Mapping the sodium channel-ankyrin interaction

by

Wei Wong

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Pharmacology
University of Toronto

© Copyright by Wei Wong, 1998
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author’s permission.

L’auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-34081-3
Voltage-gated sodium channels (Na⁺ channels) mediate the initial depolarization event during impulse conduction in excitable tissues. In neurons, the polarized and clustered distribution of Na⁺ channels at the nodes of Ranvier is critical for the efficient transmission of the action potential along the axon shaft. It has been suggested that the association of Na⁺ channels with ankyrin, a cytoskeletal linker protein, may be involved in the maintenance of the specialized Na⁺ channel distributions. While progress has been made in mapping the Na⁺ channel binding site on ankyrin, nothing is known about the ankyrin binding site on Na⁺ channel.

In this thesis, the ability of fusion proteins corresponding to the intracellular domains of rat brain Na⁺ channel IIa (rbNIIa) to bind purified human erythrocyte ankyrin was tested using photo-activated cross-linking experiments, affinity precipitations and enzyme-linked immunosorption assays (ELISAs). These assays revealed that both the N-terminus (NT) and the C-terminus (CT) of rbNIIa bound specifically to ankyrin, although the NT-ankyrin interaction displayed higher affinity by at least an order of magnitude. Identification of the intracellular domains of rbNIIa involved in forming the ankyrin binding site is critical for defining the molecular basis of the Na⁺ channel-ankyrin interaction, and in understanding basic mechanisms underlying diseases such as multiple sclerosis and muscular dystrophy.
Acknowledgements

My deepest gratitude, first and foremost, to Dr. Owen Jones, for being the type of supervisor who brings out the best in his graduate students. Any success I experience in my research career will owe an extremely large debt to his guidance, enthusiasm and belief in my abilities.

The financial support of NSERC is gratefully acknowledged. Without NSERC’s support, I would not have had the range of possibilities that were open to me.

Several individuals — Denis Jugloff, Maria Cosentino, Andrew Demjen, Geoff Goodfellow and most especially Marcus Law — have generously provided technical advice and assistance during the course of this research. Many thanks to these individuals, as well as to the other members of the Jones lab for useful discussions, science-related and otherwise.

I am incredibly grateful to my parents, who have always encouraged and supported my choices and not tried to impose their own visions onto me. My sisters, by being who they are, keep me amused and remind me constantly how fortunate I am.

Finally, I cannot thank Steve enough for his emotional and financial support during this endeavour.
Table of contents

1 INTRODUCTION ........................................................................................................... 1
  1.1 Overview .................................................................................................................. 1
  1.2 Role of the cytoskeleton in neurons ........................................................................ 4
  1.3 Ankyrins .................................................................................................................. 5
    1.3.1 ANK1 ................................................................................................................ 11
    1.3.2 ANK2 ................................................................................................................ 11
    1.3.3 ANK3 ................................................................................................................ 12
  1.4 The Na⁺ channel-ankyrin interaction ....................................................................... 14
    1.4.1 Significance of the Na⁺ channel-ankyrin interaction ........................................ 15
    1.4.2 Unresolved questions about the Na⁺ channel-ankyrin interaction .................. 15
    1.4.3 Structure of the Na⁺ channel ............................................................................. 16
  1.5 Hypothesis and specific aims .................................................................................. 20

2 METHODS .................................................................................................................... 22
  2.1 Generation of various cDNAs for fusion protein expression .................................. 22
  2.2 Expression of bacterial fusion proteins .................................................................. 27
  2.3 Purification of bacterial fusion proteins ................................................................ 30
  2.4 Purification of human erythrocyte ankyrin ............................................................. 30
  2.5 Purification of rat brain Na⁺ channel .................................................................... 32
    2.5.1 Preparation of adult rat synaptic membranes ............................................... 32
    2.5.2 Preparation of ankyrin-free Na⁺ channel ....................................................... 33
    2.5.3 Preparation of the wheat-germ agglutinin column ........................................ 33
  2.6 Gel electrophoresis and immunoblotting ............................................................... 34
  2.7 Determination of protein concentration ................................................................ 36
2.8  Protein labelling .............................................................................................................37
  2.8.1  Photolabelling ...........................................................................................................37
  2.8.2  Biotinylation .............................................................................................................38
2.9  Photo-activated cross-linking experiments .................................................................38
2.10 Affinity precipitations .................................................................................................41
2.11 Characterization of the Na⁺ channel-ankyrin interaction by enzyme-linked immunosorption assay (ELISA) 43

3  RESULTS ..........................................................................................................................45
  3.1  Expression of bacterial fusion proteins ........................................................................45
  3.2  Purification of bacterial fusion proteins ......................................................................50
  3.3  Purification of human erythrocyte ankyrin .................................................................50
  3.4  Ankyrin depletion of rat brain Na⁺ channel .................................................................56
  3.5  Photo-activated cross-linking experiments ..................................................................56
  3.6  Affinity precipitations of Na⁺ channel constructs using ankyrin-coated beads ..............59
  3.7  Analysis of the binding kinetics of the Na⁺ channel construct-ankyrin interactions using ELISAs... 61

4  DISCUSSION .....................................................................................................................65
  4.1  Summary of results .......................................................................................................65
  4.2  Rationale for using His-tagged fusion proteins ..........................................................65
  4.3  Rationale for the different expression schemes ..........................................................66
  4.4  Purification of His-tagged constructs .........................................................................68
  4.5  Purification of ankyrin-depleted rat brain Na⁺ channel and human erythrocyte ankyrin ......70
  4.6  Identification of NT and CT as the ankyrin binding site ................................................71
  4.7  The molecular significance of the Na⁺ channel-ankyrin interaction .............................75
  4.8  The role of the Na⁺ channel-ankyrin interaction in the neuron ....................................78
  4.9  Implications of the Na⁺ channel-ankyrin interaction for voltage-gated ion channel biology... 82

5  FUTURE STUDIES ............................................................................................................84

6  REFERENCES ...................................................................................................................86
## List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABTS</td>
<td>Azidino-di-(3-ethyl-benzthiazoline-sulfonate)</td>
</tr>
<tr>
<td>AE</td>
<td>Anion exchanger</td>
</tr>
<tr>
<td>ANK</td>
<td>Ankyrin</td>
</tr>
<tr>
<td>BIS</td>
<td>N,N'-methylenebisacrylamide</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>Amount of BIS crosslinker expressed as a percentage of the sum of acrylamide monomer and BIS</td>
</tr>
<tr>
<td>Ca²⁺ channel</td>
<td>Voltage-gated calcium channel</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CNBr</td>
<td>Cyanogen bromide</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CT</td>
<td>Carboxy terminus</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>Cupric sulfate</td>
</tr>
<tr>
<td>CWFG</td>
<td>Cold-water fish gelatin</td>
</tr>
<tr>
<td>DAR</td>
<td>Donkey anti-rabbit</td>
</tr>
<tr>
<td>DEB</td>
<td>Denaturing Elution Buffer</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>Dn</td>
<td>Subdomain n of ankyrin membrane binding domain</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyxynucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleoside triphosphate</td>
</tr>
<tr>
<td>DWB 5.3</td>
<td>Denaturing Wash Buffer, pH 5.3</td>
</tr>
<tr>
<td>DWB 6.0</td>
<td>Denaturing Wash Buffer, pH 6.0</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorption assay</td>
</tr>
<tr>
<td>EPSP</td>
<td>Excitatory postsynaptic potential</td>
</tr>
<tr>
<td>FPR</td>
<td>Fluorescence photobleach recovery</td>
</tr>
<tr>
<td>GAM</td>
<td>Goat anti-mouse</td>
</tr>
<tr>
<td>gw</td>
<td>Specific gravity</td>
</tr>
<tr>
<td>H₂O</td>
<td>Water</td>
</tr>
<tr>
<td>HEPES-Na⁺</td>
<td>2-[(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid, sodium salt</td>
</tr>
<tr>
<td>His-tag</td>
<td>Hexahistidine tag</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloride</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>iP₃</td>
<td>Inositol 1,4,5-triphosphate</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactoside</td>
</tr>
<tr>
<td>K⁺ channel</td>
<td>Voltage-gated potassium channel</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>Dipotassium hydrogen phosphate</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>Potassium dihydrogen phosphate</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>Kds</td>
<td>Kaleidoscope</td>
</tr>
<tr>
<td>LI-II</td>
<td>Linker between Na⁺ channel transmembrane domains I and II</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LII-III</td>
<td>Linker between Na(^+) channel transmembrane domains II and III</td>
</tr>
<tr>
<td>LIII-IV</td>
<td>Linker between Na(^+) channel transmembrane domains III and IV</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>MgCl(_2)</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney</td>
</tr>
<tr>
<td>N-CAM</td>
<td>Neural cell adhesion molecule</td>
</tr>
<tr>
<td>Na(^+) channel</td>
<td>Voltage-gated sodium channel</td>
</tr>
<tr>
<td>Na(^+)K(^-) tartrate</td>
<td>Sodium potassium tartrate</td>
</tr>
<tr>
<td>NaCO(_3)</td>
<td>Sodium carbonate</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaHCO(_3)</td>
<td>Sodium bicarbonate</td>
</tr>
<tr>
<td>Na(_2)S</td>
<td>Sodium azide</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>nb</td>
<td>Normoblastosis</td>
</tr>
<tr>
<td>NFDM</td>
<td>Non-fat dry milk</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>NT</td>
<td>Amino terminus</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PDZ</td>
<td>Named after PSD-95, Dlg (disc-large protein) and ZO-1 (zonula occludens-1)</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonyl fluoride</td>
</tr>
<tr>
<td>PSD</td>
<td>Post-synaptic density</td>
</tr>
<tr>
<td>R(_{1-17})</td>
<td>Repeats 1-17 of ankyrin membrane binding domain</td>
</tr>
<tr>
<td>R(_{10-22})</td>
<td>Repeats 10-22 of ankyrin membrane binding domain</td>
</tr>
<tr>
<td>rATP</td>
<td>Ribo-adenosine triphosphate</td>
</tr>
<tr>
<td>rbNIIa</td>
<td>Rat brain Na(^+) channel IIa</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>rSkM-1</td>
<td>Rat skeletal muscle Na(^+) channel</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>SAP</td>
<td>Synapse associated protein</td>
</tr>
<tr>
<td>SH3</td>
<td>Src-homology-3</td>
</tr>
<tr>
<td>SB</td>
<td>Sample buffer</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>S(_n)</td>
<td>Segment (n) of Na(^+) channel transmembrane domain</td>
</tr>
<tr>
<td>T</td>
<td>Acrylamide monomer and N,N'-methylenebisacrylamide (BIS) crosslinker</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylenediamine</td>
</tr>
<tr>
<td>TB</td>
<td>Terrific Broth</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TTBS</td>
<td>Tween-TBS</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume/volume</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight/volume</td>
</tr>
<tr>
<td>WGA</td>
<td>Wheat-germ agglutinin</td>
</tr>
<tr>
<td>Z</td>
<td>Benzophenone</td>
</tr>
</tbody>
</table>
1 Introduction

1.1 Overview

Neurons are highly specialized cells designed for the transfer of information, which they transmit chemically and interpret electrically (Kandel et al., 1991). The electrical impulses that traverse a neuron from dendrite to axon to presynaptic terminal are triggered by precisely timed and directionally oriented ion fluxes, which in turn are produced by integral pore-forming proteins known collectively as ion channels (Catterall, 1984; Hille, 1991).

Two general types of ion channels exist, each of which is categorized by its mode of activation. The first type are those channels which open in response to the binding of activating ligands (agonists), such as the common neurotransmitters acetylcholine and glutamate (Dingledine et al., 1990; Hollenberg, 1987). The second type of channels are those which allow the selective passage of ions in response to changes in membrane potential (voltage) (Catterall, 1993; Catterall, 1995). It is this latter class of ion channels that form the central part of this thesis.

At least three major classes of voltage-gated channels exist, each corresponding to the major electrophysiologically central ions: Ca$^{2+}$, K$^-$ or Na$^+$. While the focus of this thesis is on the voltage-gated Na$^+$ channel, early electrophysiology (Aldrich et al., 1983; Armstrong & Bezanilla, 1977), pharmacology (Catterall, 1993; Catterall, 1995; Narahashi, 1992) and more recently molecular and cellular biology studies (Catterall, 1993; Catterall, 1995) reveal that Na$^+$ channels share many features with Ca$^{2+}$ and K$^-$ channels. Consequently, Na$^+$ channels have served as excellent models with which to determine general principles of ion channel biology. Conversely, information gleaned from other ion channels has proved enormously beneficial in understanding Na$^+$ channel biology (Catterall, 1993; Catterall, 1995; Goldin, 1995).

In general, the function of voltage-gated ion channels is to allow the selective passage of ions in
response to changes in the membrane potential. Thus, voltage-gated ion channels have several basic properties, including:

a) a channel pore through which ions flow (Goldin, 1995),

b) a mechanism for sensing changes in membrane voltage (Goldin, 1995; Guy & Durell, 1996; Guy & Seetharamulu, 1986), and

c) a mechanism for coupling the voltage sensor to channel opening (Goldin, 1995).

A less intuitive property common to most ion channels is the presence of mechanisms to inactivate the channel upon prolongation of the activating voltage stimulus (Aldrich et al., 1983; Goldin, 1995). In the case of Na\(^+\) channels, changes in membrane potential that cause the cell membrane to depolarize (i.e. become more positive under physiological conditions) induce a conformational change in the protein which opens a transmembrane pore, allowing sodium ions to flood into the neuron so triggering the "initial depolarization event during impulse conduction" (Goldin, 1995).

Owing to their pivotal role in determining the electrophysiology of neurons and other electrically excitable cells, how voltage-gated ion channels operate has been the subject of intense study for several decades (Hille, 1991). One of the most significant breakthroughs in ion channel biology has been the identification of large concentrations of Na\(^+\) channel in the electric organ of the eel *Electrophorus electricus* (Moore et al., 1982). In conjunction with the description of toxins capable of specifically blocking such channels, it has subsequently become possible to obtain large enough amounts of pure Na\(^+\) channel protein sufficient for biochemical characterization (Hartshorne & Catterall, 1981), sequencing (Noda et al., 1984) and molecular cloning (Kayano et al., 1988; Noda et al., 1986; Noda et al., 1984). The resulting molecular information has allowed the identification of Na\(^+\), Ca\(^{2+}\) and K\(^-\) channel proteins and genes in diverse species and is proving critical in identifying the molecular basis for diverse pathological disorders ranging from epilepsy (Lombardo et al., 1996; Willow et al., 1986) to heart failure (Keating et al., 1991).

In spite of the enormous progress that has been made in understanding the physiological and
pharmacological properties of discrete voltage-gated ion channels, one key question remains - how do such channels determine the integrated electrical properties of neurons?

While it is fairly apparent that the specific biophysical properties of ion channels such as the activation voltage, rates of activation and inactivation ultimately underlie neuronal electrophysiology, a second factor, their distribution on the nerve cell surface, is equally important. In order for synaptic transmission to occur, for example, it is insufficient that neurons simply express those ion channels which mediate neurotransmitter release; the ion channels must also be targeted correctly to the appropriate locations at the presynaptic axon terminal (Cohen et al., 1991; Haydon et al., 1994; Katz, 1969; Robitaille et al., 1990). The importance of channel distribution is not merely restricted to transmitter release, however. Numerous examples of asymmetric (i.e. non-random) distributions of discrete ion channels on the nerve cell surface, and their functional correlates, are now known to exist (Hell et al., 1993; Linas, 1988; Linas & Yarom, 1981; Maletic-Savatic et al., 1995; Mills et al., 1994; Sheng et al., 1993; Sheng et al., 1992; Westenbroek et al., 1990). Thus, non-random channel distributions underlie saltatory conduction in myelinated axons (Salzer, 1997), integration of excitatory postsynaptic potentials (EPSPs) (Christie et al., 1996) and their translation into action potentials by the axon hillock (Waxman & Ritchie, 1985). Moreover, the location and state of channel aggregation (clustering) may serve to increase the diversity of neuronal signaling pathways by localizing electrical or chemical signaling to restricted regions of the neuron (Froehner, 1993; Linas & Yarom, 1981) or by shaping the waveform of action potentials (Johnston et al., 1996). In many cases, the contribution of ion channels to nerve function may be determined simply by the proximity of discrete ion channels to each other in the cell (Mills et al., 1994; Robitaille et al., 1990). The possibility that ion channel co-localization allows for functional cooperativity has been suggested by computer modelling studies (Mills et al., 1994) and has been postulated to be a mechanism underlying substrates of learning and memory such as long-term potentiation and depression.
Indeed, it is hard to conceive of any models of learning and memory based on the weighting of individual synapses (Hebbian models) (Hebb, 1949) where the weighted components, especially the ion channels, are not maintained at those reinforced synapses (Siegel et al., 1994).

In spite of their significance to integrated nerve function, the mechanisms responsible for generating asymmetric distributions of ion channels are essentially unknown. Localization of ion channels in a specific area of the nerve membrane requires two processes: (1) sorting (Chung et al., 1989; Griffith & Simons, 1986; Matter & Mellman, 1994; Rodriguez-Boulan & Powell, 1992), which directs the ion channels to the proper destination and (2) maintenance (Froehner, 1993), which keeps the ion channels at those locations (Craig & Banker, 1994). The existence of maintenance processes for neuronal ion channels is supported by fluorescence photobleach recovery (FPR) experiments (Angelides, 1986; Angelides et al., 1988; Jones et al., 1989) which show that most ion channels are not free to diffuse through the lipid bilayer. This result runs contrary to the Singer-Nicholson model of membrane structure (Singer & Nicholson, 1972), and suggests these channels must be fixed in position somehow.

1.2 Role of the cytoskeleton in neurons

Maintenance of any membrane protein in specific locations can be achieved in at least three different ways. One way would be through differential membrane composition in distinct regions of the neuron such that the resulting lipid microdomains might selectively include or exclude proteins (Simons & Ikonen, 1997). Although the formation of lipid microdomains has been suggested for epithelial cells (Simons & van Meer, 1988), there is no evidence for their existence in neurons.

Another way to maintain channels in place has been referred to as “corraling” and requires the presence of actual physical barriers to prevent the diffusion of membrane proteins beyond the boundaries of local domains (Jacobson et al., 1987; Sako & Kusumi, 1994; Sako & Kusumi, 1995). Such
corrauls usually operate on the micron scale and have been observed in several cell types (Sako & Kusumi, 1994; Sako & Kusumi, 1995). Perhaps the most extreme example of corrauling occurs in epithelial cells, where tight junctions demarcate the apical and basolateral surfaces and thus separate the membrane proteins into two distinct populations (Almers & Stirling, 1984; Simons & Fuller, 1985). Whether such a mechanism exists in neurons is highly controversial and the evidence contradictory. Based on parallels between the targeting mechanisms in epithelia and neurons (Craig & Banker, 1994; Dotti & Simons, 1990; Matter & Mellman, 1994; Pietrini et al., 1994), one logical location for physical barriers analogous to the tight junction in the neuron is at the axon hillock, which is situated at the axon-somatodendrnte boundary. Initially, the failure of axonally applied fluorescent lipids to penetrate the somatodendritic domain appeared to confirm the presence of such a barrier (Kobayashi et al., 1992). However, subsequent experiments have shown that similar lipids can diffuse gradually throughout the cell irrespective of whether they are applied to axons or dendrites (Winckler & Poo, 1996).

Finally, membrane proteins may be maintained in their proper locations through an interaction, either direct or indirect (i.e. through linker proteins), with the underlying cytoskeleton (Burgoyne, 1991). Of the three maintenance mechanisms described here, cytoskeletal associations appear potentially to be a prominent maintenance mechanism for ion channels in neurons. The strongest evidence for this statement comes from pioneering studies (Srinivasan et al., 1988) showing that voltage-gated Na⁺ channels interact with a linker protein named ankyrin (Bennett, 1979), a known component of the spectrin-based cytoskeleton.

1.3 Ankyrins

Ankyrins are a family of proteins characterized by their diversity. They (or related proteins) have been described in organisms ranging from primitive organisms such as fruit flies (Dubreuil & Yu, 1994)
and worms (Otsuka et al., 1995) to vertebrates such as mice, rats and humans (Bennett, 1992). In vertebrates, ankyrin expression has been detected in multiple cell types and tissues, including erythrocytes (Bennett & Stenbuck, 1980), brain (Otto et al., 1991), epithelia (Peters et al., 1995) and skeletal muscle (Peters et al., 1995). Depending on the tissue type, ank:'rrns may exhibit different cellular localizations, with the most common location being peripheral to the plasma membrane (Bennett, 1992); however, discrete ankyrin isoforms have also been localized to the sarcoplasmic reticulum (Zhou et al., 1997) and Golgi apparatus (Devarajan et al., 1996).

Within these many organisms and tissues, ank_yrns usually serve to link diverse integral proteins of disparate structures (Table 1) to a spectrin-based cytoskeleton (Bennett, 1992) (Figure 1). Ankyrin function is manifested by a multitude of isoforms generated by multiple genes and alternative splicing (Bennett, 1992). In humans, ank_yrns are the products of at least three different genes termed \( \text{ANK}^1 \) (Gallagher et al., 1997; Lambert et al., 1990; Lux et al., 1990), \( \text{ANK}^2 \) (Otto et al., 1991) and \( \text{ANK}^3 \) (Kordeli et al., 1995); each gene product (referred to as \( \text{ANK}^1 \), \( \text{ANK}^2 \) and \( \text{ANK}^3 \)) undergoes alternative splicing to give rise to numerous isoforms (Lambert et al., 1990; Lux et al., 1990; Otto et al., 1991). Similar genes and isoforms have been discovered in mice (Birkenmeier et al., 1993; Bourguignon et al., 1986; Devarajan et al., 1996; Drenckhahn & Bennett, 1987; Kapfhamer et al., 1995; Peters et al., 1995; Piepenhagen et al., 1995; White et al., 1990; Zhou et al., 1997) and in rats (Babitch, 1993; Devarajan et al., 1996; Drenckhahn & Bennett, 1987; Drenckhahn et al., 1985; Kordeli et al., 1990; Kunimoto, 1995; Kunimoto et al., 1991; Lambert & Bennett, 1993; Zhou et al., 1997).

Each ankyrin gene product has a similar overall basic structure (Figure 2). The N-terminal domain is characterized by an 89kDa region which consists of 24 contiguous copies of so-called ankyrin repeats (Lux et al., 1990; Michaely & Bennett, 1993). Each repeat is typically 33 amino acids
<table>
<thead>
<tr>
<th>Membrane protein</th>
<th>Anion exchanger (isoforms 1 and 3)</th>
<th>Cardiac Na-, Ca$^{2+}$ exchanger</th>
<th>GPR55(C34A)</th>
<th>Inositol 1,4,5-trisphosphate (IP$_3$) receptor</th>
<th>Na$^+$, K$^+$-ATPase</th>
<th>Neuronal cell-adhesion (neurofascin, L1, NrCAM, NgCAM, neuroglian)</th>
<th>Voltag-geg Na$^+$ channels</th>
</tr>
</thead>
<tbody>
<tr>
<td>References</td>
<td>Ding et al., 1994; Ding et al., 1996</td>
<td>Lai et al., 1993</td>
<td>GGNGF/VEDRKPSEI</td>
<td>GGYQDLRRPS</td>
<td>ALLK/ALLK</td>
<td>K$^+$ (neurofascin, L1)</td>
<td>Unknown</td>
</tr>
<tr>
<td>Two regions required for ankyrin binding</td>
<td>(1) the 70 NH$_2$-terminal amino acid residues</td>
<td>(2) amino acids 155-195</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

Table 1: Membrane proteins which are known to bind to ankyrin. Binding motifs, if known, are provided.
Figure 1. Schematic diagram illustrating the attachment of a membrane protein's to the spectrin-based membrane skeleton through ankyrin.
(Redrawn from Lambert and Bennett, 1991; Michaely and Bennett, 1995)
Figure 2. Schematic diagrams of ankyrin demonstrating its linear (A) and three-dimensional (D) structure, as well as the organization of the membrane binding domain into four subdomains (B) and the organization of a subdomain into six ankyrin repeats (C). Abbreviations for D: RD, regulatory domain; MBD, membrane binding domain; SBD, spectrin binding domain.
in length and has a consensus sequence of \(-G\text{-TPLH-}AA\text{-}GH\text{-}V\text{-}A\text{-}LL\text{-}GA\text{-}N\text{-}D\text{-}\) (Lux et al., 1990). Based on binding and circular dichroism studies, it is currently believed that six of these ankyrin repeats form an independently folded subdomain; the four subdomains, in turn, are arranged in a square planar or tetrahedral configuration (Michael & Bennett, 1993). Because ankyrin-binding membrane proteins invariably associate with the N-terminal region (Davis et al., 1991; Morgans & Kopito, 1993), it is also termed the membrane binding domain. Closely related repeats have been noted in a diverse range of proteins such as cell cycle control proteins, transcription factors and a toxin from black widow spider venom (Bork, 1993; Lux et al., 1990; Michael & Bennett, 1992), where they bind seemingly unrelated target proteins. These observations have led to the proposal that ankyrin repeats are generalized protein binding motifs (Michael & Bennett, 1992). Interestingly, binding sites in target membrane proteins that interact with ankyrin do not display any noticeable degree of conservation, as shown in Table 1 (p. 7).

The central portion of ankyrin is thought to comprise the spectrin binding domain, which consists of an acidic N-terminal region and a basic C-terminal region (Bennett, 1992). While removal of the N-terminal region abolishes 93% of normal spectrin binding (Davis & Bennett, 1990), this region is also less highly conserved between ANK1 and ANK2 than the basic region (Bennett, 1992). It therefore seems likely that variations in the acidic region account for the preferential association of given ankyrin isoforms with specific spectrin isoforms (Bennett et al., 1982).

The most C-terminal region of ankyrin has been termed the regulatory domain since this region confers intramolecular modulation of the affinities of both the membrane binding and spectrin binding domains (Hall & Bennett, 1987). The level of modulation, in turn, is affected by alternative splicing of this region. For example, a truncated version of ANK1 known as protein 2.2, which is missing 163 residues from the regulatory domain, exhibits a higher affinity for spectrin compared to whole ANK1
(Hall & Bennett, 1987; Lux et al., 1990). Since the regulatory domain is the least conserved region among the ankyrin isoforms, this region is likely to play a crucial role in refining the specificity of ankyrin-protein interactions (Bennett, 1992).

1.3.1 ANK1

ANK1 (also referred to as ankyrinR or ANKα) was the first member of the ankyrin gene family to be characterized. In erythrocytes, ANK1 serves as a linker between spectrin and the cytoplasmic domain of the anion exchanger (AE1 or Band 3) (Bennett & Stenbuck, 1980), an association that stabilizes the erythrocyte membrane (Costa et al., 1990; Hanspal et al., 1991; Lux et al., 1990). Expression of ANK1 is also observed in the nervous system, specifically the Purkinje and granule cells of the cerebellum, motor neurons of the spinal column, and a small subset of hippocampal neurons (Lambert & Bennett, 1993). In these neurons, ANK1 is produced postmitotically and is localized to the plasma membrane (Lambert & Bennett, 1993), suggesting a possible role in the formation of specialized membrane domains. Deficiencies in ANK1 are clinically important, since mutant mice with normoblastosis (nb/nb) produce less than 10% of normal levels of ANK1 and suffer from severe hemolytic anemia and neurological deficits such as ataxia and tremor mediated at least in part by progressive degeneration of Purkinje cells (White et al., 1990).

1.3.2 ANK2

The product of the ANK2 gene (also referred to as ankyrinβ or ANKβ) is more broadly expressed in neurons and glia than ANK1 (Kordeli et al., 1991; Kordeli et al., 1990; Kunimoto et al., 1991) and thus has been widely referred to as brain ankyrin. The ANK2 gene product undergoes alternative
splicing to produce a 220kDa and a 440kDa form (Kunimoto et al., 1991). Although these isoforms possess identical membrane binding and spectrin binding domains, the 440kDa form contains a large domain inserted between the membrane/spectrin-binding domains and the carboxy-terminus (Kunimoto et al., 1991). Thus, the 220kDa may be called "a naturally occurring deletion mutant of the 440kDa form" (Kunimoto, 1995) (Figure 3). These two isoforms exhibit distinct temporal and spatial expression patterns in the rat brain. The 440kDa form is a neuron-specific isoform which is localized to unmyelinated axons during development (Kunimoto, 1995). Its expression reaches a peak at postnatal day 10 during axogenesis and declines as the axon matures and becomes fully myelinated (Kunimoto et al., 1991). Binding of this isoform to L1, a developmentally regulated cell adhesion molecule, may provide a method for mechanically stabilizing unmyelinated axons (Kunimoto, 1995). In contrast, the 220kDa form is found in the dendrites and cell bodies of mature neurons, as well as in glial cells (Kunimoto, 1995). It seems likely, then, that the inserted domain in the 440kDa isoform contains targeting information that allows for the differential distribution of this isoform (Kunimoto, 1995). The precise role of the ANK2 isoforms in neurons and glial cells has not yet been determined and is a subject of intense interest.

1.3.3 ANK3

The most widely distributed ankyrin, ANK3 (also referred to as ankyrinα or ANKα), is expressed in diverse alternatively spliced forms in multiple tissues. It is the major ankyrin in the kidney and other epithelial tissues (Peters et al., 1995). Interestingly, the localization of ANK3 in kidney cells coincides with that of the Na⁺,K⁺-ATPase (Katz et al., 1979), a known-ankyrin binding protein (Devarajan et al., 1994; Nelson & Veshnock, 1987), implying a role for ANK3 in the polarized distribution of this ion.
Figure 3. Structural comparison of the ANK2 isoforms. 
440kDa ANK2 contains an inserted 220kDa domain between the membrane/spectrin-binding domains and the regulatory domains. (Redrawn from Chan et al., 1993)
pump. Cardiac, smooth and skeletal muscle also express ANK3, as do certain specialized cells such as the Leydig cells of the testis, megakaryocytes and macrophages (Peters et al., 1995). In neurons, ANK3 is expressed as 480 kDa and 270 kDa forms that contain an unusual 46 kDa serine-rich domain between the spectrin-binding domain and the tail domain (Kordeli et al., 1995). This serine-rich domain exhibits sequence similarity to mucins and glycoproteins (Kordeli et al., 1995), and undergoes glycosylation resulting in single O-linked N-acetylgalcosamine residues (Zhang & Bennett, 1996). The 480 kDa ANK3 is closely structurally related to 440 kDa ANK2, but is expressed in mature myelinated axons where it is localized to the initial segment of the axon and the nodes of Ranvier (Kordeli et al., 1990).

1.4 The Na\(^+\) channel–ankyrin interaction

Based on its copurification with Na\(^+\) channels, ankyrin has been proposed to be a major mode of immobilization of Na\(^+\) channels in neurons (Srinivasan et al., 1988). The binding of Na\(^+\) channels to ankyrin in vitro occurs in a dose-dependent manner, with high affinity (K\(_a\)=22nM) and with an equimolar stoichiometry (Srinivasan et al., 1988; Srinivasan et al., 1992). Most importantly, ankyrin binds to Na\(^+\) channels selectively, as it fails to co-immunoprecipitate with, or bind to, either the neuronal \(\gamma\)-aminobutyric acid receptor or the dihydropyridine-sensitive Ca\(^2+\) channel (Srinivasan et al., 1988). Likewise, substitution of other cytoskeletal proteins such as neurofilaments or actin for ankyrin in these assays does not result in Na\(^+\) channel binding (Srinivasan et al., 1988). Important evidence in support of an interaction between Na\(^+\) channels and ankyrin has been provided by the observation that the distribution of Na\(^+\) channels in neurons, and especially in the myelinated axon, closely correlates with that of ANK3 (Kordeli et al., 1990).
1.4.1 Significance of the Na\(^+\) channel-ankyrin interaction

Fluorescence photobleach recovery data indicate that neuronal Na\(^+\) channels are freely mobile only on the cell body and are highly immobile elsewhere (Angelides et al., 1988). Thus, some mechanism must be present at regions such as the axonal hillock, presynaptic terminal and focal points along the axon to anchor Na\(^+\) channels that is absent in the cell body (Angelides et al., 1988). The shared distribution patterns of ANK3 and Na\(^+\) channels in the myelinated axon strongly suggest a role for ANK3 in this mechanism. It has long been recognized that the density of Na\(^+\) channels is extremely high at nodes of Ranvier and much lower in intervening axonal regions (Ritchie et al., 1976; Ritchie & Rogart, 1977). This distribution is thought to be one part of a more complex mechanism which allows for the rapid transmission of action potentials down axons via a process termed saltatory conduction (Salzer, 1997). Impairment of the mechanism of saltatory conduction would result in a marked decrease in the velocity of the action potential (Johnston et al., 1996), with resulting clinical manifestations similar to those seen in demyelinating diseases (Deerinck et al., 1997; Moll et al., 1991; Salzer, 1997; Waxman & Ritchie, 1993; Westenbroek et al., 1992).

1.4.2 Unresolved questions about the Na\(^+\) channel-ankyrin interaction

Despite the potential significance of the Na\(^+\) channel-ankyrin interaction, many fundamental questions remain. Central issues include how the specificity of Na\(^+\) channel-ankyrin interaction might be generated, and whether ankyrins serve to target Na\(^+\) channels to discrete locations. In addition, mechanisms that regulate this interaction, if regulation indeed exists, have not been investigated. In order to resolve these issues, it is necessary to define the structural basis for the Na\(^+\) channel-ankyrin interaction. While the Na\(^+\) channel binding site on ankyrin has been broadly localized to the last two
subdomains of the membrane binding domain (D3 and D4, Figure 2) (Srinivasan et al., 1992), the ankyrin binding site on the Na⁺ channel as not yet been determined.

1.4.3 Structure of the Na⁺ channel

Biochemical and transfection studies indicate that all the essential features of the voltage-gated sodium channel, such as voltage sensing and gating, are encapsulated within a single polypeptide of molecular weight 260kDa (Hartshorne & Catterall, 1981; Hartshorne et al., 1986). Termed the α subunit, this polypeptide is ubiquitous in excitable tissues in higher vertebrates (Goldin, 1995). However, the precise distribution pattern of the α subunits reflects the relative levels of expression of discrete genes encoding several tissue-specific isoforms (Suzuki et al., 1988). Depending on the tissues from which they are purified, the α subunits are associated with one or two smaller subunits which have been designated β₁ (36kDa) and β₂ (33kDa) (Hartshorne & Catterall, 1981; Hartshorne & Catterall, 1984). In adult rat brain, the predominant channel is a complex comprised of an α subunit encoded by the Na⁺ channel type IIa gene and both β₁ and β₂ subunits (Hartshorne & Catterall, 1981; Sarao et al., 1991). However, the precise role of these auxiliary subunits is unclear since injection of only the mRNA for the α subunit into Xenopus oocytes results in the synthesis of fully functional Na⁺ channels (Moorman et al., 1990; Noda et al., 1986). Nevertheless, co-expression of the auxiliary subunits appears to have significant effects on the activation and inactivation kinetics and enhances levels of expression of the α subunits (Patton et al., 1994).

More intriguing is the homology, albeit low, of the β subunits to members of the cell adhesion molecule superfamily (Isom & Catterall, 1996; Vaughn & Bjorkman, 1996), an observation that has led Catterall and co-workers to postulate that the β subunits may serve to steer Na⁺ channels to or from sites
of cell-cell contact (Isom et al., 1995). While it is possible that the β subunits mediate the interaction between Na⁺ channels and ankyrin, a direct association with the α subunit seems more likely since purified Na⁺ channel preparations first used to demonstrate interaction with ankyrin are reported to be devoid of β subunits (Srinivasan et al., 1988). How ankyrin might interact with the α subunit is unclear but any direct interaction must be through association with regions of the Na⁺ channel exposed to the cytoplasm - an observation consistent with the abolition of ankyrin binding by tryptic digestion of Na⁺ channels reconstituted into lipid vehicles (Srinivasan et al., 1988). Fortunately, the identification of such regions has been greatly simplified by the availability of structural models for the membrane topology of the Na⁺ channel (and other members of the voltage-gated channel superfamily) (Guy & Durell, 1996; Guy & Seetharamulu, 1986; Noda et al., 1984) that have persisted over a decade of intense scrutiny at the biochemical, electrophysiological, pharmacological and cell biological levels.

On a gross structural scale, the α subunit consists of 4 homologous domains termed I-IV which are 40-60% identical to each other at the amino acid level (Figure 4) (Noda et al., 1984). Each domain contains 6 segments which are thought to form membrane-spanning α-helices; 5 of these segments are hydrophobic (S1, S2, S3, S5, and S6) while the remaining segment (S4) is amphipathic and is characterized by positively charged residues generally localized at every third position (Noda et al., 1984). S4 has been identified as the voltage sensor, and the pore region is thought to be contained within the region between S5 and S6 (Catterall, 1992; Catterall, 1993; Catterall, 1995).

The homologous domains are joined by cytoplasmic linker regions which we designate as LI-II (the linker between domains I and II), LI-III (the linker between domains II and III) and LI-IV (the linker between domains III and IV). In addition, domains I and IV are attached to the amino terminus (N-term or NT) and carboxy terminus (C-terminus or CT) respectively. Because of its movement during
Figure 4. Schematic diagram of the voltage-gated Na' channel (see text for a detailed description of Na' channel structure).
inactivation which causes it to move toward the lipid bilayer (Vassilev et al., 1988; Vassilev et al., 1989). LIII-IV is the least likely region to contain a site for interaction with a cytoskeletal linker protein such as ankyrin. A more attractive candidate for the ankyrin binding site is LI-II, since this region contains several protein kinase A (PKA) consensus phosphorylation sites (Murphy et al., 1996; Smith & Goldin, 1996) whose phosphorylation could potentially affect and thus regulate interactions with other proteins. The remaining linker region (LII-III) has not been assigned a specific function or role, and thus may also be involved in forming the ankyrin binding site.

Several lines of evidence argue strongly, however, that the most likely regions to interact with ankyrin are the NT and CT. Until recently, the role of these regions has largely been speculative since neither the NT nor the CT in the Na⁺ channel confer any essential electrophysiological property to channel function (Goldin, 1995). Nevertheless, the existence of considerable diversity between the NT and CT regions (especially the CT) of discrete Na⁺ channel isoforms is a seductive argument that these regions are carefully designed for an important, non-electrophysiological (i.e. cellular role) such as interaction with the cytoskeleton. A non-electrophysiological role for such regions in Na⁺ channels is supported by recent evidence that clustering of structurally-related K⁺ channels is mediated by interaction between motifs in their carboxy termini and the recently identified SAP-90/PSD-95 protein family (Kim et al., 1995; Scannevin et al., 1996). It is certainly noteworthy that such regions often represent the major ankyrin binding domains in other ankyrin-binding membrane proteins such as the anion exchangers (Ding et al., 1996).

Recently, studies on the rat skeletal muscle Na⁺ channel (rSkM-1), a voltage-gated ion channel found in innervated adult skeletal muscle, allude to a possible role for the CT and NT regions in interaction with cytoskeletal elements. Depending on its localization, rSkM-1 is labelled by different
antibodies (Haimovich et al., 1987), both of which recognize epitopes residing within the NT region of the \( \alpha \) subunit (Cohen & Barchi, 1992). Several lines of evidence strongly suggest that these antibodies recognize epitopes on the same protein, implying that the differential interaction of the epitope-containing region with proteins involved in the maintenance process causes the differential antibody labelling and cellular localization (Cohen & Barchi, 1992). Furthermore, the NT region of rSkM-1 may associate with the CT region, thus implicating the CT region in the maintenance of this ion channel in its proper locations (Sun et al., 1995). Because of the similarity between the rSkM-1 ion channel and the rat brain Na\(^+\) channel (Goldin, 1995), it seems likely that the CT and NT regions of the rat brain Na\(^+\) channel form analogous structures and perform analogous functions.

The availability of the cDNA sequences for both rat brain Na\(^+\) channel IIa (rbNIIa) (Goldin, 1995; Noda et al., 1986) and rat ANK2 (Cosentino & Jones, 1995) has made it possible to dissect the basis of the Na\(^+\) channel-ankyrin interaction at the molecular level. I now outline how I have used such information to prepare fusion protein constructs corresponding to the intracellular regions of rbNIIa and to the membrane binding domain of rat ANK2 to identify the ankyrin-binding site on the Na\(^+\) channel.

### 1.5 Hypothesis and specific aims

The central hypothesis of this thesis is that either or both of the NT and CT regions of the Na\(^+\) channel form the ankyrin-binding site. To address this hypothesis, I plan to focus on one particular isoform of the rat brain Na\(^+\) channel, RIIa, since it is the major isoform expressed in the adult rat brain (Sarao et al., 1991). Similarly, I have chosen to focus on the major isoform of ankyrin in brain, ANK2, as its role in neurobiology remains unknown (Otto et al., 1991). As well, the membrane binding domain is
highly conserved (~70% identity) among the ankyrin isoforms (Kordeli et al., 1995; Otto et al., 1991).

My specific objectives were:

1. To develop a bacterial expression and purification system to produce fusion proteins corresponding to regions within the membrane binding domain of ankyrin and the intracellular regions of the Na^+ channels

2. To develop assays that confirm or deny an interaction between ankyrin and the NT and CT regions of Na^+ channel
2 Methods

2.1 Generation of various cDNAs for fusion protein expression

The rat ANK2 isoform had not been isolated prior to the commencement of these studies; therefore, our laboratory cloned and sequenced the membrane binding domain of this isoform. Using the primers indicated in Table 2 and the ANK2 membrane binding domain clones (Genbank Accession No. U65916) obtained by our laboratory, two cDNAs corresponding to overlapping segments of the membrane binding domain (ankyrin repeats 1-17, designated R1-17 and ankyrin repeats 10-22, designated R10-22; Table 3 and Figure 5) were generated by polymerase chain reaction (PCR). This, and all subsequent PCRs, were conducted in a Perkin Elmer Cetus DNA Thermal Cycler. The identity of these cDNAs were confirmed by sequencing (United States Biochemical Sequenase version 2.0 DNA Sequencing Kit).

The cDNAs corresponding to the various cytoplasmic regions of the α subunit of rhNIIa were generated by polymerase chain reaction using full-length α subunit cDNA kindly provided by Dr. Alan L. Goldin (University of California at Irvine; Irvine, CA) and the primers indicated in Table 2. Further details about these constructs are provided in Table 3 and Figure 6.

PCR amplification (38 cycles) was conducted under the following conditions: 10 ng template DNA, 12.5 μM of each primer, 2.5 mM of each dNTP, 2.5 units of Pfu DNA polymerase (Stratagene), 500 mM KCl, and 15 mM MgCl2. Following an initial 2 min denaturation at 95°C, denaturation was performed at 93°C for 1 min, hybridization at 50-65°C for 1 min, and polymerization at 72°C for 2 min. A final polymerization step was performed at 72°C for 5 min. Use of Pfu polymerase instead of Taq polymerase reduced the possibility of PCR-related mutations, since the Taq polymerase has an error rate of 1:1,000 bases (Uemori et al., 1993).
<table>
<thead>
<tr>
<th>Construct</th>
<th>Sequences of sense and antisense primers</th>
</tr>
</thead>
</table>
| **R<sub>17</sub>** | Sense: ATG GAC TAC AAG GAC GAC GAT GAC AAG CTC CGT GCT GCC AGA GCA GGC  
|               | Antisense: TCA CGG GGT AAG GCC GTT CTT CCC TGC                                                      |
| **R<sub>10.22</sub>** | Sense: ATG GAC TAC AAG GAC GAC GAT GAC AAG CAC ATG GCT GCC CAG GGA GAC  
|               | Antisense: TCA CTG AGC GGC CTG GTG CAA AGG                                                        |

<table>
<thead>
<tr>
<th>Construct</th>
<th>Sequences of sense and antisense primers</th>
</tr>
</thead>
</table>
| **NT**        | Sense: AAA GGA TCC ATG GCA CGG TCA GTG  
|               | Antisense: T TTA AGC TTA CTT AAT AGC TAA TTT                                                        |
| **LI-I**      | Sense: AAA GGA TAC TAC GAG GAA CAG AAC  
|               | Antisense: T TTA AGC TTA ATT GAC AAC GTG TTT                                                         |
| **LI-III**    | Sense: AAA GGA TCC AGT TCT TTC AGC TCA  
|               | Antisense: TTT CTG CAG TTA CTT GTA GCA CGT TTT                                                        |
| **LI-IV**     | Sense: AAA GGA TCC CTC TAC ATG TAC CTT  
|               | Antisense: TTT AAG CTT AAT CAA AGA CCA TCC C                                                        |
| **CT**        | Sense: ATG GAG AAC TTC AGC GTC GCC ACC  
|               | Antisense: TTA GCC GGC CTT TTT ACT TTC CCT GAT ATG                                                    |

Table 2. Sequences of the primers used to generate the various constructs. All sequences are given 5' to 3'. The forward primers for R<sub>17</sub> and R<sub>10.22</sub> contain a sequence encoding the FLAG epitope.
<table>
<thead>
<tr>
<th>Construct</th>
<th>Position of cDNA (length of cDNA)</th>
<th>Length of protein construct (# amino acids)</th>
<th>Expected MW (Da)</th>
<th>pI</th>
</tr>
</thead>
<tbody>
<tr>
<td>R₁,₁⁻</td>
<td>1⁻ 564 (564)</td>
<td>622</td>
<td>67,008</td>
<td>8.31</td>
</tr>
<tr>
<td>R₁₉⁻,₂₂</td>
<td>302-735 (434)</td>
<td>492</td>
<td>53,450</td>
<td>8.22</td>
</tr>
<tr>
<td>NT</td>
<td>1-124 (124)</td>
<td>160</td>
<td>18,155</td>
<td>6.97</td>
</tr>
<tr>
<td>LI-II</td>
<td>428-753 (326)</td>
<td>362</td>
<td>40,696</td>
<td>8.00</td>
</tr>
<tr>
<td>LII-III</td>
<td>984-1203 (220)</td>
<td>256</td>
<td>28,726</td>
<td>4.62</td>
</tr>
<tr>
<td>LIII-IV</td>
<td>1474-1526 (53)</td>
<td>89</td>
<td>10,384</td>
<td>4.62</td>
</tr>
<tr>
<td>CT</td>
<td>1777-2005 (229)</td>
<td>284</td>
<td>32,270</td>
<td>5.45</td>
</tr>
</tbody>
</table>

Table 3. Position and sizes of the cDNA and corresponding protein constructs. *Position 1 defined as amino acid #7 in the most recent nomenclature of Michaely & Bennett (1993).
Figure 5. Schematic diagram of the ankyrin constructs.
Figure 6. Schematic diagram of the Na⁺ channel constructs.
2.2 Expression of bacterial fusion proteins

Amplified cDNAs were subcloned in frame in the pRSET-A expression vector (Invitrogen), which generates recombinant protein constructs fused to an N-terminal hexahistidine tag (His-tag). Both cDNAs and vector were digested with the appropriate restriction enzymes (see Table 4) at 37°C overnight. Constructs were ligated into the vector overnight under the following conditions: ratio of cDNA insert to pRSET vector varying between 1:6 to 1:20, 2μl One-Phor-All PLUS buffer (Pharmacia), 7-8.5 units of T4 ligase (Pharmacia) and 2μl rATP (Pharmacia). The identities of all the pRSET constructs were verified initially by restriction mapping and subsequently by DNA sequencing. The pRSET constructs were then used to transform E. coli BL21(DE3) (Novagen) via the heat shock method (Sambrook et al., 1989). In this transformation procedure, the expression vector was incubated with competent bacteria for 30 mins on ice prior to a 90 s incubation at 42°C. To each aliquot of heat-shocked bacteria, 400μl of SOC (20g/L tryptone, 5g/L yeast extract, 0.5g/L NaCl, 186.0mg/L KCl, 0.2M MgCl₂, 5% glucose, pH 7.5) was added. The mixture was then shaken at 225rpm in a 37°C incubator for 30 mins and consequently spread on a LB plate containing 100μg/ml ampicillin (LB-amp plate; 10g/L tryptone, 5g/L yeast extract, 5g/L NaCl, 15g/L agar, pH 7.0) to grow for 18 hr at 37°C. Colonies on this plate were individually grown overnight in 3ml sterile Terrific Broth (TB; 12g/L tryptone, 24g/L yeast extract, 4ml/L glycerol, pH 7.0-7.5) containing a final concentration of 100μg/ml ampicillin and frozen at -80°C as a 15% glycerol stock.

Fusion protein expression was conducted in two stages: first, through an initial pilot screen, and second, through large-scale preparations under optimally defined conditions. For pilot studies,
<table>
<thead>
<tr>
<th>Construct</th>
<th>Restriction enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>R&lt;sub&gt;1.17&lt;/sub&gt;</td>
<td>EcoRI</td>
</tr>
<tr>
<td>R&lt;sub&gt;10.22&lt;/sub&gt;</td>
<td>EcoRI</td>
</tr>
<tr>
<td>NT</td>
<td>BamHI, PstI</td>
</tr>
<tr>
<td>LI-II</td>
<td>BamHI, HindIII</td>
</tr>
<tr>
<td>LII-III</td>
<td>BamHI, PstI</td>
</tr>
<tr>
<td>LIII-IV</td>
<td>BamHI, HindIII</td>
</tr>
<tr>
<td>CT*</td>
<td>SacI, PstI</td>
</tr>
</tbody>
</table>

Table 4. Restriction enzymes used for ligation of cDNA constructs into pRSET expression vector.

*CT was initially cloned into SacI-digested pCRII for sequence verification, then ligated into PstI-digested pRSET-A.
glycerol stocks were streaked onto LB-amp plates that were incubated overnight at 37°C. Colonies on these plates were used to inoculate 50ml sterile TB containing a final concentration of 100μg/ml ampicillin. This culture was then placed in a 37°C shaker (300rpm). When the OD<sub>600</sub> of the bacterial culture was greater than 0.5 but less than 0.6, isopropyl-β-D-thiogalactoside (IPTG: Bioshop) was added to a final concentration of 0.4mM and ampicillin to a final concentration of 100μg/ml. The OD<sub>600</sub> was monitored by periodically removing small (<1ml) aliquots from the culture for measurement of optical density scattering in a Perkin Elmer UV/VIS Spectrophotometer Lambda. At 0.5, 1, 2 and 3 hr post-induction, 1ml of culture was removed, centrifuged at 9,500g (10,000rpm in an IEC MicroMax microfuge) for 3 min and processed for SDS-polyacrylamide gel electrophoresis as described in 2.6. The resulting blots indicated the length of post-induction growth required to produce the maximal amount of fusion protein. Once the optimal expression parameters were determined, large-scale cultures with a typical volume of 500ml TB were grown for purification purposes.

In some cases (3.1) fusion proteins could not be grown in sufficient quantities for purification using the above conditions. In such cases, expression was optimized by varying induction point (e.g. OD<sub>600</sub> of 0.5 vs. 1.0), post-induction temperature (e.g. room temperature (25°C) vs. 37°C) and bacterial strain (e.g. BL21(DE3) vs. BL21(DE3)pLysS). Selection of the pLysS plasmid required adding chloramphenicol to a final concentration of 34μg/ml to the growth medium. In all other respects, these expression studies were conducted in a manner similar to that described above.
2.3 Purification of bacterial fusion proteins

Upon completion of post-induction growth, bacteria were sedimented at \( \sim 6,000g_w \) (6,000 rpm, Sorvall GSA rotor) for 10 min at \( 4^\circ C \) and resuspended in 50 ml cold Denaturing Binding Buffer (DBB; 8 M urea, 20 mM sodium phosphate pH 7.8, 50 mM NaCl). This suspension was then subjected to four freeze-thaw cycles using a dry ice/methanol slurry and a 37°C water bath. To limit proteolysis, PMSF (final concentration of 100 \( \mu \)M) and benzamidine (final concentration of 1 mM) was added prior to the freeze-thaw cycles and after each subsequent thaw. Upon completion of the freeze-thaw cycles, the suspension was incubated overnight at \( 4^\circ C \) to solubilize any His-tagged fusion protein present in inclusion bodies.

The following day, the suspension was passed through an 18-gauge needle six times to shear nucleic acids and then centrifuged at \( 12,000g_w \) (10,000 rpm, Sorvall SS-34 rotor) to pellet any insoluble material. The supernatant was rotated with 2 ml ProBond resin (Invitrogen) pre-equilibrated with 14 ml deionized water and 21 ml DBB for 20 min. The resin was then washed with 16 ml each of DBB, Denaturing Wash Buffer 6.0 (DWB 6.0; 8 M urea, 20 mM sodium phosphate pH 6.0, 50 mM NaCl) and Denaturing Wash Buffer 5.3 (DWB 5.3; 8 M urea, 20 mM sodium phosphate pH 5.3, 50 mM NaCl) prior to elution with Denaturing Elution Buffer (DEB; 8 M urea, 20 mM sodium phosphate pH 4.0, 50 mM NaCl). Eluted material was collected in 0.5 mL fractions using a fraction collector (Pharmacia) and tested for the presence of His-tagged material as described in 2.6.

2.4 Purification of human erythrocyte ankyrin

Human erythrocyte ankyrin was isolated and purified using a modified version of the protocol described in Bennett (1983). Blood was obtained from volunteers among laboratory
personnel and treated separately to prevent agglutination. All subsequent steps were performed at 4°C. To each set of donor blood, 1/7 volume of acid-citrate-dextrose solution (75mM trisodium citrate, 42mM citric acid, 140mM glucose) and 4 volumes of sedimentation buffer (150mM NaCl, 5mM sodium phosphate pH 7.5, 0.75% (w/v) dextran 500) were added and the erythrocytes were allowed to settle for 1.5 hr. After aspirating the top layer, fresh sedimentation buffer was added and the erythrocytes were allowed to settle for an additional 2 hr. The erythrocytes were then washed twice with 20 volumes of saline solution (150mM NaCl) and sedimented after each wash by centrifuging for 5-10 min at 2,000g, (4,400rpm, Sorvall GSA rotor). Lysis of the erythrocytes was accomplished by adding 6.67 volumes of lysis buffer (7.5mM sodium phosphate pH 7.5, 1mM EDTA with protease inhibitors: 100μM PMSF, 1mM pepstatin A, 1mM benzamidine) to each pellet, centrifuging the mixture for 30 min at 38,000g (19,000rpm, Sorvall GSA rotor) and repeating this step twice more.

The resulting erythrocyte ghosts were extracted by a 15 min incubation with 10 volumes of ankyrin buffer 1 (100mM KCl, 7.5mM sodium phosphate pH 7.5, 1mM EDTA, 0.4mM dithiothreitol (DTT), 0.5% (v/v) Triton X-100, plus protease inhibitors). Following centrifugation for 25 min at 38,000g, the ghosts were re-extracted as described and then re-extracted with ankyrin buffer 1 lacking Triton X-100. Next, the ghosts were incubated with ankyrin buffer 2 (1M KCl, 7.5mM sodium phosphate pH 7.5, 1mM EDTA, 0.2mM DTT) for 30 mins. Any non-extracted material was pelleted with a 30 min 29,000g (20,000rpm, Sorvall A-841 rotor) spin and the resulting supernatant dialyzed against a large volume (4L) of dialysis buffer (7.5mM sodium phosphate pH 7.5, 1mM EDTA, 0.2mM DTT, 0.1mM sodium azide, plus protease inhibitors) overnight. The next day, the dialysate was concentrated through exposure to dialysis buffer.
containing 10% (w/v) sucrose for 2 hr, after which the concentrated dialysate was loaded onto a DEAE cellulose column pre-equilibrated with dialysis buffer lacking DTT and azide (column buffer). The column was washed with 10 column volumes of column buffer and column material was eluted with a gradient of 0 to 500mM KCl in column buffer with 2% (w/v) sucrose, collected in 1ml fractions. The position of ankyrin in the elution was determined by immunoblotting using anti-human erythrocyte ankyrin (Calbiochem; 1:800 dilution) as described in 2.6. The relevant fractions were pooled. After adding 1mM azide, 0.2% Tween-20 and 0.2mM DTT, the pooled material was frozen and stored in aliquots at -80°C.

2.5 Purification of rat brain Na⁺ channel

2.5.1 Preparation of adult rat synaptic membranes

Adult rat synaptic membranes (7mg/ml) were prepared as described in Hartshorne & Catterall (1984). After sacrifice by CO₂ asphyxiation, the brains of 10 Wistar adult rats were dissected out of their skulls and homogenized by 8 strokes of a Potter-Elvejhem tissue grinder in 4.5ml of cold Buffer A (0.32M sucrose, 5mM Tris-HCl pH 7.4). The homogenate was briefly centrifuged (700g, spin (3,000rpm, Sorvall AH-650 rotor) for 10 min at 4°C) to pellet any large particulate matter and the supernatant was then centrifuged at 27,000g₅₀ (19,000rpm, Sorval AH-650 rotor) for 45 min at 4°C. The resulting pellet was resuspended by homogenizing in lysis buffer B (5mM Tris-HCl pH 8.2, 1mM EDTA) and incubated on ice for 15 min. Next, the lysate was spun at 27,000g₅₀ for 45 min at 4°C, and the resulting pellet was resuspended in Buffer C (5mM HEPES-Na⁺ pH 7.4, 0.32M sucrose). To minimize proteolytic degradation, protease inhibitors at the following concentrations were added to each buffer immediately prior to use: PMSF (100μM), O-phenanthroline (1mM), leupeptin (1μM), aprotinin
(1µg/ml), benzamidine (1mM) and iodoacetamide (1mM). The adult rat synaptic membranes were stored in aliquots in liquid nitrogen. The concentration of membrane proteins in these preparations was determined by Lowry assay (2.7).

2.5.2 Preparation of ankyrin-free Na⁺ channel

Rat brain Na⁺ channel was crudely purified using a modified version of the method described in Hartshorne & Catterall (1981) and stripped of any interacting ankyrin according to Srinivasan et al. (1988). Briefly, 8ml of Buffer A (20mM HEPES pH 7.4, 1mM EDTA, 10⁻⁶ (v/v) glycerol, 100µM PMSF, 1mM pepstatin and 1.56mM benzamidine), 1ml 10⁻⁶ (v/v) Triton X-100 (Boehringer-Mannheim) and 1ml of adult synaptic membranes were mixed together and incubated on ice for 1 hr. The solubilization mixture was centrifuged at 100,000gₑ (33,000rpm, Sorvall AH-650 rotor) for 90 min at 4°C and the resulting supernatant was applied to a 2ml-bed wheat-germ agglutinin (WGA) column (prepared as in 2.5.3), which was pre-equilibrated at 4°C with 5 column volumes of Buffer A and 6 column volumes of Buffer B (0.1% (v/v) Triton X-100 and 0.02% (w/v) L-α-phosphatidylcholine (Sigma) in Buffer A). After incubating the solubilized membranes with the WGA resin for 2 hr at 4°C, the column was drained and washed with 20 column volumes of 500mM KCl in Buffer B. Glycoprotein bound to the column was eluted by adding 10 column volumes of Buffer B containing 100mM N-acetylglucosamine and 150mM KCl and collected in 1ml fractions. Those fractions containing the highest amount of protein as determined by the Bradford assay (2.7) were pooled.

2.5.3 Preparation of the wheat-germ agglutinin column

WGA was coupled to CNBr-activated Sepharose as follows. CNBr-activated Sepharose (Sigma)
was first washed with ice cold 1mM HCl for 15 min, then incubated with 10mg WGA dissolved in 0.5ml ice cold coupling buffer (0.5M NaCl, 0.1M NaHCO₃, pH 8.3 and 0.1M N-acetylglucosamine) overnight at 4°C. Unreacted sites were quenched by rotating the gel with 10ml 0.1M Tris pH 8.0 for 2 hr room temperature. The gel was then washed with the following buffers: 10ml 0.5M NaCl, 10ml 0.1M citric acid adjusted to pH 5.4 with NaOH, 10ml 0.5M NaCl and 10ml NaHCO₃, pH 8.0. The above washing was repeated after each Na⁺ channel purification. The gel was stored in phosphate-buffered saline (PBS; 137mM NaCl, 8.6mM K₂HPO₄, 1.5mM KH₂PO₄, pH 7.4) containing 0.04% sodium azide at 4°C.

2.6 Gel electrophoresis and immunoblotting

To assay for the presence of His-tagged fusion protein in the aliquots of bacteria generated by the pilot expression studies, 1ml of culture was sedimented as described previously, resuspended in 133μl 1X electrophoresis sample buffer (0.0625M Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS), 5% (v/v) 3-mercaptoethanol, 0.001% (w/v) bromophenol blue) and frozen at -20°C. Upon completion of the time course, all the time point samples were thawed and boiled (100°C) for 5 mins. 10-20μl of these mixtures were loaded on polyacrylamide gels. To assay for the presence of His-tagged fusion protein in the various flowthroughs and eluted fractions generated by each purification, 16μl of each sample was mixed with 24μl of 2X electrophoresis sample buffer and the entire volume of the resulting mixture was loaded on polyacrylamide gels following boiling. All other samples were diluted at least 6.25 fold in 1X electrophoresis sample buffer before boiling.

Discontinuous polyacrylamide gels (80mm x 1.5mm x 7.3mm) of the following composition were assembled in a Mini-Protean II gel apparatus (Bio-Rad): stacking gel - 4%T, 2.7%C (where %T represents the total percentage (w/v) of acrylamide monomer and N,N-methylenebisacrylamide (BIS)
crosslinker

and O:OC represents the amount of BIS crosslinker expressed as a percentage of the sum of acrylamide monomer and BIS), 0.125 M Tris-HCl pH 6.8, 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate, 0.05% (v/v) TEMED; separating gel - 7.5% T, 2.7% C or 10% T, 2.7% C or 13% T, 2.7% C, 0.375 M Tris-HCl pH 8.8, 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate and 0.05% (v/v) TEMED. Electrophoretic separation using a Mini-Protean II gel apparatus attached to a Model 1000/500 power supply (Bio-Rad) was done at 100-150 V constant voltage for 60-90 min in electrophoresis tank running buffer (0.025 M Tris-HCl pH 8.3, 0.192 M glycine and 0.1% (w/v) SDS). Kaleidoscope (Kds) pre-stained molecular mass standards (Bio-Rad) containing myosin (~208 kDa), β-galactosidase (~144 kDa), bovine serum albumin (BSA; ~87 kDa), carbonic anhydrase (~44 kDa), soybean trypsin inhibitor (~33 kDa), lysozyme (~18 kDa) and aprotinin (~7 kDa) were also separated on each gel. Each standard lane contained 5 µl of Kds diluted in sample buffer 6.25-fold.

The separated proteins were transferred electrophoretically to nitrocellulose sheets (Bio-Rad) by the method of Towbin et al. (1979). Prior to transfer, nitrocellulose sheets and gels were equilibrated in transfer buffer (0.025 M Tris-HCl pH 8.3, 0.192 M glycine, 20% (v/v) methanol) for at least 15 min. Transfer occurred at 100 V in transfer buffer for 1 hr in a Mini Trans-Blot Electrophoretic Transfer Cell attached to a Model 200/2.0 power supply (Bio-Rad). Efficiency of transfer was assessed by briefly (2-3 min) staining the blot with a solution containing 0.2% (w/v) Ponceau S, 0.18 M trichloroacetic acid and 0.12 M sulfosalicylic acid and destaining with deionized water.

Following transfer, excess protein binding sites were blocked using 5% (w/v) dry skim milk (Carnation)/tris-buffered saline (TBS; 20 mM Tris pH 7.5, 500 mM NaCl)/0.01% thimerosal (5% NFDM/TBS) overnight at 4°C. The next day, the blots were washed as follows: 3 short (15 s) washes in Tween-TBS (TTBS; TBS with 0.05% Tween-20), then 3 long (5 min) washes in TTBS, and finally 3
short and 3 long washes in TBS at room temperature (RT). Blots were incubated for 1.5-2 hr at RT in primary antibody (His-probe antibody (Santa Cruz Biotechnologies) diluted 1:1,000 in 5% NFDM/TBS: anti-chicken-erythrocyte-ankyrin (Calbiochem) diluted 1:3,000; anti-human-erythrocyte-ankyrin (Calbiochem) diluted 1:800; anti-rat brain Na+ channel (Upstate Biotechnology) at 1μg/ml), and then washed extensively as described above. Blots were then incubated for 1 hr at RT in horseradish peroxidase conjugated to donkey-anti-rabbit (HRP-DAR; Amersham) diluted 1:4,000 (all blots except those initially probed with anti-human-erythrocyte-ankyrin) or horseradish peroxidase conjugated to goat-anti-mouse (HRP-GAM; Zymed) diluted 1:3,000 (anti-human-erythrocyte-ankyrin) in 5% NFDM/TBS, following which was extensive washing as described above.

Enhanced chemiluminescence (ECL) was performed by mixing equal volumes of Amersham ECL detection reagents and incubating the mixture on the blots (0.125ml of mixture/cm² of nitrocellulose) for 1.5-2 min. The sheets were drained, covered in plastic wrap (Saran Wrap), placed in autoradiography cassettes (Fisher Biotech, FBXC 810, 8x10") and exposed to Kodak scientific imaging film (X-Omat AR, 8x10") or Amersham Hyperfilm ECL (8x10"). When greater sensitivity was required, Amersham Hyperfilm ECL was used and prefilled with a Sensitize Pre-flash Unit (Amersham). The films were developed using equipment in the Toronto Western Hospital radiology unit. Blots were scanned and displayed pictorially using PhotoShop 4.0 (Adobe).

2.7 Determination of protein concentration

Protein concentration was determined using the Lowry (1951) or Bradford (1976) assays. Regardless of which assay was performed, protein solutions, including the BSA standards, were prepared in H2O in a total volume of 100μl at various dilutions, and calculations of sample concentrations were
based on the BSA standard curve.

The Lowry assay was carried out as follows. To prepare Lowry solution 1, one part of solution B (2% (w/v) Na\textsuperscript{+}K\textsuperscript{−} tartrate/H\textsubscript{2}O) was mixed with one part of solution C (1% (w/v) CuSO\textsubscript{4}/H\textsubscript{2}O), and then added to 100 parts of Solution A (2% (w/v) Na\textsubscript{2}CO\textsubscript{3}, 0.4% (w/v) NaOH/H\textsubscript{2}O). 1ml of this Lowry solution 1 was added to each sample and incubated for 10 min. 100\mu l of Lowry solution 2 (Folin-Ciocalteu’s phenol reagent (Fluka) diluted 1:1 in H\textsubscript{2}O) was then added to each sample while mixing. Absorbances were read 45 min later at 750nm.

The reagent for the Bradford assay was prepared by diluting Bio-Rad Protein Assay Dye Concentrate (Bio-Rad) 1:4 in H\textsubscript{2}O and filtering the mixture. 4.5ml of this Bradford reagent was added to each sample, and absorbances were read at 595nm after 10 min.

2.8 Protein labelling

Prior to labelling, the His-tagged constructs were renatured and pH-adjusted through dialysis. Renaturation was accomplished by dialyzing the constructs against a continuous gradient of urea from 0.6M to 0M in 50mM sodium phosphate pH 7.4, 0.1M NaCl, 0.1% Tween-20, 1mM azide for 7 hr. To raise the pH of the constructs to a level conducive to the labelling reactions, the constructs were next dialyzed against 0.1M NaHCO\textsubscript{3}, pH 8.0 for 2 hr.

2.8.1 Photolabelling

His-tagged constructs were labelled with the photoreactive group benzophenone (Hermanson, 1996) at a stoichiometry of 10:1 using benzophenone-NHS ester (Molecular Probes) dissolved in 5% DMSO to give a concentration of 1.5mM. The final reaction mixture contained 0.1M sodium
bicarbonate pH 8.2/10% DMSO and allowed to proceed for 2 hr under light-protected conditions. The labelled constructs were exhaustively dialyzed in the dark against PBS/0.1% Tween-20 and stored in aliquots at -80°C.

2.8.2 Biotinylation

His-tagged constructs and erythrocyte ankyrin were biotinylated at a stoichiometry of 4:1 using sulfo-NHS-LC-biotin (Pierce). If necessary, 0.15M sodium bicarbonate pH 8.5 was added until the protein pH was between 8.0 and 8.5. Sulfo-NHS-LC-biotin was dissolved in deionized water to give a concentration of 50μM and immediately added to the reaction mixture with gentle vortexing. The reaction was allowed to proceed for 10 min at room temperature, then at least 50 min on ice, and was light-protected at all times. Glycine was then added to a final concentration of 1mM to quench the reaction. The biotinylated proteins were stored in aliquots at -80°C.

2.9 Photo-activated cross-linking experiments

Each photolabelled Na⁺ channel construct was mixed with human erythrocyte ankyrin in a buffer containing 50mM sodium phosphate pH 7.4, 0.1M NaCl and 0.1% Tween-20. The total reaction volume was 100μl and the final concentrations of Na⁺ channel construct and ankyrin were 2μM and 0.5μM respectively. The mixtures were placed in inverted silanized vial caps and irradiated (360nm) for 40 min using a UV lamp (Blak-Ray, UV Products). Controls included no irradiation of a separate set of mixtures, irradiation of Na⁺ channel constructs only and irradiation of ankyrin only. Following the irradiation period, all samples were electrophoretically separated on polyacrylamide gels, transferred to nitrocellulose and probed with His-probe antibody as described in 2.6.
Figure 7. Structural diagram of benzophenone illustrating how it can cross-link interacting proteins.
Figure 8. Flowchart outlining the photo-activated cross-linking experimental protocol.
2.10 Affinity precipitations

Affinity precipitations were carried out essentially as described in Michaely & Bennett (1995). Epoxy-activated latex beads with a diameter of 0.4\(\mu\)m (Bangs Laboratories) were reacted with NeutrAvidin (Pierce) during end-over-end mixing for 72 hr at 4°C in coupling buffer (50mM sodium phosphate pH 8.0, 1M NaCl, 1mM Na\(_2\)S). To quench unreacted sites, the beads were then washed two times with 100mM Tris pH 8.0 and further incubated with this quenching buffer overnight. Next, nonspecific protein sites on the beads were blocked by incubating the beads with 0.5% (v/v) cold-water fish gelatin (CWFG; Amersham) for 2 hr. After the beads were washed three times with coupling buffer, they were conjugated with biotinylated human erythrocyte ankyrin overnight at 4°C. Unbound protein was removed by washing three times with buffer containing 10mM sodium phosphate pH 7.2, 1M NaCl, 1mM Na\(_2\)S, 1mM DTT and 0.05°o (v/v) Tween-20.

The Na\(^+\) channel constructs were diluted to a final concentration of 0.1\(\mu\)M in binding buffer (10mM sodium phosphate pH 7.2, 100mM NaCl, 1mM Na\(_2\)S, 1mM DTT, 0.1°o (v/v) Tween-20) and incubated with 100\(\mu\)l ankyrin-conjugated beads in silanized tubes overnight. Control tubes also contained 500nM unbiotinylated ankyrin. The next day, the beads were washed two times with wash buffer (10mM sodium phosphate pH 7.2, 1mM azide, 1mM DTT, 0.1°o (v/v) Tween-20, 0.5°o (v/v) CWFG) and once with 25mM Tris pH 7.5, then boiled in 50\(\mu\)l of sample buffer. The supernatants were electrophoretically separated on polyacrylamide gels and transferred to nitrocellulose and the resulting blots were probed with His-probe antibody (2.6).
Na\(^+\) channel constructs

probe precipitates for His-tag immunoreactivity

Figure 9. Flowchart outlining the affinity precipitation protocol.
2.11 Characterization of the Na\(^+\) channel–ankyrin interaction by enzyme-linked immunosorption assay (ELISA)

\(\text{Na}^+\) channel constructs (50\(\mu\)l 0.01mg/ml in PBS/0.2\(\%\) (w/v) azide) were adsorbed onto the ELISA plate (Corning) overnight at 4\(^\circ\)C. All subsequent steps were carried out at room temperature. The next day, the wells were aspirated and blocking solution (300\(\mu\)l 2\(\%\) (v/v) CWFG) in PBS/thimerosal was added to each microwell. After 3 hr, the blocking solution was aspirated and 50\(\mu\)l biotinylated ankyrin (0-128nM final in 1\(\%\) (v/v) CWFG/PBS/thimerosal) was added. A parallel set of wells also contained unbiotinylated 500nM ankyrin in the same volume. After 3 hr, the wells were washed three times with 0.1\(\%\) (v/v) CWFG blocking solution and 75\(\mu\)l avidin peroxidase (1:2,000 in 0.1\(\%\) (v/v) CWFG blocking solution; Bio-Rad) was added to each well. The reactions were incubated for 2 hr, after which the wells were aspirated, washed three times with 0.1\(\%\) (v/v) CWFG blocking solution and once with PBS alone. The plates were developed by adding 75\(\mu\)l/well of the peroxidase substrate azidino-di-(3-ethyl-benzthiazoline-sulphonate) (ABTS; Boehringer Mannheim) at 0.5mg/ml in ELISA buffer (25mM borate, 25mM phosphate, 25mM citrate pH 5.0 containing 0.05\(\%\) (v/v) \(\text{H}_2\text{O}_2\). The absorbances were then read at 405nm, 22\(^\circ\)C periodically using an ELISA plate reader (UV Thermomax, Molecular Devices). The maximum absorbance used was 1.0 and any further readings were terminated to preclude any non-linearity. All measurements were conducted in triplicate.

Specific binding was determined by subtracting signals seen in the presence of 500nM ankyrin from those obtained in the absence of competing ankyrin. The resulting data was then analyzed using a non-linear least squares analysis (Levenberg, 1944; Marquardt, 1963).
Figure 10. Flowchart outlining the EJLISA protocol.

- Biotinylated ankyrin + unbiotinylated ankyrin
- Na⁺ channel construct
- EJLISA well
- Probe for biotin
3 Results

3.1 Expression of bacterial fusion proteins

Pilot studies were conducted as described in 2.2 prior to large-scale expression cultures in order to optimize expression parameters. Immunoblots were used to analyze the post-induction expression of His-tagged constructs under defined parameters and revealed that the majority of the fusion proteins, specifically CT, NT, R117, and R10-22, were successfully expressed under "standard" conditions involving (1) the use of BL21(DE3) strain, (2) induction at OD₆₀₀ of 0.5-0.6 and (3) a post-induction growth period of 3 hr. A blot of a representative time course is given in Figure 11.

Three of the fusion proteins, namely LI-II, LII-III and LIII-IV, could not be grown in sufficient quantities for purification under standard conditions. Various expression strategies were employed to determine the optimal expression parameters for these constructs. In the case of LII-III, maximal production in BL21(DE3) occurred when the culture was grown at 25°C post-induction for 6 hr, with induction at an OD₆₀₀ of 0.5 (Figure 12). Production of the LIII-IV construct necessitated transforming the expression vector into the BL21(DE3)pLysS strain, which was then induced at OD₆₀₀ of 1.0 and incubated for 1 hr post-induction at 37°C (Figure 13).

Several expression schemes were attempted for LI-II (detailed in Table 5), none of which yielded any detectable production of this construct.
Figure 11. Time course of \( R_{10.22} \) expression under standard conditions (see 3.1 for description).
Lane A, pre-induction; lane B, 0.5 h post-induction; lane C, 1 h post-induction; lane D, 2 h post-induction; lane E, 3 h post-induction. Samples were electrophoretically separated on a 7.5% acrylamide gel, transferred to nitrocellulose membrane and probed with His-tag antibody.
Figure 12. Time course of LII-III under non-standard conditions (see 3.1 for description).
Lane A, pre-induction; lane B, 1 h post-induction; lane C, 2 h post-induction; lane D, 3 h post-induction; lane E, 4 h post-induction; lane F, 5 h post-induction; lane G, 6 h post-induction; lane H, 7 h post-induction; lane I, 8 h post-induction. Samples were electrophoretically separated on a 10% acrylamide gel, transferred to nitrocellulose membrane and probed with His-tag antibody.
Figure 13. Time course of LIII-IV under non-standard conditions (see 3.1 for description).
Lane A, pre-induction; lane B, 1 h post-induction, lane C, 2 h post-induction; lane D, 3 h post-induction; lane E, 4 h post-induction; lane F, 5 h post-induction; lane G, 6 h post-induction. Samples were electrophoretically separated on a 13% acrylamide gel, transferred to nitrocellulose membrane and probed with His-tag antibody.
<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>OD&lt;sub&gt;600&lt;/sub&gt; at induction</th>
<th>Post-induction temperature (°C)</th>
<th>Post-induction period (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL21(DE3)</td>
<td>0.5</td>
<td>37</td>
<td>3</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>0.5</td>
<td>30</td>
<td>6</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>0.5</td>
<td>25</td>
<td>6</td>
</tr>
<tr>
<td>BL21(DE3)pLysS</td>
<td>0.5</td>
<td>37</td>
<td>3</td>
</tr>
<tr>
<td>BL21(DE3)pLysS</td>
<td>1.0</td>
<td>37</td>
<td>3</td>
</tr>
<tr>
<td>BL21(DE3)pLysS</td>
<td>0.5</td>
<td>25</td>
<td>6</td>
</tr>
<tr>
<td>BL21(DE3)pLysS</td>
<td>1.0</td>
<td>25</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 5. Description of expression schemes attempted for LI-II production.
3.2 Purification of bacterial fusion proteins

The His-tagged constructs were purified by a denaturing scheme utilizing 8M urea as the denaturant. Initially, washes of pH 6.0 and pH 5.3 were employed, per the manufacturer’s instructions, which resulted in the presence of His-tagged construct in the first 4-5ml of eluting buffer (Figure 14). When the pH 5.3 wash was omitted from purification procedures, His-tagged construct was detected in the 3rd-4th ml of eluting buffer (Figure 15). Since the constructs have no endogenous activity that could be used to monitor their purification, purity was ascertained by the proportion of His-tagged protein and the relative absence of extraneous bands, as determined by Ponceau staining of nitrocellulose membranes following transfer. Although other bands were evident, the bands corresponding to the His-tagged proteins were clearly the predominant proteins in the eluted fractions; the limitations to these criteria are explored further in 4.4.

Typical yields for each of the constructs after denaturing purification is given in Table 6. The apparent molecular weights of the Na+ channel constructs and the ankyrin constructs are illustrated in Figures 16 and 17, respectively, and are compared against expected molecular weights are given in Table 7.

3.3 Purification of human erythrocyte ankyrin

Ankyrin was selectively extracted from erythrocyte ghosts in two stages. First, the ghosts were extracted with a low concentration of Triton X-100 to deplete spectrin. The Triton-X-100-extracted ghosts were incubated in 1M KCl in the absence of detergent, which solubilized ankyrin only. Following this preferential solubilization procedure, ankyrin was purified to near-homogeneity by DEAE chromatography. Surprisingly, ankyrin eluted in a biphasic peak although it appeared homogenous on
Figure 14. Affinity purification of NT with the pH 5.3 wash.
Lane A, pre-column material; lanes B and C, unbound material; lane D, pH 7.8 wash flowthrough; lane E, pH 6.0 wash flowthrough; lane F, pH 5.3 wash flowthrough; lanes G-M, elution fractions 1, 3, 5, 7, 9, 11 and 13. Samples were electrophoretically separated on a 7.5% acrylamide gel, transferred to nitrocellulose membrane and probed with His-tag antibody.

Figure 15. Affinity purification of LIII-IV without the pH 5.3 wash.
Lane A, pre-column material; lanes B and C, unbound material; lane D, pH 7.8 wash flowthrough; lane E, pH 6.0 wash flowthrough; lanes F-L, elution fractions 1, 3, 5, 7, 9, 11 and 13. Samples were electrophoretically separated on a 13% acrylamide gel, transferred to nitrocellulose membrane and probed with His-tag antibody.
<table>
<thead>
<tr>
<th>Construct</th>
<th>Yield (mg) from a 500ml bacterial culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R_{10.17}$</td>
<td>1.6</td>
</tr>
<tr>
<td>$R_{10.22}$</td>
<td>0.6</td>
</tr>
<tr>
<td>NT</td>
<td>1.05</td>
</tr>
<tr>
<td>LII-III</td>
<td>1.13</td>
</tr>
<tr>
<td>LIII-IV</td>
<td>0.53</td>
</tr>
<tr>
<td>CT</td>
<td>1.65</td>
</tr>
</tbody>
</table>

Table 6. Typical yields of His-tagged constructs following purification using Ni$^{2+}$ resin.
Figure 16. Na⁺ channel constructs. 4 µg protein was loaded per lane. Lane A, CT; lane B, LIII-IV; lane C, LII-III; lane D, NT. Samples were electrophoretically separated on a 10% acrylamide gel, transferred to nitrocellulose membrane and probed with His-tag antibody.

Figure 17. Ankyrin constructs. 2.4 µg of protein was loaded per lane. Lane A, R₁₋₁; lane B, R₁₋₂₂. Samples were electrophoretically separated on a 10% acrylamide gel, transferred to nitrocellulose membrane and probed with His-tag antibody.
<table>
<thead>
<tr>
<th>Construct</th>
<th>Expected molecular weight (kDa)</th>
<th>Apparent molecular weight (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R&lt;sub&gt;1-17&lt;/sub&gt;</td>
<td>67</td>
<td>79.4</td>
</tr>
<tr>
<td>R&lt;sub&gt;10-22&lt;/sub&gt;</td>
<td>53</td>
<td>62.5</td>
</tr>
<tr>
<td>NT</td>
<td>18</td>
<td>15.4</td>
</tr>
<tr>
<td>LII-III</td>
<td>29</td>
<td>52.9</td>
</tr>
<tr>
<td>LIII-IV</td>
<td>10</td>
<td>7.1</td>
</tr>
<tr>
<td>CT</td>
<td>32</td>
<td>45.2</td>
</tr>
</tbody>
</table>

Table 7. Comparison of expected versus apparent molecular weights of His-tagged constructs. Apparent molecular weights were calculated against a standard curve obtained by plotting log distance travelled by the Kds pre-stained molecular mass standards against their molecular weights.
Figure 18. Purification of human erythrocyte ankyrin
10µl of each sample was mixed with 25µl 2X sample buffer, electrophoretically separated on 7.5% acrylamide gels, transferred to nitrocellulose membrane and probed with anti-human-erythrocyte-ankyrin antibody. Upper blot: Lane A, erythrocyte ghosts; lanes B-ß, eluted fractions 1-25. Lower blot: Lanes A-ß, eluted fractions 27-53.
3.4 Ankyrin depletion of rat brain Na\(^+\) channel

Rat brain Na\(^+\) channel was crudely purified using an abbreviated version of the protocol described in Hartshorne & Catterall (1981), then stripped of interacting ankyrin by washing with a highly ionic buffer (Srinivasan et al., 1988). Analysis of the purification through immunoblotting reveals an enrichment of Na\(^+\) channel, which appears as a broadly diffuse band of relative molecular mass 220-260kDa (Figure 19, upper blot). Absence of ankyrin in the eluted fractions containing Na\(^+\) channel was also confirmed through immunoblotting (Figure 19, lower blot).

3.5 Photo-activated cross-linking experiments

Initial attempts to localize the ankyrin-binding site on the Na\(^+\) channel focused on identifying the specific intracellular domain(s) involved through photo-activated cross-linking. The principle on which these experiments were based is the ability of benzophenone to cross-link and incorporate covalently molecules that are in extremely close proximity (van der Waals distance) to each other. Owing to the presence of multiple lysine residues within each Na\(^+\) channel fragment, it was possible to generate benzophenone adducts of each fragment. The absence of any visible precipitation suggested that the labelling conditions chosen did not result in overmodification with this hydrophobic label. When the benzophenone-labelled Na\(^+\) channel fragments were incubated with ankyrin in the absence of any incident UV light and the reactions resolved by immunoblotting with His-tag antibody, no cross-linked adducts were detected (lanes I-L, Figure 20). However, immunoblotting of the irradiated Na\(^+\) channel-ankyrin mixtures revealed that the NT and CT constructs formed broad bands corresponding to the
Figure 19. Preparation of ankyrin-free Na\(^+\) channel. Samples were electrophoretically separated on 5% acrylamide gels and transferred to nitrocellulose as described in 2.6. The upper blot was probed with anti-Na\(^+\) channel and the lower blot was probed with anti-chicken erythrocyte ankyrin as described in 2.6. Lane A, solubilized membranes; lane B, unbound material; lane C, high salt wash; lanes D-H, eluted fractions 3, 5, 7, 9 and 11.
Figure 20. Photo-activated cross-linking experiments. 15μl of each reaction mixture was mixed with 25μl of 2X sample buffer and electrophoretically separated on 7.5% acrylamide gels, transferred to nitrocellulose membrane and probed with His-tag antibody. The prefix "Z" before a construct name indicates that it is conjugated to benzophenone. Lanes A and J, Z-NT and ankyrin; lanes B and K, Z-LII-III and ankyrin; lanes C and L, Z-LIII-IV and ankyrin; lanes D and M, Z-CT and ankyrin; lane E, Z-NT alone; lane F, Z-LII-III alone; lane G, Z-LIII-IV alone; lane H, Z-CT alone; lane I, ankyrin alone. All the preceding reaction mixtures were irradiated as described in 2.9 except for those in lanes K-M.
anticipated high molecular weight adducts (>200kDa) with ankyrin (lanes A-D, Figure 20). The primary band centered around 220kDa for the NT-ankyrin adduct and 232kDa for the CT-ankyrin adduct. The intensity of these bands was greatly reduced when ankyrin was omitted (lanes E-H, Figure 20), consistent with primary adduct formation through interaction with ankyrin. Interestingly, some adduct formation was seen in the absence of ankyrin for NT, CT and LII-III, albeit at much lower levels than seen in the presence of ankyrin.

3.6 Affinity precipitations of Na⁺ channel constructs using ankyrin–coated beads

To further investigate the Na⁺ channel-ankyrin interaction, ankyrin was attached to latex beads and used as an affinity matrix in a series of precipitation reactions. The strategy employed was identical to that used by Michaely & Bennett (1995) to probe anion exchanger-ankyrin interactions and involved the prior derivatization of ankyrin with biotin and its conjugation to an avidin analog (NeutrAvidin) covalently attached to epoxy-functionalized latex beads. The resulting ankyrin-coated beads were then used to probe interactions with NT, LII-III, LIII-IV or CT using an immunoprecipitation format. To test for specificity the reactions were conducted in the presence or absence of competing excess free erythrocyte ankyrin. Immunoblot analysis of proteins extracted from the precipitates clearly revealed the presence of only the NT fragment (lane B, Figure 21). This band was almost completely eliminated in the presence of excess competing ankyrin (lane F, Figure 21). The other constructs (CT, LII-III, LIII-IV) could not be precipitated in this assay, suggesting their interaction with ankyrin is either non-existent or of low affinity under the conditions employed.
Figure 21. Affinity precipitation with ankyrin-coated latex beads.
-40μl of each bead precipitate was electrophoretically separated on a 7.5% acrylamide gel, transferred to nitrocellulose membrane and probed with His-tag antibody. The ankyrin-coated beads were exposed to the following conditions: Lane A, no construct added to beads; lane B, beads incubated with NT; lane C, beads incubated with LII-III; lane D, beads incubated with LIII-IV; lane E, beads incubated with CT; lane F, beads incubated with NT and free ankyrin; lane G, beads incubated with LII-III and free ankyrin; lane H, beads incubated with LIII-IV and free ankyrin; lane I, beads incubated with CT and free ankyrin; lane J, beads incubated with free ankyrin alone.
3.7 **Analysis of the binding kinetics of the Na\(^+\) channel construct-ankyrin interactions using ELISAs**

As a final test of the existence and specificity of the Na\(^+\) channel-ankyrin fragment interaction, ELISAs were used. Such assays provide exquisite sensitivity and, under the appropriate conditions, can be used to determine the binding kinetics of protein-protein interactions. In these assays, the binding of ankyrin to adsorbed Na\(^+\) channel fragment was examined. Binding was observed for all constructs tested, but was most marked for NT and CT (Figure 22, A and D). More significantly, only binding of ankyrin to adsorbed NT and CT could be displaced by excess competing ankyrin (Figure 22, A and D).

Based on the assumption that the competing ankyrin was at a concentration sufficient enough to eliminate specific interactions, it is possible to replot the data taking into account non-specific binding. Since the curves obtained in the presence and absence of unlabelled (unbiotinylated) ankyrin for LII-III and LIII-IV overlapped at all but the highest ligand concentrations (Figure 22, B and C), the binding of ankyrin to these constructs could be regarded as non-specific binding over this range.

The resulting plots for specific binding seen with NT and CT are shown in Figure 23, lower panel and inset. The data for the NT construct could be accurately fit using a simple equilibrium binding equation through nonlinear regression analysis (Levenberg, 1944; Marquardt, 1963). From these data, it was deduced that NT binds ankyrin with a \(K_\text{d}\) of 23±4nM. Unfortunately, data for the CT could not be analyzed in a similar fashion, since there was no evidence of saturation of binding. However, since the dissociation constant, \(K_\text{d}\), is given as the concentration of ligand at which 50% of maximal binding occurs, it is clear that the \(K_\text{d}\) of CT binding to ankyrin must be at least an order of magnitude higher than that for NT.
Figure 22. Binding of ankyrin to the Na\textsuperscript{+} channel constructs.

The curves connecting the filled shapes represent binding of biotinylated ankyrin to the Na\textsuperscript{+} channel constructs (total binding curves). Although each of these total binding curves represents a single set of data points, the salient features of the total binding curves were obtained in two separate experiments under essentially identical conditions. The curves connecting the unfilled shapes represent binding of biotinylated ankyrin to Na\textsuperscript{+} channel constructs in the presence of competing unbiotinylated ankyrin (non-specific binding curves). Biotinylated ankyrin was applied to the Na\textsuperscript{+} channel constructs at 0, 2, 4, 8, 16, 32, 64 and 128nM; 500nM unbiotinylated ankyrin was used.
Figure 23. Further analysis of ankyrin binding to the Na\(^+\) channel constructs. The binding of biotinylated ankyrin at a fixed concentration in the presence and absence of competitor (500nM unbiotinylated ankyrin) was analyzed in quadruplicate. The fixed concentration chosen (32nM) corresponds to the midpoint of the binding curves in Figure 22. The proportion of biotinylated ankyrin that specifically bound to the Na\(^+\) channel constructs is represented in this histogram (bars represent means ± standard deviation).
Figure 24. Specific binding curves of the NT-ankyrin interaction (large graph) and the CT-ankyrin interaction (inset graph).

The specific binding curves were derived by subtracting the non-specific binding curves from the total binding curves (Figure 22). Ankyrin binding to NT displayed evidence of saturation and its $K_d$ was calculated to be $23 \pm 4 \text{nM}$ according to non-linear least squares analysis. Binding of ankyrin to CT did not display evidence of saturation in the concentration range used in this experiment.
4 Discussion

4.1 Summary of results

In this thesis I have described the successful preparation of fusion protein constructs corresponding to the NT, LII-III, LIII-IV and CT regions of the rat brain Na⁺ channel IIa and to regions within the membrane binding domain of ANK2. These fusion protein constructs were identified immunologically by employing a His-probe antibody to identify the constructs on immunoblots. To identify Na⁺ channel-ankyrin interactions, the Na⁺ channel constructs were conjugated to benzophenone, a photoreactive crosslinking reagent and irradiated while in solution with human erythrocyte ankyrin. The immunoblots of the irradiated mixtures revealed that both NT and CT regions interact with ankyrin, a result that was confirmed by ELISA overlay assays. In addition, ELISAs demonstrated the specificity of these interactions.

4.2 Rationale for using His-tagged fusion proteins

cDNA inserts corresponding to the intracellular regions of the Na⁺ channels and to regions within the membrane binding domain of ANK2 were cloned into the bacterial expression vector, pRSET, which adds a N-terminal His-tag to the expressed recombinant protein (Kroll et al., 1993). Such a system offers several advantages over more lengthy biochemical preparations. First, bacteria often produce milligram amounts of recombinant protein (Guarente et al., 1980; Guarente et al., 1980) and compared to expression schemes utilizing mammalian cells, bacteria can be quickly and inexpensively cultured in large quantities. The second advantage offered by this system is the affinity tag engineered into each recombinant protein, thus allowing the application of a relatively standard purification method to a variety of proteins (4.4). A further advantage is that the association between His-tag and Na⁺
remains stable even under denaturing conditions involving up to 8M urea and 6M guanidinium (Hochuli, 1988; Hochuli et al., 1987) without the requirement for the proteins being in their native confirmation. In addition, the hexahistadine tag is small (only 3.5-4.9kDa) compared to other affinity tags such as glutathione S-transferase (26kDa; Pharmacia), maltose binding protein (47kDa; New England Biolabs) and transcarboxylase complex from Propionibacterium shermanii (13kDa; Promega); consequently, it is rarely necessary to remove the His-tag due to interference with protein function or antigenicity.

While the His-tagged approach is very powerful, there are certain limitations, some of which were experienced during the course of this work. The most salient disadvantage was the propensity for virtually all constructs to form inclusion bodies within the bacteria. This problem is likely less marked when expressing constructs fused to large water-soluble affinity tags, such as thioredoxin (LaVallie et al., 1993; Novy et al., 1995). A further problem was the persistent presence of a band likely corresponding to bacterial superoxide dismutase, as well as other minor contaminants, following purification of the pRSET constructs on Ni²⁺ resin alone (Kroll et al., 1993). While these contaminants could be readily removed by further purification on ion exchange resin, their presence was surprising but has been noted elsewhere. For most procedures, the constructs were considered sufficiently pure, so that in conjunction with the specificity of the His-probe antibody in recognizing His-tagged constructs, further purification was deemed unnecessary. It should be noted that there are very few examples in the literature where additional purification of pRSET constructs has been required.

4.3 Rationale for the different expression schemes

The development of a standard protocol for the preparation of pRSET constructs was extremely valuable in obtaining the majority of fusion constructs. It was interesting that the remaining constructs
each required specific conditions for their expression. Several possible explanations exist for why the Na\textsuperscript+ channel intracellular linker domains (LI-II, LII-III and LIII-IV) could not be expressed under “standard” conditions. Improper folding of these fusion proteins could make them more susceptible to intracellular protease activity, which could explain why lowering post-induction growth temperature for LII-III led to the production of detectable levels of this construct. Since this measure did not aid in the production of LI-II and LIII-IV, other factors must lie behind their lack of expression under “standard” conditions. Expression of these constructs was attempted in BL21(DE3)pLysS, a bacterial strain which contains a plasmid encoding the T7 lysozyme, an inhibitor of the T7 RNA polymerase (Moffatt & Studier, 1987; Studier, 1991). Use of this strain suppresses T7 RNA polymerase activity prior to induction, thus preventing pre-induction expression of the fusion protein. Such a measure is useful if the fusion protein is toxic to the host bacteria, as it allows those bacteria carrying the expression vector to reach the induction point without negative selection pressure acting against them. Highly hydrophobic regions in fusion proteins could also be toxic to bacteria as they can cause puncturing of the cell membrane (Lama & Carrasco, 1992; Yike et al., 1996). Toxicity may also arise if the fusion protein strongly resembles a bacterial protein - it may bind to enzymes, substrates or other molecules that factor prominently in key cellular processes and make these substances unavailable to the bacteria. Toxicity appears to be the reason behind the inability of LIII-IV to be expressed under “standard” conditions, since expression of this fusion protein in BL21(DE3)pLysS was high enough to permit purification.

Manipulation of induction point, post-induction temperature and host bacterial strain failed to yield an expression scheme for LI-II. Since this region contains several consensus sites for protein phosphorylation (Murphy et al., 1996; Smith & Goldin, 1996), it is conceivable that phosphorylation of these sites in the fusion protein construct interferes with crucial phosphorylation-dependent events,
consequently disrupting bacterial growth. One way to circumvent this problem may require the use of alternative expression systems such as those which exploit eukaryotic host cells (e.g. baculovirus-infected Sf9 cells or transfected S2 cells). Whether these systems would be less sensitive than bacteria to the cytotoxic effects of LI-II seems unlikely but may be worth testing. An alternative route to expressing this construct in bacteria might be to express it in fragments in the hope that each individual section would have a less deleterious effect.

4.4 Purification of His-tagged constructs

Because of the selective binding of stretches of multiple consecutive histidine residues to divalent cations, the recombinant His-tagged proteins can be retained on a matrix conjugated to Ni²⁺ (Hochuli et al., 1987). In theory, most intracellular bacterial proteins do not bind to such a matrix and those that do, do so because they contain short consecutive histidine stretches. By virtue of their weaker interaction with the Ni²⁺ resin than hexahistidine-containing constituents, such non-specifically bound proteins can be removed by washes of progressively lower pH. Decreasing the buffer pH causes protonation of the imidazole ring in the histidine residues, making interaction with Ni²⁺ incompatible; by decreasing the buffer pH to 4.0, elution of the His-tagged constructs can be achieved.

The purification protocol was designed such that both the soluble and insoluble intracellular proteins were applied to the Ni²⁺ resin under denaturing conditions. The soluble and insoluble fractions were not separated so as to prevent loss of fusion protein, since certain constructs partitioned into both fractions. The fusion proteins were purified under denaturing conditions for several reasons. The ankyrin constructs were present in the bacteria as inclusion bodies, necessitating solubilization with a strong denaturant such as 8M urea prior to purification. After inclusion body solubilization, keeping 8M
urea in all the buffers used during the purification afforded two advantages, which also applied to the purification of soluble fusion proteins. Maintaining the fusion proteins in a denatured, linear conformation ensured the accessibility of the His-tag, which could be masked in the native conformation, for binding to the Ni$^{2+}$ resin. Furthermore, the presence of 8M urea limits the activity of most proteolytic enzymes, obviating the need for special measures to limit proteolytic activity, such as protease inhibitors and carrying out the purification at 4°C.

The purified constructs were electrophoretically separated by SDS-PAGE, transferred to nitrocellulose and probed with His-probe antibody to verify their identities. Although the apparent molecular weights of NT and LIII-IV agreed with the expected molecular weights, the other constructs exhibited apparent molecular weights ranging from 10-30kDa above the expected molecular weights (Table 7, p. 50). This phenomenon has been reported elsewhere, and is attributed to the constructs’ abnormally low or high isoelectric points (Table 3, p. 24), which has been known to cause anomalous protein migration through polyacrylamide gels (Coombs et al., 1978; Cremel et al., 1985; Goward et al., 1990; Huner & Macdowall, 1979; Kumagai et al., 1981). An important consideration is the criteria used to ascertain purity. Because the fragments have no endogenous activity by which to monitor their purification, purity was determined by the fraction of total protein that is His-tagged construct. Unfortunately, this can lead to several errors. For example, one source of error arises through differences in the efficiency of transfer of proteins during blotting, leading to an inability to detect those proteins that are not captured on the nitrocellulose membrane. Another source of error may be the inability of protein to migrate in the gel through aggregation, which was deemed unlikely and found to be minimal due to the inclusion of SDS and reducing agents in the loading buffer. The only true measure of purity in reality is the presence of a single amino-terminus for each of the constructs, which can be
determined through amino acid sequencing. However, even this method has limitations, the most prominent being the propensity of amino-termini to undergo blocking reactions during purification which prevent sequencing (Findlay & Geisow, 1989). An alternative strategy that will be undertaken in future studies would be to determine the amino acid composition of the purified protein, which should also provide confirmation of the identity of the expressed construct.

Although the ankyrin constructs were not utilized in the experiments described in this thesis, they will be important in future experiments characterizing the Na⁺ channel-ankyrin interaction.

4.5 Purification of ankyrin-depleted rat brain Na⁺ channel and human erythrocyte ankyrin

Because the rat brain Na⁺ channel undergoes post-translational glycosylation (Hartshorne & Catterall, 1981), it can be crudely purified by isolating the glycoprotein fraction in synaptic membranes using WGA, a carbohydrate-binding protein which avidly binds N-acetylglucosamine or sialic acid residues in glycoproteins (Roth, 1978). Since the interaction of glycoproteins such as the Na⁺ channels with WGA is stable at high ionic strengths (Hartshorne & Catterall, 1981), we exploited this phenomenon to strip interacting ankyrin from the WGA-bound Na⁺ channel simply using a high ionic strength wash (Srinivasan et al., 1988). The advantage of this procedure is that it effects complete removal of ankyrin and afforded ready isolation of Na⁺ channel enriched glycoprotein through simple elution with free N-acetylglucosamine (Hartshorne & Catterall, 1981; Srinivasan et al., 1988). Rat brain Na⁺ channel was not purified to homogeneity because of the lengthy, time-consuming and costly measures required, in addition to the deterioration of the channel in detergent-containing solutions (Hartshorne & Catterall, 1981). Furthermore, the antibody used to detect rat brain Na⁺ channel afforded
specific detection of this protein in the glycoprotein fraction.

Ankyrin was purified from human erythrocytes to near-homogeneity according to the method of Bennett (1983), with certain modifications. Purification of ankyrin from erythrocytes rather than brain tissue offers several advantages, the first and foremost being that ankyrin represents a higher fraction of total protein in human erythrocyte ghosts (4.7%) (Bennett & Stenbuck, 1980) than in pig brain membranes (1-2%) (Davis & Bennett, 1984). Large amounts of brain tissue (400g) (Davis & Bennett, 1984) are therefore required to obtain milligram amounts of ankyrin. Furthermore, brain tissue in sufficient quantities for ankyrin purification is more difficult to obtain than blood, and requires special processing steps such as freezing and storage in liquid nitrogen within a short period after death (Davis & Bennett, 1984). Another major disadvantage of purifying ankyrin from brain tissue is the greater difficulty, cost and time-consumption required when compared to its purification from erythrocytes (Davis & Bennett, 1984). Also, and perhaps most importantly, brain tissue contains a multitude of proteases, thus very rigorous precautions must be used to guard against proteolytic degradation (Bennett et al., 1986). Finally, the region of interest in ankyrin, namely the membrane binding domain, is so highly conserved among the isoforms (Kordeli et al., 1995; Otto et al., 1991) that, as shown in past studies, the membrane binding domain of ANK1 can be substituted for that of ANK2 (Srinivasan et al., 1988; Srinivasan et al., 1992).

4.6 Identification of NT and CT as the ankyrin binding site

Our fundamental goal has been to identify the precise site(s) within the voltage-gated Na+ channel that might interact with the cytoskeletal linker protein, ankyrin. Our first attempts to identify such interactions were done through cross-linking experiments using a photo-reactive label,
benzophenone. The advantage of this approach was the ease with which benzophenone-labelled fragments could be generated and the low amounts of material required to detect any interactions. A further advantage of cross-linking is its ability to detect transient, low-affinity interactions which might go unnoticed using conventional methods such as binding assays. Benzophenone affords several advantages over other photolabels. The most significant advantage is its high efficiency of incorporation, i.e. reactivity, with target molecules. This efficiency, which can reach as high as 100% (Campbell & Gioannini, 1979), is due to the ability of benzophenones to generate triplet free radicals on absorption of incident ultraviolet light (Walling & Gibian, 1965). The resulting free radical is highly reactive, but unlike conventional photolabels such as azides, does not react well with water (Dorman & Prestwich, 1994; Galardy et al., 1973; Turro, 1978). In addition, decomposition of the excited state of benzophenone leads to regeneration of the unexcited benzophenone, rather than any photodecomposition (Dorman & Prestwich, 1994). The result is that, on maintained UV radiation, benzophenones are able to cycle between ground and excited states until they react with their target molecules. Conventional photolabels invariably decompose after absorption of UV radiation, leading to a smaller time frame within which they can react with the target molecule. They therefore have a much lower efficiency of incorporation than benzophenones. The only real disadvantage of using benzophenones is their low polarity. However, at low label:protein ratios, such as those used here, and with their attachment to much larger protein molecules, any adverse effects of benzophenone labelling such as protein precipitation are negated.

The Na⁺ channel constructs were labelled with the photoreagent, benzophenone NHS-ester, and UV-irradiated in the presence of ankyrin. Immunoblots of these mixtures revealed that both NT and CT formed adducts with ankyrin. This result is consistent with the formation of an ankyrin-binding site by both of these regions as proposed in the original hypothesis (1.5). Interestingly, some high molecular
weight adducts were observed to form in the absence of ankyrin for NT, CT and LII-III. The origin of these adducts is unclear, but most likely reflect low-level nonspecific binding of the constructs to minor contaminants in the reactions, or possibly to each other. The salient feature of the cross-linking experiments is that adduct formation is predominantly through ankyrin-NT and ankyrin-CT interactions.

Although there was no marked laddering effect (where adducts of consecutively higher molecular weights than expected for a 1:1 interaction between proteins arise), some smearing was observed, suggesting that multiple NT or CT fragments may bind to a single ankyrin molecule. The possibility that multiple membrane proteins can interact with a single ankyrin membrane binding domain has recently been suggested (Michaely & Bennett, 1995).

Further evidence for an interaction between cytoplasmic domains of Na\(^+\) channel and ankyrin was provided by affinity precipitation experiments using a procedure identical to that described in Michaely & Bennett (1995). This method involved immobilizing biotinylated ankyrin on avidin-bearing latex beads and using the resulting ankyrin-coated beads as an affinity matrix to test the possible interactions with discrete Na\(^+\) channel fragments. The use of the latex beads, rather than conventional matrices such as Protein A-agarose or sepharose, provided several advantages. The lower surface porosity of the latex beads compared to agarose or sepharose eliminated trapping of low molecular weight fragments within the affinity matrix (Michaely & Bennett, 1995). Such trapped fragments would contribute to unnecessarily high levels of background binding. A further advantage of the avidin-coated beads was their versatility since they could be easily coated with any biotinylated protein.

Surprisingly, the affinity precipitation methodology suggested that the predominant interaction between Na\(^+\) channel and ankyrin was mediated exclusively by the NT. The absence of detectable binding by the CT could have several origins. The most trivial explanation, that CT was present in
amounts too low to be detectable, was inconsistent with the amounts used in the immunoblotting. A much more likely explanation is that the interaction between ankyrin and CT is of lower affinity than that between ankyrin and NT. In the absence of a high affinity interaction, the CT would readily dissociate from the ankyrin under the repeated washings required in the affinity precipitation protocol. Based on earlier reports, the interaction of ankyrin and Na\(^+\) channel is thought to occur with a \(K_d\) of approximately 20mM (Srinivasan et al., 1992). Such a low dissociation constant would certainly be consistent with the results obtained for the NT. Whether the CT plays an important role in the association of Na\(^+\) channels with ankyrin is unclear, and is likely to be best confirmed using strategies capable of measuring weak or transient protein-protein interactions such as the photolabelling method described above and the yeast two-hybrid system (Bartel et al., 1993).

An important issue is whether the interaction of NT and CT with ankyrin shows any degree of specificity. Based on the failure of several fragments (LI-III, LIII-IV) to interact with ankyrin, it seems likely that some measure of specificity exists. Conventionally, specific binding is demonstrated as that remaining after displacement with a competing ligand (Boeynaems & Dumont, 1980). To test for such specificity, ankyrin was coated onto ELISA plates and the interaction of Na\(^+\) channel fragments determined in the presence and absence of competing free ankyrin. The rationale for these experiments was that the free unlabelled ankyrin would complex with the adsorbed Na\(^+\) channel fragments and thereby reducing the binding of biotinylated ankyrin to these constructs. Of the Na\(^+\) channel fragments tested in this assay, only the NT and CT displayed convincing evidence of ankyrin displaceable binding and thus specific interaction with ankyrin. These data not only argue that the CT region is indeed capable of binding to ankyrin, along with the NT, but support the contention that the CT-ankyrin interaction is of low affinity. Presumably, our ability to detect binding of CT in the ELISA compared to
the affinity precipitations is due to the greater sensitivity of this method compared to immunoblotting and the less extensive washing steps involved. An important aspect of these ELISAs is the possible presence of non-specific protein-protein interactions. This is most readily determined by coating ELISA wells with proteins unrelated to the target protein of interest. In effect this was achieved by assaying the ELISA signal from coated with blocking solution (1% CWFG). The resulting signals were extremely low, typically <5% of those seen in the presence of the true target proteins (Na⁺ channel constructs). However, it should be noted that when BSA was used rather than CWFG, non-specific binding was marked. The reason for this appears to be the presence of contaminating ankyrin in the BSA preparations, an observation that was confirmed through immunoblotting but has not been reported previously.

4.7 The molecular significance of the Na⁺ channel-ankyrin interaction

The results presented here are compatible with the structural data gleaned from studies involving the analogous voltage-gated Na⁺ channel from skeletal muscle, rSkM-1 (Cohen & Barchi, 1992; Haimovich et al., 1987; Sun et al., 1995). In their studies, Barchi et al. used antibodies to determine the intracellular topology of the rSkM-1. On the basis of their results, these investigators suggested that the NT region played a role in the differential localization of rSkM-1 (Cohen & Barchi, 1992). Further experiments suggested that the NT and CT regions lay in close proximity to each other, a result which was subsequently confirmed by demonstrating that fusion proteins corresponding to these regions interacted in vitro (Sun et al., 1995). Together, these data are consistent with the hypothesis that both the NT and CT somehow act together to fulfill some undetermined aspect of Na⁺ channel function. One such function could be the formation of a binding site for the recognition and attachment of cytoskeletal
proteins such as ankyrin. An alternative possibility is that ankyrin interacts primarily with the NT region of the Na+ channel, but the CT region serves as a negative regulator of this interaction. Indeed, it is interesting that a major precedent for such a modulation of binding affinity by a spatially distinct protein region is given by ankyrin itself (Davis et al., 1992; Hall & Bennett, 1987). The ability of ankyrin to bind with the cytoskeleton and membrane proteins appears to be regulated by the carboxy terminal region. Such regions in ankyrin are subject to considerable variability (Bennett, 1992). Similarly, the CT region is the most variable region between Na+ channel isoforms (Goldin, 1995); thus, certain parallels may exist between CT regions of ankyrin and Na+ channel.

While our data provides evidence for an interaction between NT/CT and ankyrin, we cannot exclude the possibility that some other region of the Na+ channel interacts with ankyrin. Due to the non-existent level of LI-II expression, it was not possible to test the LI-II region in the interaction assays and therefore the prospect that this region participates in forming the ankyrin-binding site cannot be ruled out. Our results with the NT and CT, however, demonstrate that although the LI-II region may contribute, it is unlikely to form the ankyrin-binding site by itself.

An interesting possibility that was considered was that Na+ channel subunits other than the α subunit contained the ankyrin-binding site. To date, the only subunits shown to interact with the α subunit are β1 and β2, in brain (Hartshorne & Catterall, 1984) and skeletal muscle (Casadei et al., 1986). Thus, it is always possible that the α subunit is attached indirectly to ankyrin through the association of one or both of the auxiliary subunits with ankyrin. The most compelling argument for a linking role for the β subunits is provided by their homology to cell adhesion molecules (Isom et al., 1995), some of which are known to bind to ankyrin (Davis & Bennett, 1994). However, inspection of the transmembrane topology of the cell adhesion molecules reveals that the regions of greatest homology to
the β subunits are entirely extracellular (Isom et al., 1994). Moreover, the recently identified ankyrin binding motif in cell adhesion molecules is not duplicated in β subunits (or α subunits) (Davis & Bennett, 1994). A further argument against the role of β subunits in ankyrin binding is provided by the inconsistency of their co-expression with Na⁺ channels. Indeed, the Na⁺ channel in rat myocytes, which is not accompanied by β subunits, has been demonstrated to interact with the cytoskeleton, although it is unclear if this is via ankyrin (Undrovinas et al., 1995).

A final consideration concerns the stoichiometry of the Na⁺ channel-ankyrin interaction. In their original papers, Srinivasin et al. (1988, 1992) concluded that the interaction of Na⁺ channels and ankyrin occurred with a 1:1 stoichiometry, although they recognized the limitations of the simple binding models used for their assays. However, recent studies by Michaely & Bennett (1995) have found evidence that ankryins may simultaneously associate with more than one target protein. This raises the interesting possibility that Na⁺ channels may co-cluster with each other and also with other ankyrin-binding proteins. An excellent candidate for such co-clustering would be the neural cell adhesion molecules (N-CAMs), known to be localized at the nodes of Ranvier (Davis et al., 1996). The stoichiometry of the Na⁺ channel-ankyrin interaction was not analyzed in detail in this work. Indeed, such analysis is not likely to be trivial, since the most direct binding studies would require the generation of radiolabelled NT (or its corresponding ankyrin partner), the determination of rate constants for the interaction and the identification and mathematical description of any cooperativity (Boeynaems & Dumont, 1980). These issues are fundamental and will be addressed in future studies.
4.8 The role of the Na\(^+\) channel–ankyrin interaction in the neuron

Based on their results in *Aplysia* neurons, Johnston *et al.* (1993) hypothesized that clustering of voltage-gated Na\(^+\) channels may optimize action potential conduction in one of two (or both) ways. Clusters may speed the transmission of the action potential. In demyelinated preparations, distinct clusters of Na\(^+\) channels continue to conduct action potentials (England *et al.*, 1990; Smith *et al.*, 1982). This implies that saltatory conduction can be supported by clustered Na\(^+\) channels, irrespective of the presence of the myelin sheath. Another way in which clusters may promote efficient action potential conduction is through a reduction in the number of Na\(^+\) channels required. Recent computer modeling based on the *Aplysia* neuron indicate that 40% fewer Na\(^+\) channels must be present in a clustered state versus an even distribution along the axon to support conduction of action potentials (Johnston *et al.*, 1996).

Regardless of the mechanism by which Na\(^+\) channel clusters optimize action potential conduction, it is clear that maintenance of these clusters is necessary for proper neuronal function and suggests alternative mechanisms for diseases such as multiple sclerosis and muscular dystrophy, whose clinical manifestations have been attributed mainly to demyelination (Deenck *et al.*, 1997; Moll *et al.*, 1991). Of course, the possibility exists that anchoring of Na\(^+\) channels to the cytoskeleton is only one step in a complex signaling pathway whose cues are provided by myelinating cells. Such pathways would be reminiscent of the mechanisms underlying synaptogenesis (Colman *et al.*, 1997) and clustering of acetylcholine receptors at the neuromuscular junction (Hoch *et al.*, 1994). An interesting possibility is that cell-cell contact between myelinating cells (oligodendrocytes or Schwann cells) and neurons serves concentrate to N-CAMs at the nodes of Ranvier (Froehner, 1993). The resulting aggregation of these N-CAMs might serve as a cue leading to the concentration of ankyrin and any associated Na\(^+\) channels in
those same regions (Davis et al., 1996; Dubreuil et al., 1996; Kaplan et al., 1997). A further implication of the Na\(^+\) channel-ankyrin interaction is its dependence on the fidelity of the cytoskeleton. For example, if the cytoskeleton in neurons degenerates with age, as it does in erythrocytes (Galletti et al., 1983; Suzuki & Dale, 1989), it is not inconceivable that some of the effects attributed to aging may actually be in fact due to the breakdown of the mechanism which maintains Na\(^+\) or indeed other ion channels in their clustered conformations (Bennett & Lambert, 1991). Understanding the molecular basis of the Na\(^+\) channel-ankyrin interaction provides a means of investigating the role of this interaction in both normal and pathological states.

A fundamental issue is what molecular determinants specify the Na\(^+\) channel-ankyrin interaction. Numerous proteins are known to interact with ankyrin; however, many of these proteins are not co-localized within the same subcellular region. It therefore seems unlikely that interactions with the Na\(^+\) channel are generated at random or unregulated. Our results are thus only the beginning in a more lengthy series of experiments to define precise binding sites on both the Na\(^+\) channel and ankyrin. Such mapping will undoubtedly rely heavily on molecular approaches such as alanine scanning and deletion mutagenesis. The search for ankyrin-binding motifs is complicated by two factors: first, the lack of any real ankyrin-binding consensus sequences (Michaely & Bennett, 1995), and second, the possibility that multiple Na\(^+\) channel regions come together to form the ankyrin-binding site (Ding et al., 1996). The observation that the NT represents a major site of high affinity binding should certainly simplify the future search for such motifs. The interaction of ankyrin with NT is likely to be significant in determining ankyrin binding specificity since the NT is known to differ between various Na\(^+\) channels (Goldin, 1995). Indeed, the relative affinities of ankyrin for the various Na\(^+\) channel isoforms may prove to be a powerful tool in mapping the ankyrin binding sites. Based on the data presented here and
elsewhere (Srinivasan et al., 1992) it seems likely that NT interacts with motifs in the ankyrin repeats 10-22 of the membrane binding domain. How such an interaction occurs is unclear. Very recently, crystallographic studies have revealed the three-dimensional structure of a complex formed by the interaction of the p53 tumor suppressor with an ankyrin repeat in 53BP2 (Gorina & Pavletich, 1996). These data confirm the earlier contention that each ankyrin repeat is comprised of two α-helical domains and show that interaction with their target protein is dictated by a few residues at appropriate, non-contiguous locations (Michaely & Bennett, 1993). Were this situation to apply to the Na⁺ channel-ankyrin interaction, it would be necessary to identify single or clustered residues somewhere within the R₁₀₋₂ region that mediate multi-point attachment to NT or other Na⁺ channel regions. The identification of non-contiguous residues is not a trivial task, and not amenable to a purely mutagenic approach. One possibility would be to map sites of protein-protein interaction using newly described protein footprinting methodologies (Matsudaira, 1994; Newham et al., 1997; Tytgat et al., 1995). An even more powerful approach would be to co-crystallize the NT and R₁₀₋₂ regions and determine sites of interaction crystallographically.

While the NT regions may dictate which Na⁺ channel isoforms can interact with ankyrin, they may also be responsible for determining which ankyrin isoform interacts with the Na⁺ channel. This issue is critical, since it is hard to see how the very similar membrane binding domains displayed by the various ankyrin isoforms found in brain (Kordeli et al., 1995) can determine the specificity of the interaction. Our experiments utilized the ANK2 isoform, which displays developmentally regulated splicing and localization: the 440kDa isoform appears in unmyelinated axons during development (Otto et al., 1991) and the 220kDa isoform in cell bodies post-myelination (Otto et al., 1991) (1.3.2). Both isoforms have identical membrane binding domains (Otto et al., 1991), which have been shown in this
thesis to interact with the voltage-gated Na\(^+\) channel. It therefore seems likely that these isoforms play specific roles in anchoring Na\(^+\) channels during the course of development. The 440kDa isoform may maintain Na\(^+\) channel clusters in the axon before myelination occurs, after which time it is replaced by ANK3 (1.3.3). It is interesting to note that the apparent targeting domain in ANK3 is significantly smaller than in 440kDa ANK2 (46kDa versus 220kDa) (Chan et al., 1993; Kordeli et al., 1995; Otto et al., 1991), suggesting that the neuron may switch to production of ANK3 post-myelination because it is metabolically less expensive. Another possible reason may be due to predominant expression of rat brain Na\(^+\) channel II, not IIa, during development (Sarao et al., 1991), to which 440kDa ANK2 may have a greater affinity than ANK3. It follows, then, that ANK3 might bind better to rat brain Na\(^+\) channel IIa compared to ANK2; however, this remains extremely speculative as differential Na\(^+\) channel-ankyrin affinities have not been investigated.

An interesting issue is the extent to which the interaction of Na\(^+\) channels with specific ankyrin isoforms determines their subcellular distribution. Although Na\(^+\) channels are found primarily on axons in mature neurons, certain cell types such as cerebellar Purkinje cells (Sarao et al., 1991), neocortical pyramidal cells (Stuart & Sakmann, 1994), sensory neurons (Turner et al., 1994), substantia nigra neurons (Hausser et al., 1995) and CA1 pyramidal cells (Spruston et al., 1995) also exhibit dendritic Na\(^+\) channels. Indeed, the presence of such dendritic Na\(^+\) channels appears to be critical for the recently discovered phenomenon of back propagation where action potentials generated in the soma are relayed back into the dendrites whose responsiveness to incoming signals is subsequently modified (Jung et al., 1997; Regehr et al., 1992). The presence of the 220kDa isoform of ANK2 in the cell bodies of mature neurons would certainly be consistent with a role in anchoring these dendritic Na\(^+\) channels. How ankyrin isoforms might differentially target Na\(^+\) channels is unknown, but could occur either by selective
concentration of Na\(^+\) channels at sites where the appropriate ankyrin isoform is itself targeted or by association with Na\(^+\) channels prior to trafficking. In this latter role, the ankyrin would serve as the targeting signal for the complex. Powerful support for this model comes from studies on the anion-exchanger-ankyrin interaction, where ankyrin associates with its anion exchanger early in its biosynthetic pathway (Gomez & Morgans, 1993).

### 4.9 Implications of the Na\(^+\) channel-ankyrin interaction for voltage-gated ion channel biology

It is thought that the voltage-gated ion channels constitute a superfamily descended from a single gene which has undergone duplication in order to fulfill different functional roles (Jan & Jan, 1992). This is most evident when examining the structures of the voltage-gated ion channels. The functional unit of Ca\(^{2+}\) and Na\(^+\) channels consists of a single polypeptide containing four transmembrane domains, with each domain composed of six probable \(\alpha\)-helices designated S1 to S6 (Noda et al., 1984; Tanabe et al., 1987). The functional K\(^-\) channel consists of four separate gene products, each of which resembles one of the transmembrane domains in the Ca\(^{2+}\) and Na\(^+\) channel (MacKinnon, 1991; Noda et al., 1984). Certain structure-function relationships have also been conserved, namely pore formation by the S5-S6 linker and voltage sensing by S4 segments (Catterall, 1993; Catterall, 1995). It stands to reason, therefore, that despite their dissimilar sequences, the CT and/or NT regions could play a role not only in the maintenance of Na\(^+\) channels, but also of Ca\(^{2+}\) and K\(^-\) channels in their proper locations on the neuronal surface. Powerful support for an interaction between Ca\(^{2+}\) channels and the cytoskeleton has been obtained through FPR studies which show that these channels are immobilized on dendrites in a manner similar to Na\(^+\) channels (Jones et al., 1989). Nevertheless, data obtained in our laboratory have failed to
find evidence of an interaction between Ca\(^{2+}\) channels and ankyrin, although it does not exclude the possibility of interactions with other cytoskeletal elements. While the interaction of cytoskeletal elements such as ankyrin with NT and CT of Na\(^{+}\) and Ca\(^{2+}\) channels is structurally compatible, the situation is more complicated for the more simple K\(^{-}\) channels. Here, the focus of a possible cytoskeletal interaction has been on the CT rather than the NT, since the NT region mediates fast inactivation in the K\(^{-}\) channel (at least for the Shaker subfamily) (Hoshi et al., 1990; Isacoff et al., 1991; Zagotta et al., 1990). Indeed, these and other studies indicate that the NT region of K\(^{-}\) channels operates in a manner analogous to the LIII-IV inactivation region in the Na\(^{+}\) channel. Recently, Sheng and colleagues, using the CT of the Shaker K\(^{-}\) channel as bait in a yeast two-hybrid screen, have identified proteins responsible for clustering these channels (Kim et al., 1995). Owing to the presence of multiple protein-protein interaction domains (PDZ, SH3) within such clustering proteins, it is certainly possible these proteins have a dual role: first, to induce clustering of channels and second, to cause attachment to as yet undetermined cytoskeletal proteins. Further support for a non-electrophysiological role for the CT has been obtained very recently by the laboratories of Trimmer (Scannevin et al., 1996) and Rudy (Ponce et al., 1996). These workers have identified a cytoplasmic domain in Kv2.1 or Kv3.1 K\(^{-}\) channels that is responsible for generating both a polarized and clustered distribution of these channels when expressed in MDCK cells. Mutational studies by Trimmer’s group suggest at least part of the subcellular segregation of Kv2.1 is determined by interactions with the cytoskeleton (Scannevin et al., 1996).

Future studies will undoubtedly reveal the importance of cytoskeletal interactions in ion channel biology. It is my contention that a definition of the interaction between Na\(^{+}\) channel and ankyrin will serve as a powerful model system with which to define how such interactions are generated, maintained and regulated.
5 Future Studies

The \textit{in vitro} identification of the amino- and carboxy-terminal regions as the ankyrin-binding site on the Na\(^+\) channel represents an important step in defining the Na\(^+\) channel-ankyrin interaction. The next step is to confirm the \textit{in vitro} studies by reconstituting the interaction of the NT and CT regions with ankyrin \textit{in vivo}. One avenue would be to use the yeast two-hybrid system, a particularly attractive option for the CT region, as it appears to interact with ankyrin with low affinity (3.6). Another method of accomplishing this goal would involve co-transfecting either NT or CT (or both) with ankyrin into a mammalian cell line and demonstrating an ability to immunoprecipitate these constructs as complexes. The latter strategy has been utilized with great success by Kopito and colleagues in identifying the ankyrin-binding site on the anion exchanger (Ding \textit{et al.}, 1994; Ding \textit{et al.}, 1996).

Future studies into the molecular basis of the Na\(^+\) channel-ankyrin interaction benefit greatly from the identification of the ankyrin-binding site in Na\(^+\) channel described in this thesis. Bacteria and yeast are more likely to generate large amounts of small protein fragments compared to large membrane-spanning proteins for the purposes of producing constructs for \textit{in vitro} assays and two-hybrid assays respectively. One issue that can be investigated in this manner is the role of isoform specificity in Na\(^+\) channel-ankyrin interactions and how this specificity, if it exists, might be created at the molecular level. A further important application arising from the ability to generate large amounts of the components involved in the Na\(^+\) channel-ankyrin interaction is the possibility of determining the three-dimensional structure of the interacting partners through co-crystallization (Gorina & Pavlentich, 1996). Identification of the ankyrin-binding site in Na\(^+\) channels also allows investigation of the functional effects of the Na\(^+\) channel-ankyrin interaction. By microinjecting large amounts the NT or CT regions (or both) into neurons, this interaction could be disrupted and any electrophysiological effects measured.
The Na⁺ channel-ankyrin interaction may have purposes beyond that of maintaining these ion channels in specific locations. It has been proposed that the association of Na⁺ channels with ankyrin might target them to their proper destinations (Bennett, 1992; Srinivasan et al., 1988; Srinivasan et al., 1992). One way of investigating this would be to transf ect Na⁺ channels into Madin-Darby canine kidney (MDCK) cells. Transfection of neuronal membrane proteins into MDCK cells suggests that neurons and epithelia may share similarities in their trafficking mechanisms. For example, axonal membrane proteins usually appear on the apical surface of MDCK cells, while somatodendritic membrane proteins are expressed on the basolateral surface, and vice versa (Ahn et al., 1996; Dotti & Simons, 1990; Pietrini et al., 1994). In accordance with this analogy, neuronal Na⁺ channels would be expected to be localized at the apical surface of MDCK cells. Interestingly, however, ankyrin is concentrated only at the basolateral surface (Drenckhahn & Bennett, 1987; Morrow et al., 1989). Thus, a basolateral distribution for the neuronal Na⁺ channel would be expected if ankyrin association targets this ion channel, whereas an apical localization would be anticipated if targeting of Na⁺ channels is determined by their interaction with some other protein.
6 References


Kunimoto, M (1995). A neuron-specific isoform of brain ankyrin, 440-kD ankyrinB, is targeted to the


Lux, SE, John, KM & Bennett, V (1990). Analysis of cDNA for human erythrocyte ankyrin indicates a repeated structure with homology to tissue-differentiation and cell-cycle control proteins. Nature 344,
36-42.


Zagotta, WN, Hoshi, T & Aldrich, RW (1990). Restoration of inactivation in mutants of Shaker potassium channels by a peptide derived from ShB. Science 250, 568-571.


IMAGE EVALUATION
TEST TARGET (QA-3)

1.0  1.1  1.25  1.4  1.6

1.0  1.1  1.25  1.4  1.6

1.0  1.1  1.25  1.4  1.6

1.0  1.1  1.25  1.4  1.6

1.0  1.1  1.25  1.4  1.6

APPLIED IMAGE, Inc.
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/658-5989

© 1993, Applied Image, Inc., All Rights Reserved