17) would explain why these authors found the vacuoles of V-ATPase deficient yeast to be near neutral (pH 6.9).

In summary, we found that the vacuoles of yeast incubated under acidic conditions that promote growth have a low pH and that vacuolar acidity, rather than H⁺ pumping per se is essential for yeast viability. Moreover, we found that NH₃ and NH₄⁺, which are routinely added at high concentrations to growth media as a source of nitrogen, shuttle H⁺ equivalents across the plasma and/or vacuolar membranes. The former likely permeates the lipid bilayer, while the latter possibly utilizes specific transporters such as Mep1p and Mep2p or other transporters to deliver acid equivalents to the cytosol and to the vacuole.
FOOTNOTES

1The abbreviations used in this paper are: V-ATPase, Vacuolar-type H⁺-ATPase; PMA, Plasma membrane H⁺-ATPase; pHvac, vacuolar pH; BCECF, 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein; MES, 3-(N-morpholino)ethanesulfonic acid; CCCP, carbonylcyanide m-chlorophenylhydrazone; DIC, Differential Interference Contrast.

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282


