Investigation of Rab34 and Munc13 In The Secretory Pathway: Potential Roles In Diabetic Nephropathy

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Institute of Medical Science
University of Toronto

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Potential Roles In Diabetic Nephropathy

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Doctor of Philosophy  
Institute of Medical Science  
University of Toronto  
2009

Abstract

Constitutive secretion is responsible for the targeting of transmembrane proteins to the plasma membrane, and for the secretion of extracellular matrix proteins, hormones, and other cellular products. The basic steps of secretion are well understood – proteins synthesized in the endoplasmic reticulum are transported in lipid-bound intermediates to the Golgi, and from the Golgi to the plasma membrane or cell exterior. Dysfunction of the secretory pathway – either constitutive or regulated – is involved in many disease states.

One such state is diabetic nephropathy (DN). DN is characterized by proteinuria, matrix expansion, fibrosis and progression to kidney failure, and is the leading cause of renal failure worldwide. Our lab had previously shown that munc13 is both upregulated and activated in the diabetic kidney, and that munc13 is an effector of rab34. Study of rab34 in HeLa cells revealed that rab34 is localized to the Golgi, and that it is required for the secretion of the Vesicular Stomatitis Virus glycoprotein. Colocalization experiments, as well as the use of Brefeldin A, localized the effect of rab34 to intra-Golgi transport. Further experiments indicated that glucose-induced upregulation of munc13 in rat mesangial cells increased the rate of constitutive secretion to the plasma membrane, and that this effect depended on its interaction with rab34. Finally,
munc13 and rab34 were found to be required for the high glucose-mediated stimulation of Transforming Growth Factor-β secretion from mesangial cells, placing these two proteins at a key point in a pathway of physiological significance in the pathology of DN.
Acknowledgments

I would like to thank Dr. Mel Silverman for being my supervisor and mentor for the past five years. Dr. Silverman fostered my love of science and medicine, provided me with the freedom to undertake a project of personal interest, and let me learn from my mistakes as I made them. Most importantly, I have benefited from his open door and open mind throughout my time in the lab. Dr. Silverman always had time for me, and was always there to provide perspective when things looked the worst.

I would also like to thank my thesis committee, Dr. Reinhart Reithmeier and Dr. Sergio Grinstein. There was never any doubt that my committee was on my side, and I have benefited tremendously from the vast knowledge that Dr. Reithmeier and Dr. Grinstein have brought to my project. A student couldn’t ask for better advisors, and they will always have my respect and admiration.

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Thank you to Sandy McGugan and the MD/PhD Program. You’re the only other people who understand exactly what we all go through during this long program, and your support, comic relief, and help in all facets of research life went above and beyond the call of duty.

Finally, none of this work would have been possible without the love and support of my family. Mom, Dad, Jeff, Marnie, and Jackson – you’ve never questioned this odd path that I’ve
found myself walking, and you’ve been there for all the ups and downs along the way. You’ve been my cheering section all these years. Vanessa – you’ve had to put up with all the mood swings of a fledgling scientist, and you’ve done an incredible job. You always set me straight, and I’m sure you always will.
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<tbody>
<tr>
<td>ACE</td>
<td>Angiotensin-converting enzyme</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced glycation end-product</td>
</tr>
<tr>
<td>AngII</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BFA</td>
<td>Brefeldin A</td>
</tr>
<tr>
<td>CA-GFP-Rab34</td>
<td>Constitutively active GFP-Rab34</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DDRT-PCR</td>
<td>differential-display reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>DN-GFP-Rab34</td>
<td>Dominant-negative GFP-Rab34</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EndoH</td>
<td>Endoglycosidase H</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERGIC</td>
<td>ER-Golgi intermediate compartment</td>
</tr>
<tr>
<td>EST</td>
<td>Expressed sequence tag</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FHL3</td>
<td>Familial hemophagocytic lymphohistiocytosis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>FISH:</td>
<td>Fluorescence <em>in situ</em> hybridization</td>
</tr>
<tr>
<td>GAP:</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>GDI:</td>
<td>GDP dissociation inhibitor</td>
</tr>
<tr>
<td>GEF:</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GFP:</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GGTase:</td>
<td>Geranylgeranyl transferase</td>
</tr>
<tr>
<td>GPL:</td>
<td>Glycerophospholipid</td>
</tr>
<tr>
<td>GS2:</td>
<td>Griscelli Syndrome Type 2</td>
</tr>
<tr>
<td>GTP:</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HA:</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>LAMP-1:</td>
<td>Lysosome-associated membrane protein 1</td>
</tr>
<tr>
<td>LTP:</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>M6PR:</td>
<td>Mannose 6-phosphate receptor</td>
</tr>
<tr>
<td>MC:</td>
<td>Mesangial cell</td>
</tr>
<tr>
<td>MHC:</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MHD:</td>
<td>Munc13 homology domain</td>
</tr>
<tr>
<td>MTOC:</td>
<td>Microtubule organizing centre</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NCDZ:</td>
<td>Nocodazole</td>
</tr>
<tr>
<td>NMJ:</td>
<td>Neuromuscular junction</td>
</tr>
<tr>
<td>NSF:</td>
<td>N-ethyl-maleimide-sensitive fusion protein</td>
</tr>
<tr>
<td>PDBu:</td>
<td>Phorbol 12,13-dibutyrate</td>
</tr>
<tr>
<td>PDGF:</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PDMP:</td>
<td>D-threo-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol</td>
</tr>
<tr>
<td>PH:</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>PI4K:</td>
<td>Phosphatidylinositol 4-kinase</td>
</tr>
<tr>
<td>PI4P:</td>
<td>Phosphatidylinositol 4-phosphate</td>
</tr>
<tr>
<td>PKC:</td>
<td>Protein kinase c</td>
</tr>
<tr>
<td>PKD:</td>
<td>Protein kinase d</td>
</tr>
<tr>
<td>PLC:</td>
<td>Phospholipase c</td>
</tr>
<tr>
<td>REP:</td>
<td>Rab escort protein</td>
</tr>
<tr>
<td>RFP:</td>
<td>Red fluorescent protein</td>
</tr>
<tr>
<td>RILP:</td>
<td>Rab7-interacting lysosomal protein</td>
</tr>
<tr>
<td>RNAi:</td>
<td>RNA interference</td>
</tr>
<tr>
<td>siRNA:</td>
<td>Short interfering RNA</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>SL</td>
<td>Sphingolipid</td>
</tr>
<tr>
<td>SNARE</td>
<td>Soluble NSF attachment protein receptor</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SPT</td>
<td>Serine palmitoyl transferase</td>
</tr>
<tr>
<td>STP</td>
<td>Short-term potentiation</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TGN</td>
<td>Trans-Golgi network</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-Tetradecanoylphorbol-13-acetate</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>VSVG</td>
<td>Vesicular stomatitis virus glycoprotein</td>
</tr>
<tr>
<td>wt-GFP-Rab34</td>
<td>Wild-type GFP-Rab34</td>
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</tbody>
</table>
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Chapter 1

1 Introduction

1.1 Cloning and Structural Analysis of munc13

In *C. elegans*, *unc-13* mutants exhibited uncoordinated movement and abnormal pharyngeal pumping. While the musculature of the animals was unaffected, abnormal connections existed between major interneurons, and immunohistochemistry revealed that motor and sensory neurons were found in abnormal locations (Maruyama and Brenner, 1991; Ahmed et al., 1992). Further, *unc-13* mutants were resistant to acetylcholinesterase inhibitors even though the enzyme itself was not altered. High levels of acetylcholine accumulated in the neurons as well, in the absence of any changes in acetylcholine metabolism (Hosono et al., 1987; Siddiqui, 1990; Hosono and Kamiya, 1991). These observations lead to the hypothesis that the *unc-13* gene product may somehow be involved in presynaptic neurotransmitter release within the cholinergic system.

Molecular cloning of the *C. elegans unc-13* gene by the laboratory of Sydney Brenner lead to the characterization of the *unc-13* gene product via expression of protein fragments in *E. coli*. They found a 1734 amino acid protein containing areas of homology with the protein kinase c (PKC) family that mapped to the C1 and C2 domains of PKC, and not to the kinase domain (Maruyama and Brenner, 1991). Using purified protein from *E. coli*, they were able to demonstrate phorbol ester binding in the presence of calcium, and put forward the idea that the *unc-13* product may be involved in a signal transduction pathway in the nervous system that involved diacylglycerol (DAG), but was not part of the PKC pathway (Maruyama and Brenner, 1991). Further work by Brenner and others defined the phorbol ester binding properties of the *unc-13* product: phorbol ester binding was zinc- and phospholipid-dependent, stereospecific,
and high-affinity, with a $K_d$ of 67 nM (Ahmed et al., 1992). These findings further suggested that UNC-13 could be involved in DAG- and calcium-dependent neurotransmitter release. At that time, the DAG- and calcium-dependent steps of neurotransmitter release had not been determined, but it was known that these two second messengers had profound effects on this pathway (Brose et al., 1995).

The first studies of a mammalian homologue of UNC-13 came from Nils Brose and Thomas Sudhof, who began studying mammalian UNC-13 (named munc13) in the rat (Brose et al., 1995). They screened a rat brain cDNA library, and found three distinct unc-13-related proteins – all showing a high degree of sequence identity with the C. elegans protein -- that they named munc13-1, munc13-2, and munc13-3 (Brose et al., 1995). The three isoforms of munc13 were found to have similar C-termini, and largely divergent N-termini. Their analysis confirmed the presence of a C1 domain in all three proteins, as well as two separate C2 domains in each (Brose et al., 1995). Like some other C2 domains, those present in munc13s were found to not bind calcium or phospholipid in vitro. Brose also demonstrated that all three munc13 isoforms were expressed specifically in the rat brain, and that munc13-1 appeared to be a peripheral membrane protein associated with the synaptic plasma membrane (Brose et al., 1995).

As a final structural and genomic note, Brose’s laboratory defined two consensus sequences found in all known munc13 isoforms, and named them munc13 homology domains (MHD1 and MHD2) (Koch et al., 2000). Searching an expressed sequence tag (Mostov et al.) library for these domains, they were able to identify several proteins containing MHD sequences (Figure 1). Of note, two novel munc13 isoforms were found: one was a splice variant of munc13-2 that was found to be ubiquitously expressed in all tissues tested. The second was a novel munc13 isoform, munc13-4 (Koch et al., 2000). Munc13-4 seems to be a divergent family
member, lacking the C1 domain, but containing two C2 domains and both MHD domains. While outside the scope of this review, munc13-4 was subsequently found to be mutated in the blood disorder Familial Hemophagocytic Lymphohistiocytosis (FHL3) (Feldmann et al., 2003). FHL3, like the related disease Griscelli Syndrome type 2 (GS2), is characterized by abnormal immune function whereby cytotoxic T cells are unable to properly degranulate during the immune response (Feldmann et al., 2003). In FHL3 patients, cytotoxic granules are normally targeted and docked at the plasma membrane, but are unable to fuse with the plasma membrane to release their contents to the extracellular space (Feldmann et al., 2003). Further work has demonstrated that munc13-4 is an effector of rab27a on the granule membrane, and that the munc13-4:rab27a interaction is critical for the cytotoxic T cell response, platelet degranulation, and melanosome trafficking (Shirakawa et al., 2004; Neeft et al., 2005). Rab27a is a member of the rab family of GTPases, to be discussed later in this thesis.
Figure 1. MHD-containing proteins. All munc13 family members contain a single C1 domain (except for munc13-4, not pictured), at least one C2 domain, and a single copy of the MHD1 and MHD2 domains. ubMunc13-2 is a synonym for hmunc13, and represents a ubiquitously expressed splice variant of brain-specific bMunc13-2. ce, C. elegans, r, rat. Taken from (Betz et al., 2001).
1.2 Neurotransmitter docking and release

In order to further describe the function of munc13 in the brain, a brief description of the events governing neurotransmitter vesicle release is required (Figure 2). Neurons store neurotransmitter in pre-formed vesicles at the synaptic terminal. These vesicles dock at the specialized active zone at the plasma membrane, where they undergo further maturation, termed priming. Primed vesicles make up what is termed the readily releasable vesicle pool. When the neuron is depolarized, the concentration of intracellular calcium increases, resulting in the release of the contents of primed vesicles into the synapse via exocytosis. Subsequently, vesicle membrane and proteins are recycled by endocytosis to an early endosomal compartment, from where they can bud off to form vesicles for a new round of secretion (Sudhof, 1995). In molecular terms, vesicle fusion and release rely upon the SNARE (soluble NSF attachment protein receptor, where NSF is N-ethyl-maleimide-sensitive fusion protein) core complex, consisting of synaptobrevin (on the vesicle membrane, also called VAMP), and syntaxin and SNAP-25 (on the plasma membrane) (Sudhof, 1995). Each of these three proteins form coiled-coils in their secondary structure, allowing them to adopt an extremely stable ternary complex, which is resistant to heat, SDS denaturation, protease digestion, and cleavage with Clostridium toxin (Chen and Scheller, 2001) (Figure 2). The stability of this complex means that ATP is required to dissociate it, and this is achieved primarily by the small ATPase, NSF (Chen and Scheller, 2001). Clearly, regulation of the formation and dissociation of the SNARE core complex is of critical importance in a process as carefully coordinated as neurotransmitter release.
a

Targeting

Docking

ATP

Other factors

\( \text{Ca}^{2+} \)

Fusion

Endocytosis

Priming

Triggering

Recycling

b

Synaptotagmin III

C2A

C2B

Synaptobrevin

Syntaxin

SNAP-25

SNARE complex

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Figure 2. The synaptic vesicle cycle and the SNARE core complex. A. Vesicles are loaded with neurotransmitter and are subsequently targeted to the active zone. After docking at the plasma membrane, a series of ATP-dependent steps, termed priming, occur in order to make the vesicle fusion-competent. Following an action potential, intracellular calcium spikes, resulting in exocytosis of the vesicle into the synaptic space. Neurotransmitter membrane proteins are then recycled via endocytosis for another round of the cycle. B. The SNARE core complex allows for the functional interaction of the vesicle and target membranes. The coiled-coil structures of syntaxin, synaptobrevin, and SNAP-25 can be seen in interaction with each other as detailed in the text. Reprinted from (Chapman, 2002).
1.3 Functional analysis of munc13 in the brain

1.3.1 Munc13-1 binds syntaxin and is involved in vesicle priming

One of the first notable findings in determining the function of munc13 in the brain was that munc13-1 interacts directly with syntaxin (Betz et al., 1997). Many proteins bind syntaxin, but virtually all of these interactions occur near the C-terminal coiled-coil domain of the molecule. In contrast, munc13-1 binds the N-terminal of syntaxin, and also co-immunoprecipitates with the entire SNARE core complex (Betz et al., 1997). The only other protein that was known to interact with the N-terminal of syntaxin was munc18, which produces a similar phenotype in C. elegans as munc13 when disrupted (Hata et al., 1993). This finding suggested that munc13 and munc18 might play a similar role in preparing vesicles for release at the active zone. In fact, further work on the munc13-syntaxin interaction provided insight into a potential mechanism for munc13-mediated vesicle priming. In order to form the SNARE core complex, syntaxin must be available for pairing with VAMP and SNAP-25. Solution structures of syntaxin showed that it is normally in a “closed” conformation that cannot pair with other SNARE proteins (Dulubova et al., 1999). Richmond and others were able to show that in C. elegans, mutant syntaxin that remained in an “open” conformation could bypass the need for munc13-mediated vesicle priming (Richmond et al., 2001). From the other end of the complex, Stevens and others were able to define the minimal domain of munc13-1 required for vesicle priming. This domain contained, among other sites, the binding site for syntaxin (Stevens et al., 2005). Together, these data led to the development of a model whereby syntaxin is normally held in a closed conformation that cannot form the SNARE core complex. When munc13-1 is recruited to the active zone membrane, it binds the N-terminal of syntaxin – potentially
displacing munc18 from this same site – stabilizing the open form of syntaxin which can then
form the core complex and initiate membrane fusion between the vesicle and the plasmalemma
(reviewed in (Martin, 2002)) (Figure 3). This model, with some modification, is still the
accepted standard for the mechanism of munc13-mediated vesicle priming.

The lab of Nils Brose went on to do the first functional studies of munc13-1 in
neurotransmission (Betz et al., 1998). They were able to show that munc13-1 was most highly
expressed in areas of high presynaptic density in the rat hippocampus. In light of this finding,
they proceeded to study munc13-1 function in Xenopus laevis neuromuscular junctions (NMJ)
that were overexpressing exogenous munc13-1 (Betz et al., 1998). Overexpression of wild-type
munc13-1 was found to greatly enhance the increase in evoked currents caused by phorbol ester
treatment as compared to wild-type NMJ. A munc13-1 H567K mutant that cannot bind phorbol
ester or translocate to the plasma membrane in response to phorbol ester had no effect on phorbol
ester-induced stimulation of NMJ activity, suggesting a direct role for munc13-1 in this
phenomenon (Betz et al., 1998). This was the first demonstration that munc13-1 expression
effects neurotransmitter release. Additionally, munc13-1 overexpression increased the basal
activity of NMJs, suggesting that endogenous DAG was capable of activating munc13-1 and
increasing neurotransmitter release at the active zone (Betz et al., 1998).

Knockout mice lacking endogenous munc13-1 died shortly after birth, even though
overall neural architecture remained normal (Augustin et al., 1999). Further investigation of
brain tissue from knockout animals revealed that while synapses formed normally in the absence
of munc13-1, the synaptic vesicle cycle was arrested at the maturation step in presynaptic
neurons. Glutaminergic neurons could not release neurotransmitter in response to action
potential, increased intracellular calcium, or hypertonic sucrose (Augustin et al., 1999). Similar
results were reported in double knock-out mice lacking munc13-1 and munc13-2, with the double knock-out phenotype also including GABAergic neurons (Varoqueaux et al., 2002).

Figure 3. The role of munc13 in vesicle priming. One the left side, the resting state is shown. Syntaxin is maintained in a closed conformation via its interaction with munc18. Upon stimulation of the cell, there is an increase in the DAG content of the plasma membrane, which, in concert with RIM, recruits munc13 to the active zone. Munc13 then binds syntaxin (shown on the right half of the figure), displacing munc18 and stabilizing the open conformation of syntaxin. This allows for the formation of the SNARE core complex of syntaxin, synaptobrevin, and SNAP-25 so that vesicle fusion can occur. Reprinted from (Martin, 2002).
1.3.2 **Munc13s are targeted to the active zone by RIM**

How is munc13 targeted to the active zone? In 2001, an interaction between munc13-1 and the active zone protein, RIM was described (Betz et al., 2001). RIM is an effector of rab3, which is a vesicle-associated rab protein (Wang et al., 1997). Interestingly, loss of the munc13-1:RIM interaction phenocopies munc13-1 knockout, resulting in a marked loss of the readily releasable vesicle pool and concomitant decreases in evoked potentials, suggesting a critical role for this interaction in vesicle priming (Wang et al., 1997). This model is strengthened by data showing that hippocampal neurons lacking endogenous RIM also display the same phenotype as munc13-1 knockout cells (Calakos et al., 2004). The initial data detailing the interaction between RIM and munc13-1 could be explained in one of three ways: (i) RIM targets munc13-1 to the active zone, (Yamamoto et al.) The interaction between RIM and rab3 provides a physical link between munc13-1 and vesicles, allowing for increased vesicle priming, or (iii), RIM interaction with munc13-1 somehow increases the intrinsic rate of munc13-1 “activity” (Betz et al., 2001).

Further data from the Brose lab would shed light on this mechanism. By creating a munc13-1 point mutant that does not bind RIM and expressing it in hippocampal neurons from munc13-1/munc13-2 knockout mice, they were able to show that the point-mutant munc13-1 was not properly targeted to the active zone (Andrews-Zwilling et al., 2006). Additionally, subcellular fractionation and immunoblotting showed that in RIM knockdown mice, munc13-1 and the ubiquitously expressed munc13-2 isoform are both downregulated and mistargeted in hippocampal neurons (Andrews-Zwilling et al., 2006). Taken together, these data strongly suggest that RIM is involved in the correct targeting of munc13-1 to the active zone. Additional data has shown that, in fact, munc13-1, RIM, and Rab3 form a tripartite complex that is capable of dictating the size of the readily releasable vesicle pool, and that this complex is involved in
bridging vesicle priming and a specific type of synaptic plasticity called long-term potentiation (Dulubova et al., 2005).

1.3.3 **Munc13s mediate short-term plasticity**

Cortical neurons are capable of various adaptations to repeated stimuli that are thought to form the basis of learning and memory (Malgaroli, 1999). One such change is long-term potentiation (LTP), which is an activity-dependent enhancement of synaptic strength (Malgaroli, 1999). Another adaptation based on the use of the synapse is short-term plasticity (STP). This is a presynaptic process by which a synapse adapts its transmitter release properties in response to acute phase changes in activation patterns (Rosenmund et al., 2002). STP seems to depend on the initial response of the synapse to stimulation: highly responsive synapses tend to depress after repeated stimulation, while synapses with low initial release show increases in response in the first stages of repeated stimulation (Rosenmund et al., 2002). These properties have to do with the size of the readily releasable vesicle pool at a given synapse. The increases in presynaptic efficacy are associated with increases in cytosolic calcium concentration at the synaptic membrane (Zucker, 1999). Intriguingly, different synapses within one neuron can exhibit different properties with respect to LTP and STP.

Within excitatory, glutaminergic neurons, this was found to be the case – some synapses were dependent on munc13-1, while others depended on munc13-2. Further, munc13-1-independent synapses use munc13-2 as a priming factor (Rosenmund et al., 2002). During high frequency stimulation, these two types of synapses behave differently. Wild-type synapses showed some depression during response to repeated stimulus. Munc13-1 knockout synapses, on the other hand, exhibited a 200% increase in evoked potential over the same 10-pulse period (Rosenmund et al., 2002). Since the wild-type cells contain munc13-1 and the knockout cells do
not, the different behaviour in the knockout cells can be attributed to munc13-2, suggesting that the two isoforms affect different STP characteristics in synapses (Rosenmund et al., 2002).

Additionally, the two types of synapses behave differently following repeated stimulation, not just during the stimulation phase. Wild-type synapses recover to normal levels within seconds of the completion of a pulse sequence. Synapses from munc13-1 knockouts exhibited augmentation of nearly 5-fold in comparison (Rosenmund et al., 2002). This was specific to munc13, since rescue of the knockouts with virus-driven munc13-1 restored a wild-type phenotype. These data, when taken together, suggest a model whereby munc13-1 is depressing, and munc13-2 is augmenting, showing that the two proteins have significant, yet differential roles in synaptic plasticity (Rosenmund et al., 2002). Further experiments directed at munc13-2-dependent synapses demonstrated that augmentation is due to both an increase in the readily releasable vesicle pool, and an increase in the concentration of intracellular calcium (Rosenmund et al., 2002). Interestingly, inhibitors of PKC failed to induce any change in the phenotype of munc13-1 knockout synapses, but inhibition of phospholipase c (PLC) lead to an almost complete loss of augmentation (Rosenmund et al., 2002). These results suggest that augmentation occurs independently of PKC activation, and depends on DAG production, presumably via binding to the munc13 C1 domain. Similarly, activation of munc13 isoforms with phorbol ester resulted in differential increases in augmentation depending on the munc13 isoform involved at the synapse (Rosenmund et al., 2002).

1.3.4 Munc13 binds Calmodulin to regulate synaptic plasticity

While the role of DAG in augmentation and synaptic plasticity appears to be through binding to the munc13 C1 domain, what is the role of increased intracellular calcium? An additional layer was added to the role of munc13 in synaptic plasticity when it was found that
both munc13-1 and ubiquitous munc13-2 can bind calcium/calmodulin (Junge et al., 2004). Munc13-1/munc13-2 double knockout neurons were rescued with either wild-type munc13, or a mutant that cannot bind calmodulin to investigate the effect of calmodulin binding on synaptic plasticity. It was found that calmodulin binding is induced during synaptic activity, and that this interaction stimulates munc13 function, and controls the calcium dependence of STP (Junge et al., 2004). The regulatory role of calcium/calmodulin appears to be through the modulation of the size of the readily releasable vesicle pool (Junge et al., 2004). These studies provided critical insight into a process that is at the heart of many cortical functions, and suggests that observed phenotypes are due to the interplay between munc13 isoforms, intracellular calcium, and vesicle priming mechanisms.

1.4 Munc13 and PKC – Alternate targets of DAG signaling

DAG is produced by several pathways, including de novo synthetic routes, stimulations-dependent pathways, and systems activated during pathological processes, such as the polyol pathway during diabetes (Carrasco and Merida, 2007) (Figure 4). Phorbol esters, which are pharmacological analogs of DAG, have long been studied as inducers of synaptic transmission changes in neurons. Phorbol esters are capable of mimicking repetitive stimulation to cause increased presynaptic efficacy through binding to C1 domain-containing proteins (Rhee et al., 2002). Historically, studies using inhibitors of poor specificity had lead to the conclusion that the effects of phorbol esters on neurotransmission were achieved via PKC-dependent pathways (reviewed in (Silinsky and Searl, 2003)). Upon the discovery of munc13s, these studies were called into question, since non-specific C1 domain inhibitors would have also affected munc13 proteins. To separate the roles of PKC and munc13, the Brose laboratory engineered munc13 knockout mice that expressed a mutant munc13-1 lacking a functional C1 domain (Rhee et al.,
Experiments on neural tissue from these mice revealed that the phorbol ester receptor responsible for the acute regulation of presynaptic neurotransmitter release is munc13 (Rhee et al., 2002). These studies provided the first solid evidence that munc13s were solely responsible for presynaptic adaptations to phorbol ester signaling, taking over from PKC as the prime regulators of neurotransmitter release.
Figure 4. Cellular DAG metabolism. Shown above are schematic representations of cellular sources of DAG. *De novo* pathways arise from triglyceride metabolism and glycolysis. Stimulation-dependent pathways use PLC or PLD to metabolize membrane phospholipids into DAG, and include the largest source of DAG, namely metabolism of phosphatidic acid to DAG via phosphatidic acid phosphohydrolase enzymes. Shown at the top right is the polyol pathway, which becomes important during hyperglycemia in diabetes. Sphingomyelin synthase is found in the Golgi lumen and plasma membrane, and PLC and PLD are plasmalemmal. Other pathways take place in a variety of intracellular compartments.
1.5 Munc13 is involved in pancreatic insulin secretion

1.5.1 Munc13-1 overexpression increases insulin secretion

The role of munc13s in regulated secretion does not end at neurotransmission. Pancreatic beta cells secrete insulin in a regulated fashion, and contain populations of docked and readily releasable vesicles similar to those found in neurons (Eliasson et al., 1997). Insulin secretion occurs in a biphasic pattern – the first, fast phase is triggered by calcium entry, and the second, sustained phase is regulated by DAG and calcium (Straub and Sharp, 2002). The biphasic secretion pattern is due to functionally separate pools of insulin granules – the first phase of secretion results from exocytosis of docked, primed granules, which account for only 2-3% of the total insulin granule pool within the cell (Barg et al., 2002). Patients with type 2 diabetes lack the first phase of secretion, and display a decreased second phase (Barg et al., 2002). The apparent role of regulated secretion and vesicle priming in insulin granule exocytosis makes munc13 a candidate player in this pathway.

Studies performed on human, rat, and mouse pancreatic islets, as well as on the insulinoma cell lines HIT and INS-1, demonstrated that munc13-1 is expressed in pancreatic islets, and that maximal expression is found in insulin-secreting beta cells (Sheu et al., 2003). However, in two models of diabetes – Zucker diabetic fatty rats and non-obese Goto-Kakizaki rats – munc13-1 expression was reduced when compared to non-diabetic controls (Sheu et al., 2003). Reduced expression of the munc13-1-binding partner syntaxin-1A was also found in these animals. Interestingly, these diabetic animals exhibit abnormal insulin secretion, suggesting that reduced munc13-1 levels may be involved in the pathology seen in these model systems (Sheu et al., 2003).
Insulinoma cell lines transfected with munc13-1 exhibited an increased insulin response to high glucose – transfected cells secreted 56% more insulin in response to glucose than controls. Additionally, munc13-1-transfected cells exhibited a 166% increase in insulin secretion in response to phorbol ester treatment as compared to controls (Sheu et al., 2003). In single-cell patch-clamping experiments, munc13-1 overexpression was shown to result in a 3-fold increase in the readily releasable pool of insulin granules. Munc13-1-transfected cells also displayed an increase in secretion due to increased temperature, and this effect was shown to depend on endogenous DAG production (Sheu et al., 2003). These experiments strongly suggest a role for munc13-1 in a physiological response of great importance, extending the role of munc13 in regulated secretion beyond the brain.

1.5.2 Munc13-1 deficiency reduces insulin secretion \textit{in vivo}

Since munc13-1 knockout mice die shortly after death, munc13-1 heterozygotes, which were shown to express less munc13-1 protein than wild-type animals, were used to study the role of munc13-1 in insulin secretion \textit{in vivo} (Kwan et al., 2006). While heterozygous mice had normal fasting blood glucose levels, these animals showed impaired glucose tolerance when compared to wild-type. Interestingly, mice heterozygous for mutant munc13-1 lacking a functional C1 domain (H567K) exhibited normal glucose tolerance, suggesting that the C1 domain is not required for the effect of munc13-1 on glucose tolerance (Kwan et al., 2006). Since all animals (wild-type and munc13-1 heterozygotes) responded similarly to insulin injection, this defect is in insulin secretion, not peripheral insulin sensitivity. As expected, serum insulin levels were reduced in heterozygous, munc13-1\textsuperscript{+/−} mice after a glucose load when compared to wild-type. At time points representative of both phases of insulin secretion, serum levels were significantly lower in the heterozygous mice, while only the first phase was effected in mice expressing the H567K mutant (Kwan et al., 2006). Further, islets isolated from the
munc13-1\textsuperscript{+/−} mice exhibited nearly a 50% reduction in insulin secretion in response to glucose. While some potentiation of insulin secretion was seen in islets from heterozygotes, it was markedly less than that seen in wild-type islets, suggesting that the single allele of munc13-1 was still partially functional (Kwan \textit{et al.}, 2006). Experiments carried out using the H567K mutant showed that a functional C1 domain is required for full potentiation by phorbol esters.

Patch-clamping of single beta cells revealed that loss of munc13-1 expression resulted in impairment of phorbol-ester potentiated secretion, and that this effect was due to a reduction in the size of the readily releasable vesicle pool, and an impairment of the rate of the refilling of this pool. Consistent with the \textit{in vivo} results, patch-clamping of H567K-expressing beta cells revealed an effect on the readily releasable vesicle pool size, but no effect on the rate of refilling (Kwan \textit{et al.}, 2006). However, in the face of phorbol ester pretreatment, both parameters were affected in beta cells expressing H567K. This set of experiments demonstrates that the 60% reduction of munc13-1 expression seen in munc13-1\textsuperscript{+/−} mice is sufficient to cause significant insulin secretion defects. These results are consistent with the previous study showing secretory defects in diabetic rat models (Sheu \textit{et al.}, 2003). The results in pancreatic tissue show that munc13-1 regulates vesicle exocytosis in a critically important system in human health and disease, and that munc13-1 function is generalizable beyond the nervous system.

1.6 Munc13 is expressed in the kidney, and is implicated in diabetic nephropathy

1.6.1 Munc13 is upregulated in kidney cells during hyperglycemia

Using differential-display RT-PCR (DDRT-PCR), our lab was able to show that hmunc13 (an alternate name for munc13-2) was upregulated in human mesangial cells (MC) that were cultured in high glucose (25 mM) (Song \textit{et al.}, 1998). Relative RT-PCR and Northern blot
analysis confirmed this result, showing a 70% increase in munc13-2 expression in MC cultured in high glucose-containing medium (Song et al., 1998). This study also revealed that munc13-2 was expressed in rat MC and human renal cortical epithelial cells.

1.6.2 **Munc13 is upregulated in a diabetic animal model, and may cause apoptosis in vivo**

After cloning hmunc13 from a human kidney library, our lab sought to further characterize the potential role of munc13-2 in diabetic nephropathy. In order to look at munc13 expression in vivo, the streptozotocin (STZ)-induced diabetic rat model was used. After treatment with STZ, rats develop diabetes in a well-defined manner (Song et al., 1999). After 11 days of hyperglycemia, rats were sacrificed, and expression of munc13-1 and munc13-2 was assayed using fluorescence *in situ* hybridization (FISH) on kidney sections. Compared to controls, STZ-treated rats showed high expression of munc13-1 and munc13-2 in the renal cortex, specifically in cortical epithelial cells and in some glomerular cells (Song et al., 1999). While the precise identity of the glomerular cells expressing munc13 could not be determined, our previous data suggest that these would be mesangial cells. These results complement the *in vitro* data documenting the upregulation of munc13 in cultured MC.

To investigate the function of munc13 in these cells, cell culture systems were employed. Hemagglutinin (HA)-tagged hmunc13 was constructed (munc13-HA), as well as a mutant lacking the C1 domain (C1-less), and these constructs were expressed in opossum kidney (OK) cells (Song et al., 1999). HA-tagged munc13 protein was found to be cytosolic, and treatment of the cells with the phorbol ester, phorbol 12,13-dibutyrate (PDBu), resulted in the C1 domain-dependent translocation of hmunc13 to the Golgi (Song et al., 1999). Strikingly, PDBu-treated cells that expressed hmunc13 underwent apoptosis, as shown by TUNEL staining and genomic DNA breakdown (Song et al., 1999).
These results set up an interesting model involving high glucose, DAG, munc13, and apoptosis. In the diabetic state, high ambient glucose levels result in an increase in intracellular DAG due to increased flux through the polyol pathway (Whiteside and Dlugosz, 2002). Apoptosis of renal cells, especially in the glomerulus, is also thought to have an early role in the pathogenesis of diabetic nephropathy (Shankland, 2006). Therefore, in diabetes, munc13 is upregulated in the kidney, and is activated by increased levels of DAG, resulting in apoptosis (Song et al., 1999). This model potentially places munc13 in a central role in a process of great therapeutic interest.

1.6.3 A polymorphism in the \textit{UNC13B} locus is associated with diabetic nephropathy

While diabetic nephropathy is highly prevalent, few reliable predictors of the disease exist. A large case-control study sought to find single nucleotide polymorphisms (SNPs) that associated with diabetic nephropathy in type 1 diabetes patients (Tregouet \textit{et al.}, 2008). 1,176 patients with diabetic nephropathy, and 1,323 controls were screened for SNPs in 127 candidate genes. The only SNP with a significant association with nephropathy was mapped to intron 1 in the \textit{UNC13B} locus, which codes for munc13-2 (odds ratio of 1.63) (Tregouet \textit{et al.}, 2008). While this SNP had no known functional significance, two SNPs with complete association to the one in question affect binding sites for SP1 and Upstream Stimulating Factor at the \textit{UNC13B} promoter, suggesting a potential role for this SNP in gene expression (Tregouet \textit{et al.}, 2008). Clearly, if differences in munc13-2 expression were associated with diabetic nephropathy, munc13-2 would be a potential screening tool and therapeutic target for patients with the disease.
1.7 Munc13 binds the small GTPase, rab34

1.7.1 Munc13 is an effector of rab34

In an effort to determine the mechanism by which munc13 functions in the kidney, our lab undertook a search for munc13 interacting partners in renal cells. To this end, a bacterial two-hybrid system was used to probe a human kidney cDNA library for munc13 binding partners (Speight and Silverman, 2005). This search yielded the small GTPase, rab34. Munc13 was found to bind rab34 in a GTP-dependent manner, making it an effector of rab34. Treatment of HEK-293 cells co-expressing HA-tagged munc13 and V5-tagged rab34 with PDBu resulted in the colocalization of the two proteins at the Golgi (Speight and Silverman, 2005). Further, the interaction of munc13 and rab34 was mapped to the MHD2 domain of munc13. These studies demonstrated a novel interacting partner for munc13, setting up a potential signaling axis involving DAG, munc13, and a rab protein (Speight and Silverman, 2005).

1.7.2 Rab structure and function

Rab proteins are soluble proteins that are synthesized in the cytosol and prenylated post-translationally (Alory and Balch, 2000). Upon translation, nascent, GDP-bound rabs are bound by the Rab Escort Protein (REP) (Goody et al., 2005). Two REP isoforms, REP-1 and REP-2, exist in mammalian cells. Once bound by REP, the Rab-REP complex can be recognized by Rab geranylgeranyltransferase (GGTase). GGTase then transfers lipid groups to the two C-terminal cysteine residues on the nascent rab. The requirement for REP binding stems from the lack of a consensus GGTase recognition sequence (Overmeyer et al., 2001). In other GTPases, such as ras, a CAAX motif exists which is recognized by the prenylation machinery. In the case of rabs, there is only a dicysteine motif, so the GGTase enzyme recognizes a complex of rab and REP.
Additionally, REP serves to shield the lipid groups bound to rabs from the aqueous environment of the cytosol until it is delivered to its target membrane (Goody et al., 2005).

Upon delivery to its target membrane the rab protein is activated. A guanine nucleotide exchange factor (GEF) replaces GDP with GTP, resulting in a conformational change at the switch regions, and subsequent activation of the rab protein (Zerial and McBride, 2001) (Figure 4). At this point – membrane- and GTP-bound – the rab may interact with effector proteins. Effector binding generally results in the transport of a rab to another target membrane compartment (the acceptor compartment). At this time, a GTPase activating protein (GAP) accelerates the slow intrinsic rate of rab GTP hydrolysis, resulting in the hydrolysis of GTP to GDP (Zerial and McBride, 2001). Once GDP-bound, the rab can be removed from the membrane by a GDP dissociation inhibitor (GDI), which is structurally very similar to REP. GDI again shields the lipid groups of the rab, and recycles the protein back to its original compartment for another round of activation (Overmeyer et al., 2001).
Figure 5. The rab GTPase cycle. GDP-bound rab proteins are held in the cytosol on complex with rab GDI. GEF proteins catalyze the exchange of GDP for GTP, at which time the active rab protein is deposited at its target membrane. This process is thought to involve the prenylated rab acceptor protein (PRA). The active rab can be acted upon by a GAP protein, which catalyzes the hydrolysis of GTP to GDP and the release of inorganic phosphate. Now, GDI can remove the rab protein from the membrane, and a new activation cycle can begin.
1.7.3 Rab34 binds RILP and influences lysosomal positioning

At the time that rab34 was identified as a munc13 effector, very little had been published about rab34. One report demonstrated that rab34 was a Golgi-bound protein that was involved in the repositioning of lysosomes toward the centre of the cell (Wang and Hong, 2002). Wang and Hong found that both wild-type and constitutively active rab34 were capable of moving lysosomes toward the juxtanuclear region of the cell, and showed that this phenomenon depended on the interaction of rab34 with the rab7-interacting lysosomal protein (RILP) (Wang and Hong, 2002). RILP had previously been shown to bind rab7 on the lysosomal membrane, and tether it to microtubules via interaction with the dynein/dynactin motor system (Jordens et al., 2001). It was speculated, therefore, that rab34 was interacting with RILP, which was bound to lysosomes, and moving the organelles toward the microtubule-organizing centre (MTOC) using dynein motors. However, a precise mechanism for this phenotype was difficult to define, since it was clear that rab34 was acting upon lysosomes in an “interorganellar” manner, whereby rab34 exerted its function on lysosomes while bound to the Golgi (Wang and Hong, 2002).

1.7.4 Rab34 is involved in fluid-phase uptake at plasma membrane ruffles

Another report in the literature at this time stated that rab34 was in fact a plasma membrane protein, and that its function was to assist in macropinocytosis at membrane ruffles (Sun et al., 2003). In this report, myc-tagged rab34 was found at sites of membrane ruffling, colocalizing with actin, and on the membrane of dextran-containing macropinocytotic vesicles (Sun et al., 2003). Additionally, overexpression of either wild-type or constitutively active rab34 resulted in an increase in the overall number of macropinosomes in cells, and enhanced the stimulatory effect of both phorbol esters and platelet-derived growth factor (PDGF) on macropinocytosis (Sun et al., 2003). In an attempt to determine the mechanism of rab34-
mediated enhancement of macropinocytosis, rab34 was coexpressed with dominant-negative rac1 or WAVE. Coexpression of either of these proteins with rab34 inhibited the increase in vesicle number seen with rab34 alone, suggesting that rab34 requires rac1/WAVE-mediated membrane ruffling for its action (Sun et al., 2003).

1.8 Rationale and hypothesis

1.8.1 General rationale

Diabetic nephropathy (DN) is the leading cause of end stage renal disease in Canada, Europe, the USA and Japan (Clark et al., 2000; Remuzzi et al., 2002). In Europe and the USA, the incidence of DN has increased by 150% over the past 10 years, and diabetic patients have a significantly increased mortality rate when compared to the dialysis population as a whole (Remuzzi et al., 2002). Clearly, both the financial and medical implications of this shocking rise in DN incidence warrant intense research into the mechanisms and treatment of DN. Up to 40% of patients with type 2 diabetes will develop nephropathy (Dronavalli et al., 2008), and developing sensitive and specific screening tools for nephropathy in diabetic patients will be required in order to direct prevention and treatment resources efficiently in the face of a diabetic population that is rapidly increasing in size.

1.8.2 Part 1 – Investigation of rab34 function in HeLa cells

As outlined previously, our lab has identified munc13 as a protein that is both upregulated and activated in both cell culture systems and the renal cortex of diabetic rats (Song et al., 1998, 1999). In vitro cell culture data suggest that munc13 may be involved in the induction of apoptosis in the diabetic state, making it a potential therapeutic target in patients with DN, potentially using a GTPase inhibitor, siRNA delivery, or other small molecules designed to disrupt rab34 function (Song et al., 1999). Additionally, levels of munc13 protein
expression could prove to be a viable screening tool for the development and progression of DN. Munc13 isoforms had previously shown to be involved in vesicle priming and secretion in several organ systems, including the brain, pancreatic beta cell, and several blood cell lineages (reviewed above). Consistent with this role in secretion, our lab found that munc13 interacts directly with the small GTPase, rab34, in a GTP-dependent manner (Speight and Silverman, 2005). At that time, little was known about the function of rab34. As outlined above, different reports existed placing rab34 at either the Golgi or at the plasma membrane, and suggesting that rab34 was involved in both macropinocytosis and the repositioning of lysosomes toward the cell centre (Wang and Hong, 2002; Sun et al., 2003). Before a role for the munc13-rab34 interaction could be defined, it was necessary to assess the role of rab34 in a useful cell culture system. To this end, we sought to characterize both the localization and function of rab34 in HeLa cells. We chose HeLa cells because of their ease of transfection, the presence of endogenous rab34, and their consistent geometry that facilitates microscopic analysis of cell function. Our investigations re-analyzed the claims made in the literature regarding rab34 function, and proposed our own model for the role of rab34 in HeLa cells. Our assumption was that rab34 would be involved in an aspect of vesicle structure and function, like other rab proteins, and that a possible functional interface for rab34 and munc13 would be revealed during the course of our investigations.

1.8.3 Part 2 -- The role of the munc13-rab34 interaction in secretory pathway dynamics

Once we had shown that rab34 is involved in intra-Golgi transport within the secretory pathway in HeLa cells, we sought to determine the role of the munc13-rab34 interaction in this process, and to link this function back to our original observations made in models of DN. Using our HeLa cell model, we investigated the effect of munc13 on the kinetics of transport of a
model protein cargo, temperature-sensitive VSVG-GFP, through the secretory pathway. This model was chosen because of its wide use in the literature, and because it allows for rapid, easy evaluation of the integrity of the secretory pathway. We then took this system into a cell culture model of physiological relevance, investigating secretory pathway dynamics in rat mesangial cells cultured in both normal and high (diabetic) concentrations of glucose. The effect of high glucose on the secretory pathway, as well as on munc13 expression levels were assayed, and siRNA technology was employed to assess the specific role of munc13 in constitutive secretion in mesangial cells. We hypothesized that munc13 would effect secretion in a rab34-dependent manner, and that munc13 expression levels would allow for the formation of a link between diabetes, munc13 expression, and the dysregulation of the secretory pathway.
Chapter 2

2 Golgi-Bound Rab34 is A Novel Member of the Secretory Pathway


2.1 Abstract

Golgi-localized Rab34 has been implicated in repositioning lysosomes and activation of macropinocytosis. Using HeLa cells, we undertook a detailed investigation of Rab34 involvement in intracellular vesicle transport. Immunelectron microscopy and immunocytochemistry confirmed that Rab34 is localized to the Golgi stack and that active Rab34 shifts lysosomes to the cell center. Contrary to a previous report, we found that Rab34 is not concentrated at membrane ruffles and is not involved in fluid-phase uptake in our HeLa cell model. Also, Rab34-induced repositioning of lysosomes does not affect mannose 6-phosphate receptor trafficking. Most strikingly, HeLa cells depleted of Rab34 by transfection with dominant-negative rab34 or after RNA interference, failed to transport the temperature-sensitive vesicular stomatitis virus G-protein (VSVG) fused to green fluorescent protein (VSVG-GFP) from the Golgi to the plasma membrane. Transfection with mouse Rab34 rescued this defect. Using endogenous major histocompatibility complex class I (MHCI) as a marker, an endoglycosidase H resistance assay showed that endoplasmic reticulum (ER) to medial Golgi traffic remains intact in knockdown cells, indicating that Rab34 specifically functions downstream of the ER. Further, brefeldin A treatment revealed that Rab34 effects intra-Golgi transport, not exit from the trans-Golgi network. Collectively, these results define Rab34 as a novel member of the secretory pathway acting at the Golgi.
2.2 Introduction

Rab GTPases and their effectors are involved in virtually all aspects of transport vesicle budding, movement, targeting, and fusion (Zerial and McBride, 2001). Different rab proteins have, in addition to a specific complement of effector proteins, a distinct compartmental distribution allowing them to perform numerous functions within cells (Grosshans et al., 2006). The array of rab effectors – which includes enzymes, cytoskeletal elements, SNARE proteins, and vesicle coat proteins – combines with the tightly regulated intracellular distribution of rab proteins to allow rabs to perform a wide variety of cellular functions.

Constitutive secretion is governed by a defined set of Rab GTPases. ER to Golgi transport requires Rab1 and Rab2 (Tisdale et al., 1992; Allan et al., 2000), Rab6 has been linked with intra-Golgi transport (Echard et al., 2000), and transport from the trans-Golgi network (TGN) to the plasma membrane has been shown to involve Rab8 and Rab11 (Huber et al., 1993; Chen et al., 1998). This list, however, is not exhaustive, and whether other Rab proteins and their effectors are involved in constitutive secretion remains to be seen.

Little has been written about Golgi-bound rab, Rab34. Two effectors of Rab34 have been identified – the Rab-interacting lysosomal protein (RILP), which links Rab34 to dynein microtubule motors (Wang and Hong, 2002), and hmunc13, a PKC superfamily member, which has been implicated in the induction of apoptosis at the Golgi in response to phorbol ester treatment (Speight and Silverman, 2005). By transfecting cell lines with constitutively active or dominant-negative forms of Rab34, Rab34 has been implicated in fluid-phase uptake of proteins at membrane ruffles via macropinocytosis (Sun et al., 2003) and in the shifting of lysosomes toward the microtubule organizing centre (MTOC) (Wang and Hong, 2002).
In order to clarify the role of Rab34 in mammalian cells, we have used transiently-transfected HeLa cells as a model system. Using GFP fusions of wild-type (wt-GFP-Rab34), constitutively active (CA-GFP-Rab34), or dominant-negative Rab34 (DN-GFP-Rab34), we have re-examined the existing literature pertaining to Rab34, and investigated new functional roles for Rab34 in HeLa cells. Since Rab34 is localized to the Golgi in our system, we sought to investigate Rab34 function in the context of this organelle. To this end, we employed RNA interference to knock down Rab34 expression in HeLa cells. Using this assay, as well as the constitutively active and dominant-negative Rab34 constructs used in the studies mentioned above, we re-evaluated the functions of Rab34 that have been described in the literature, and examined the role of Rab34 in the secretory pathway. In our system, we are unable to observe any enrichment of Rab34 at membrane ruffles, or any effect of Rab34 on fluid-phase uptake. Active Rab34, however, did cause lysosomes to shift to a juxtanuclear position, consistent with a previous report. The mechanism and functional implications of this phenotype are unclear, but we have determined that trafficking of the mannose 6-phosphate receptor (M6PR) is unaffected by Rab34. More strikingly, our data indicate that Rab34 is confined to the Golgi, where it is required for the exit of transport carriers traversing the secretory pathway. Our data show specifically that Rab34 is required for exit of VSVG-GFP from the Golgi stack, upstream of the trans-Golgi network (TGN). This site of action places Rab34 upstream of several other known players in the secretory pathway, including protein kinase D (PKD) and phosphatidylinositol 4-phosphate (PI4P) (Liljedahl et al., 2001; Hausser A, 2005).
2.3 Materials and Methods

Antibodies and Reagents: The following primary antibodies were used: rabbit anti-Rab34 (Santa Cruz), mouse anti-GM130 (BD Biosciences), mouse anti-mannose 6-phosphate receptor (Calbiochem), rabbit anti-GFP (Molecular Probes), mouse anti-LAMP-1 (Developmental Studies Hybridoma Bank, University of Iowa), mouse anti-Vinculin (Sigma), mouse anti-MHC Class I W6/32 (a gift from Dr. D.B. Williams). Anti-mouse-Cy3 (Jackson ImmunoResearch) was used as a secondary antibody for immunofluorescence, and anti-rabbit-HRP and anti-mouse-HRP (Santa Cruz) were used for Western blotting. Rhodamine-conjugated wheat germ agglutinin (WGA) was from Vector Laboratories, Alexa-647-conjugated WGA was from Molecular Probes. Alexa-647-conjugated dextran was from Molecular Probes. Protein A-Sepharose (GE Healthcare) was used for immunoprecipitation, and 35S-methionine was from Amersham Biosciences. Nocodazole, TPA, and brefeldin A (BFA) were from Sigma. The ceramide analog D-threo-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) was from Bio-Mol.

Plasmids and siRNA: Wild-type Rab34 fused to the C-terminal of GFP (wt-GFP-Rab34) was constructed using Gateway Technology (Invitrogen) with pcDNA-DEST40-Rab34wt (described in (Speight and Silverman, 2005)) as donor, and pcDNA6.2/N-EmGFP-DEST as the destination vector. Both the constitutively active, GTP-restricted, Q111L mutant and the dominant-negative, GDP-restricted, T66N mutants were fused to GFP using the same method (CA-GFP-Rab34 and DN-GFP-Rab34, respectively). The pEGFPdKA206K-N1-VSVG tsO45 vector encoding VSVG-GFP and pEGFPdKA206K-N1-mCherry (VSVG-Cherry) were generous gifts from Dr. J. Lippincott-Schwartz. Plasmids encoding the tail of H-Ras fused to RFP (HRas-tail-RFP), the PH domain of phospholipase c-delta (PLCδ-PH-RFP), or GPI-linked RFP (GPI-RFP) have been described previously (Varnai and Balla, 1998; Choy et al., 1999; Keller P, 2001). Stealth siRNA
directed against human Rab34 and appropriate scrambled control siRNA were synthesized by Invitrogen. siRNA targeting Rab34 was the following annealed duplex: 5’
AAUCGUUCCAUCUGAAGUCCACUC 3’ and 5’
GAGUGGACUUGCAGAUGGAACGAUU 3’.

**Cell Culture and Transfection:** HeLa cells were grown in MEM plus 10% FBS, and maintained at 37°C in 5% CO₂. Transfection of siRNA was performed using Lipofectamine 2000 (Invitrogen) according to manufacturer’s directions. Plasmid DNA was transfected using FuGene 6 Reagent (Roche) according to manufacturer’s directions, using a DNA:FuGene ratio of 3:1.

**Cryo-Electron Microscopy:** HeLa cells were grown in 10 cm dishes, and transfected with GFP-Rab34-wt. 24 hours post-transfection, cells were washed in PBS, and fixed in 4% followed by 8% paraformaldehyde. Cells were then washed in 0.15 M glycine, followed by 1% gelatin in PBS. Cells were scraped, pelleted, and resuspended in 12% gelatin. The cells were then pelleted again, and cooled to allow the gelatin to set. The pellets were cut into 1mm³ pieces and put in 2.3 M sucrose in PBS at 4°C over night. The sucrose pieces were put on metal pins, frozen in liquid nitrogen, and sectioned at -120°C at a thickness of 75 nm. Sections were picked up in a 1:1 mixture of 2% methyl cellulose and 2.3 M sucrose and transferred to formvar-coated nickel grids. For immunostaining, sections were blocked in 5% fish skin gelatin, and then incubated for 30 min. in anti-GFP. Sections were washed, and incubated with protein A-Gold, washed, and fixed in 1% gluteraldehyde. Phosphate was removed in water, and the sections were stained in methylcellulose and uranyl acetate on ice.

**Immunoblotting:** Cells were lysed in 1% NP-40, and protein concentration in lysates was determined using a Lowry assay (Bio-Rad). 50 µg of protein was run on a 10% polyacrylamide
Following transfer to nitrocellulose, filters were blocked in 5% non-fat dry milk powder in 10 mM Tris–HCL (pH 8.0), 150 mM NaCl and 0.05% Tween 20 (TBST) overnight at 4 °C. Primary and secondary antibodies were diluted in blocking buffer, and incubations were for 1 h at room temperature. Detection was performed using an ECL Advance Western Blotting Detection Kit (Amersham Biosciences).

**Membrane Ruffling and Dextran Uptake:** HeLa cells were cotransfected with wt-GFP-Rab34 and either HRas-tail-RFP or PLCδ-PH-RFP. After 24 hours, membrane ruffling was induced by treatment with 100 nM TPA for 10 minutes in HEPES-buffered MEM, and the cells were imaged live using a spinning disk confocal microscope (Leica), and the fluorescence intensity of GFP both at a ruffle and non-ruffle, as well as for RFP at a ruffle and non-ruffle was measured using ImageJ (Altan-Bonnet et al.). The ratio of ruffle to non-ruffle fluorescence for GFP was divided by the ratio of ruffle to non-ruffle fluorescence for RFP to determine whether or not Rab34 was specifically enriched at membrane ruffles.

To measure dextran uptake, HeLa cells transfected with GFP-Rab34 vectors, or with GFP alone were exposed to 200 µg/ml Alexa-647 Dextran for 10 minutes. Cells were then rinsed in ice-cold PBS, trypsinized, and analyzed by FACS. 10 000 transfected cells were counted for each experimental condition. Data were analyzed using FlowJo (Tree Star), and Alexa-647-dextran fluorescence was expressed as a percent of Alexa-647 fluorescence in cells transfected with GFP alone.

**Lysosomal Positioning Assay:** HeLa cells were transfected with Rab34 vectors, and imaged 24 hours post-transfection. Cells were serum starved for 2 hours, then were fixed in 3.7 % paraformaldehyde, permeabilized in 0.2% Triton-X-100, and blocked in 10% fat-free milk. Fixed cells were stained using anti-LAMP-1 antibody, and Cy3-conjugated secondary antibody.
Cells were visualized by confocal microscopy. Analysis was performed using the Radial Plot function in ImageJ (Altan-Bonnet et al.). Rab34 expressing cells were identified by GFP fluorescence, and using Radial Plot, concentric circles were drawn from the cell centre to the cell boundary, and integrated LAMP-1 fluorescence intensities were recorded for the area along each circle. To normalize for cell size and fluorescence, the data were binned into the inner, middle, and outer thirds of each cell, and expressed as a percent of total LAMP-1 fluorescence.

**Dominant-negative and constitutively active Rab34 Assays:** HeLa cells plated on glass coverslips in 12 well plates were transfected with one of wt-GFP-Rab34, CA-GFP-Rab34, or DN-GFP-Rab34. For M6PR experiments, cells were fixed in 3.7% paraformaldehyde 24 hours post-transfection, permeabilized, and stained with anti-M6PR and anti-mouse-Cy3. Cells were mounted and imaged using a spinning disk confocal microscope (Leica). For VSVG-Cherry experiments, cells were cotransfected with GFP-Rab34 or its mutants, as well as VSVG-Cherry at 40°C as described below.

**VSVG-GFP Secretion Assay:** HeLa cells were plated on glass coverslips in 12 well plates. The following day, cells were transfected with either scrambled siRNA, or siRNA directed against Rab34, and incubated for 48 hours. Following this, cells were transfected with VSVG-GFP, and incubated at 40°C for a further 20 hours. All subsequent incubations were done in the presence of 50 µg/ml cycloheximide (Sigma) to halt protein synthesis. For time t=0, cells were rinsed in ice-cold PBS prior to fixation. Remaining cells were returned to an incubator at 32°C for the indicated time. After incubation, cells were rinsed in ice-cold PBS, and fixed in 3.7% paraformaldehyde. For treatment with WGA, cells were incubated with 0.01 mg/ml WGA on ice for 10 minutes. For GM130 staining, cells were permeabilized with 0.2% Triton X-100, and stained using anti-GM130, followed by a Cy3-anti-mouse antibod. Cells were mounted on slides.
and imaged using a Zeiss LSM 510 Confocal microscope. Image analysis was performed using Volocity (Improvision).

**Endoglycosidase H Assay:** HeLa cells were plated in 60 mm dishes, transfected with either scrambled siRNA or siRNA against Rab34, and incubated for 72 hours. Metabolic labeling, immunoprecipitation and EndoH digestion were performed as described (Kim *et al.*, 1996). Briefly, cells were incubated for 30 minutes in RPMI lacking methionine. Metabolic labeling was performed using 150 µCi of $^{35}$S-Methionine per plate for 10 minutes. Chase incubations were done in RPMI supplemented with methionine for the indicated times. Following chase, cells were rinsed in ice-cold PBS, and lysed in buffer containing NP40. For immunoprecipitation of MHC class I molecules, each lysate was incubated in anti-MHC class I antibody W6/32, followed by Protein A-Sepharose. After washing, lysates were split, and some were incubated with endoglycosidase H (NEB) for 3 hours at 37°C. Samples were then run on 10% polyacrylamide gels.

### 2.4 Results

#### 2.4.1 GFP-Rab34 is localized to the Golgi

Wt-GFP-Rab34 was expressed in HeLa cells and several protocols were initiated to evaluate the differing claims in the literature regarding Rab34 localization. When cells are fixed and immunostained with the Golgi marker, GM130, wt-GFP-Rab34 and GM130 co-localize at the Golgi (Figure 5). Moreover, the near-complete co-localization of wt-GFP-Rab34 and GM130 persists after microtubule depolymerization with nocodazole (Figure 5). To determine the precise localization of Rab34 in the Golgi stack, we used immunogold labeling of transfected wt-GFP-Rab34 in ultrathin cryo-sections of fixed HeLa cells. Transmission electron microscopy revealed that wt-GFP-Rab34 is present throughout the Golgi stack, both in the Golgi cisternae
and on a population of Golgi-associated vesicles (Figure 5B). Transfected wt-GFP-Rab34 is seen on some cis-Golgi transport carriers, as well as on some peri-Golgi transport vesicles (arrowheads in Figure 5B). The vast majority of labeling is observed in the Golgi cisternae themselves, and there is no preference for cis-, medial-, or trans-Golgi stacks. Similarly, wt-GFP-Rab34 is relatively evenly distributed within each cisterna, with no preference for central or terminal membranes.
Figure 6. Rab34 is localized to the Golgi in HeLa cells. (A) HeLa cells transiently transfected with wt-GFP-Rab34 were treated with or without nocodazole (ncdz) to depolymerize microtubules, and were fixed and stained for the Golgi marker, GM130. Note the colocalization of wt-GFP-Rab34 and GM130 both in the absence (−ncdz) and presence (+ncdz) of nocodazole, suggesting Golgi localization. Scale bar, 10 µm. (B) HeLa cells transiently transfected with wt-GFP-Rab34 were fixed and processed for immunoelectron microscopy as described in Materials and Methods. Immunogold labeling shows wt-GFP-Rab34 is present throughout the Golgi stack (arrows), as well as on some cis-Golgi and peri-Golgi vesicular elements (arrowheads). Scale bar, 200 nm.
2.4.2 **Rab34 is not concentrated at membrane ruffles**

It has been reported previously that Rab34 is recruited to sites of membrane ruffling, where it is involved in macropinocytosis (Sun *et al*., 2003). Membrane ruffles are accumulations of lipid, protein, and cytoskeletal elements at the plasma membrane in response to several stimuli, including phorbol esters and growth factors (Kurokawa and Matsuda, 2005). Because membrane ruffles are such high density structures, and because membrane ruffles change the 3D structure of cells, it can be difficult to ascertain by confocal microscopy whether a protein is truly recruited to a membrane ruffle, or if this is an artifact that arises from an accumulation of cell density at the ruffled region. In an effort to examine a potential role for Rab34 in membrane ruffling in HeLa cells, we co-expressed wt-GFP-Rab34 with the tail of H-Ras fused to RFP (HRas-Tail-RFP). HRas-Tail-RFP inserts into the plasma membrane by three palmitate moieties, and is therefore a consistent marker for the plasma membrane (Roy *et al*., 2005).

These cells were then treated with 100nM TPA for 10 minutes to induce membrane ruffling, and live cells were subsequently analyzed by spinning disk confocal microscopy (Figure 6). For each of the GFP and RFP channels, a ratio of fluorescence intensity was taken at a membrane ruffle (Figure 6, arrowheads), and compared with an area of the plasma membrane that has not undergone ruffling (Figure 6, arrows). The Rab34 ratio was then divided by the HRas-Tail ratio to correct for membrane density changes at the membrane ruffle. If Rab34 is truly recruited to membrane ruffles, this ratio should be greater than 1. In our HeLa cell system, we found that Rab34 is not recruited to membrane ruffles, since the ratio of Rab34 signals to HRas-Tail signals was 0.98, which was not significantly different than 1 (p>0.5, n=20). To further demonstrate the lack of Rab34 recruitment to membrane ruffles, this experiment was repeated with another membrane marker. This time, the pleckstrin-homology domain of phospholipase c-delta fused to RFP (PLCδ-PH-RFP), which binds phosphoinositides in the plasma membrane (Stauffer *et al*.,
1998), was co-expressed with wt-Rab34-GFP, and the same assay and quantification was performed as described above. Similar to what was observed with HRas-Tail, the ratio of Rab34 to PLCδ-PH-RFP was not found to differ significantly from 1, strongly supporting the observation that Rab34 is not concentrated at sites of membrane ruffling in HeLa cells (ratio=1.10, p>0.3, n=20) (data not shown).
Figure 7. Golgi-bound Rab34 is not concentrated at membrane ruffles and does not participate in fluid-phase uptake. (A) HeLa cells coexpressing wt-GFP-Rab34 and HRas-Tail-RFP were treated with 100 nM TPA for 10 min, and the live cells were imaged using a spinning disk confocal microscope. Fluorescence intensities were measured for each channel at either membrane ruffles (arrowheads) or a nonruffled area of plasma membrane (arrows). Scale bar, 12 µm. (B) FACS analysis of dextran uptake. HeLa cells expressing CA-GFP-Rab34, DN-GFP-Rab34, or GFP alone were serum-starved and allowed to take up Alexa-647–labeled dextran for 10 min. Cells were harvested, and dextran uptake in Rab34-transfected cells was analyzed for 10,000 transfected (GFP-positive) cells per transfection condition. Dextran uptake was expressed as mean Alexa-647 fluorescence, normalized to Alexa-647 fluorescence for cells transfected with GFP alone (p > 0.1, n = 3). Data are shown ±SE.
2.4.3 Rab34 does not participate in fluid-phase uptake

It has also been previously reported that active Rab34 increases macropinocytosis in mouse 10T1/2 fibroblasts, even in the absence of stimuli such as phorbol esters or PDGF (Sun et al., 2003). To test whether Rab34 affects fluid-phase uptake in our HeLa cell system, we transfected HeLa cells with CA-GFP-Rab34, DN-GFP-Rab34, or GFP alone. Cells were then incubated for 10 minutes with Alexa-647-conjugated dextran as a tracer for fluid-phase uptake. In order to perform a quantitative, high throughput analysis of dextran uptake, we used FACS to determine the amount of dextran taken up by our transfected cells (Figure 6B). For each transfection condition, 10 000 transfected cells were analyzed. Contrary to the previous report, but consistent with our finding that Rab34 is not recruited to membrane ruffles in HeLa cells, we found that Rab34 did not effect dextran uptake in HeLa cells, since neither CA-GFP-Rab34 nor DN-GFP-Rab34 cells exhibited a significant change in dextran uptake when compared with HeLa cells transfected with GFP alone – mean dextran fluorescence values of 111% and 103% of uptake in cells expressing GFP alone, were found for CA-GFP-Rab34 and DN-GFP-Rab34, respectively, and were not statistically significant (p>0.05, n=3). It is possible that the finding observed by Sun et al. (2003) represents a cell-specific phenomenon.

2.4.4 Rab34 regulates lysosomal position, but does not effect the localization of the mannose 6-phosphate receptor

The other phenotype previously associated with active Rab34 was its ability to shift lysosomes toward the MTOC via its association with RILP and the dynein/dynactin system (Wang and Hong, 2002). To test this phenomenon in our HeLa cell system, HeLa cells were transfected with either CA-GFP-Rab34 or DN-GFP-Rab34, fixed, and stained with an anti-LAMP-1 antibody to visualize lysosomes. To quantitate lysosomal position relative to the nucleus, we used a system of concentric circles drawn from the cell centre, as described in
Materials and Methods (Figure 7). Lysosomal position was then calculated as the percent of LAMP-1 fluorescence in a given cell within either the inner, middle, or outer third of the cell itself. We found that CA-GFP-Rab34 caused a significant shift of LAMP-1 fluorescence to the inner third of the cell as compared with DN-GFP-Rab34 – 63% of the LAMP-1 fluorescence was found in the inner third of cells transfected with CA-GFP-Rab34, versus 35% for DN-GFP-Rab34 (p<0.01). This finding suggests that the work of Wang and Hong in NRK cells (2002) can be generalized into our HeLa cell system.

Since active Rab34 was able to reposition lysosomes in HeLa cells, we next asked whether this positional change might be indicative of a change in TGN to lysosome trafficking. To examine this possibility, we used an antibody to the cation-independent mannose 6-phosphate receptor (M6PR). The M6PR is responsible for the trafficking of acid hydrolases from the TGN to the endosome (Ghosh et al., 2003). Once in the endosome, the M6PR and its ligand travel to the lysosomal compartment, where acidic pH results in the dissociation of ligand from the receptor, at which time the M6PR recycles back to the TGN (Ghosh et al., 2003). HeLa cells that had been transfected with either CA-GFP-Rab34 or DN-GFP-Rab34 were fixed and immunostained with an M6PR antibody (Figure 7D). In untransfected cells, the M6PR resides in both a juxtanuclear compartment as well as a punctate, endosomal compartment. This localization did not significantly change in HeLa cells expressing either Rab34 mutant, suggesting that Rab34 does not have a role in TGN to lysosome trafficking (Figure 7D). Therefore, the repositioning of lysosomes by active Rab34 is unlikely to be involved in the transport of acid hydrolases to the late endosome/lysosome.
Figure 8. Active Rab34 shifts lysosomes toward the MTOC, but does not effect the M6PR. (A) HeLa cells transfected with either CA-GFP-Rab34 or DN-GFP-Rab34 were fixed and stained with a mAb against LAMP-1 to mark lysosomes. Cells were imaged by confocal microscopy. Scale bar, 10 µm. (B) To quantitate the position of lysosomes relative to the cell center, the Radial Plot plugin was used, as described in Materials and Methods. This plugin records the integrated lysosomal fluorescence (black dots) along circles of increasing radius from the nucleus (N). To normalize for variable cell size and fluorescence intensities, these data were binned into one of the inner, middle, or outer thirds of the cell, and expressed as a percent of total LAMP-1 fluorescence. (C) Quantitated data from the method described in B. CA, CA-GFP-Rab34; DN; DN-GFP-Rab34. n = 20 cells were analyzed per condition; *p < 0.01, p < 0.05. Results are shown ±SE. Scale bar, 10 µm. (D) HeLa cells were fixed directly, or transiently transfected with either CA-GFP-Rab34 (CA-Rab34) or DN-GFP-Rab34 (DN-Rab34), serum-starved, fixed, and stained for M6PR. Cells were imaged using a spinning disk confocal microscope. The GFP signal was used to locate transfected cells, but is not shown for the purpose of clarity. All cells in these representative fields were transfected with the indicated GFP-Rab34 constructs. HeLa cells transfected with either CA- or DN-GFP-Rab34 showed no significant changes from controls, suggesting that Rab34 does not effect M6PR trafficking. Scale bar, 12 µm.
2.4.5 **Rab34 is required for secretion of VSVG-GFP at the Golgi**

Since Rab34 is localized to the Golgi, we wished to test whether Rab34 may be involved in the secretory pathway. As a reporter for constitutive secretion, we used the ts045 temperature-sensitive mutant of the VSV glycoprotein fused to one of GFP (VSVG-GFP), or monomeric Cherry (VSVG-Cherry). The temperature-sensitive mutant is retained in the ER at 40°C, and is released into the secretory pathway upon temperature shift to 32°C. To study the potential role of Rab34 in the secretory pathway, HeLa cells were either transfected with VSVG-Cherry alone, or cotransfected with VSVG-Cherry, and one of wt-GFP-Rab34, CA-GFP-Rab34, or DN-GFP-Rab34 at 40°C. The following day, protein synthesis was inhibited with cycloheximide, and at t=0 the cells were shifted to the permissive temperature of 32°C for various times. In cells expressing VSVG-Cherry alone or in combination with wt-GFP-Rab34, the VSVG protein was found in the ER at t=0, predominantly at the Golgi at t=30min, with a small amount of protein still in the ER, and entirely at the plasma membrane at t=180min (Figure 8). Only a very small number of cells co-expressing VSV-Cherry and wt-GFP-Rab34 exhibited a delay of VSVG-Cherry transport to the plasma membrane. In contrast, HeLa cells expressing DN-GFP-Rab34 exhibited a marked decrease in VSVG-Cherry transport from the Golgi to the plasma membrane (Figure 8). Only 17% of cells expressing DN-GFP-Rab34 had transported all VSVG-Cherry to the plasma membrane, compared to 83% of cells expressing wt-GFP-Rab34, or 85% of cells expressing VSVG-Cherry alone (p<0.001) (Figure 8B). These results strongly suggest that Rab34 is required for secretion of VSVG-Cherry from the Golgi. Cells expressing CA-GFP-Rab34 also exhibited limited inhibition of VSVG-Cherry secretion to the plasma membrane, with 57% of cells completely transporting VSVG to the cell surface (Figure 8B). The effect of CA-GFP-Rab34 on VSVG-Cherry secretion suggests that there is a requirement for Rab34 to maintain the ability to cycle between the GTP- and GDP-bound states for normal trafficking of
VSVG-Cherry to the plasma membrane. This phenomenon has been observed for other small GTPases as well, including Rac and Arf6 (Arrieumerlou et al., 2000; Klein et al., 2006).

To further study the role of Rab34 in the secretory pathway, we employed RNA interference (Varnai and Balla) by transfecting HeLa cells with short interfering RNA (siRNA) to deplete cells of endogenous Rab34. 72 hours post-transfection, significant knock down of endogenous Rab34 was observed in HeLa cells transfected with siRNA targeting Rab34, as compared to cells transfected with scrambled siRNA (Figure 8C). HeLa cells that had been transfected with either siRNA targeting Rab34 or a scrambled control siRNA were then transfected with VSVG-GFP, incubated at 40°C for 20 hours, and then treated with cycloheximide. After 180 minutes at the permissive temperature of 32°C, control HeLa cells transfected with scrambled siRNA had completely transported VSVG-Cherry to the plasma membrane (Figure 8D). Strikingly, HeLa cells transfected with siRNA directed against Rab34 exhibited an arrest of VSVG-Cherry transport. The vast majority of VSVG-Cherry remained in the Golgi, although there was a minor leak to the cell surface (Figure 8D). To further establish the specificity of our siRNA treatments, we sought to rescue the defect seen in siRNA-transfected cells. To this end, we used a GFP-fusion of wild-type mouse Rab34 (GFP-mRab34). The mouse Rab34 cDNA sequence differs from that of human Rab34 over the region of our siRNA, so this construct should be resistant to silencing by our siRNA transfection. HeLa cells that had been treated with siRNA to deplete endogenous Rab34 were co-transfected with VSVG-Cherry and GFP-mRab34 after 48 hours. Using our standard protocol, these cells showed a marked rescue of VSVG-Cherry transport to the cell surface as compared to cells that were not transfected with mRab34 (Figure 8D). While not all cells showed a complete rescue, it was clear that the majority of VSVG-Cherry transport to the cell surface had been restored by GFP-
mRab34. Taken together, these results define Rab34 as a novel member of the secretory pathway, acting at the Golgi.
Figure 9. Dominant-negative Rab34 or siRNA inhibit VSVG-Cherry transport from the Golgi to the plasma membrane. (A) HeLa cells were cotransfected with VSVG-Cherry and one of wt-, CA-, or DN-GFP-Rab3, and incubated overnight at 40°C. Cells were treated with cycloheximide and were shifted to 32°C at t = 0 min to allow VSVG transport to occur. Cells were fixed at the indicated times and imaged using a spinning disk confocal microscope. Scale bar, 12 µm. (B) Quantification of the results in A. Cells treated as in A were scored at t = 180 min for the proportion of cells showing complete transport of VSVG-Cherry to the plasma membrane. At least 20 cells were counted per condition for each of three independent experiments. The conditions examined were VSVG-Cherry alone (VSV), or VSVG-Cherry plus one of wt-GFP-Rab34 (WT), CA-GFP-Rab34 (CA), or DN-GFP-Rab34 (DN). Note the marked inhibition of Golgi to plasma membrane transport in cells expressing DN-GFP-Rab34 (p < 0.001 compared with wild type). (C) Knockdown of endogenous Rab34 in HeLa cells. HeLa cells were transfected with siRNA directed against human Rab34 or scrambled control siRNA. After 72 h, cells were lysed and analyzed by Western blot with a polyclonal anti-Rab34 antibody. Shown is a representative gel from three experiments. As a loading control, membranes were probed for vinculin. (D) HeLa cells transfected with scrambled siRNA (control) or siRNA against Rab34 (siRNA) were transfected with VSVG-Cherry 48 h later and incubated overnight at 40°C. Cells were shifted to the permissive temperature and fixed after 180 min. Note that Rab34 siRNA arrests VSVG-Cherry transport at the Golgi. In the third panel, cells transfected with Rab34 siRNA were then cotransfected with VSVG-Cherry and GFP-mRab34. Note that GFP-mRab34 is capable of rescuing the defect seen in siRNA cells, demonstrating the specificity of the Rab34 siRNA treatment. Scale bar, 12 µm.
To further define the precise site of action of Rab34, we performed a time course experiment along with quantitative colocalization of VSVG-GFP at either the Golgi or the plasma membrane. To this end, HeLa cells transfected with control siRNA or siRNA against Rab34 were transfected with VSVG-GFP, and followed at the permissive temperature for either 0, 30, or 180 minutes. At each time point, cells were fixed and either immunostained for the Golgi with an anti-GM130 antibody, or stained for the plasma membrane with rhodamine-conjugated WGA. Immediately after a temperature shift to 32°C, all VSVG-GFP was found in the ER in both control and knock down cells, and after 30 minutes, the majority of VSVG-GFP had traveled to the Golgi (Figure 9A, panels i-iv, vii-x). In control cells, at 180 minutes post-temperature shift, virtually all VSVG-GFP was found at the plasma membrane (Figure 9A, panel v). In contrast, in cells depleted of Rab34, very little VSVG-GFP was found at the plasma membrane, and the majority of the protein was retained in the Golgi (Figure 9A, compare panels v and vi). At 180 minutes post-temperature shift, Pearson’s coefficients for VSVG-GFP and WGA for control cells and knock-down cells of 0.82 and 0.44, respectively, show that while the majority of VSVG-GFP in control cells colocalized with the plasma membrane, much less of the GFP signal colocalized with WGA in cells depleted of endogenous Rab34 (p<0.001) (Figure 9B). Furthermore, while in control cells very little GFP signal was seen colocalizing with GM130 at the Golgi (Pearson’s coefficient of 0.16), a significant amount of GFP signal remains in the Golgi in cells depleted of Rab34 3 hours after release from the ER (Pearson’s coefficient of 0.47, p<0.001) (Figure 9C, and 9B compare panels xi and xii). The large increase in Pearson’s coefficients from 0 to 30 min post-temperature shift shows that VSVG-GFP is efficiently transported to the Golgi in both control and knockdown cells (for example, Pearson’s coefficient for GFP and GM130 increases from 0.23 to 0.73, and from 0.29 to 0.78 in control and knock-down cells, respectively). Since Pearson’s coefficients for 0 min and 30 min time points
do not differ significantly between control cells and those lacking Rab34, it appears that ER to Golgi transport is not affected by Rab34 knock-down, suggesting that Rab34 functions at a post-Golgi step in the secretory pathway. The small proportion of VSVG-GFP that is still transported to the plasma membrane in knock-down cells is probably due to either incomplete knock-down of Rab34 in these cells, or the existence of both Rab34-dependent and Rab34-independent pathways from the Golgi to the plasma membrane.
Figure 10. Rab34 is necessary for exit of VSVG-GFP from the Golgi. (A) VSVG-GFP secretion assay. HeLa cells were transfected with siRNA directed against Rab34 (siRNA) or scrambled control siRNA (control). Forty-eight hours later, cells were transfected with plasmid encoding VSVG-GFP and incubated at 40°C for 20 h. After cycloheximide treatment, the temperature was shifted to 32°C, and cells were fixed at the indicated time points and stained with a monoclonal anti-GM130 antibody to mark the Golgi or with WGA to mark the plasma membrane. Scale bar, 10 µm. Images are representative of greater than three experiments. (B) Colocalization of VSVG-GFP and WGA. Pearson’s coefficients were calculated for 18 cells per condition from at least three experiments. Data are shown ±SE. *p < 0.001. (C) As in B, but for colocalization of VSVG-GFP and GM130.
2.4.6 **Rab34 depletion arrests intra-Golgi transport of VSVG-GFP, not Golgi to TGN transport**

Several molecules have been implicated in VSVG-GFP release from the TGN, including PKD and PI4P (Liljedahl *et al.*, 2001; Hausser A, 2005). Using conventional light microscopy and colocalization techniques it is difficult to determine whether a protein is found in the trans-Golgi cisternae or in the TGN, which lies distal to the trans-Golgi itself in the secretory pathway. To explore the precise location of the effect of Rab34 on VSVG-GFP secretion, we exploited the toxin Brefeldin A (BFA). BFA treatment is known to result in the retrograde transport of Golgi-resident proteins into the ER (Lippincott-Schwartz J, 1990). The TGN, on the other hand, in response to BFA treatment, condenses in the juxtanuclear region (Chege and Pfeffer, 1990). This differential effect of BFA on trans-Golgi versus TGN-resident proteins allows for careful dissection of these two compartments. To this end, we performed our VSVG-GFP secretion assay in HeLa cells transfected with siRNA directed against Rab34 or the appropriate controls. At either t=30min or t=180min, cells were treated with 5µg/ml BFA for 30 minutes prior to fixation and imaging by spinning disk confocal microscopy (Figure 10). At t=30min, both control cells (30 min ctrl) and knockdown cells (30 min siRNA) show VSVG-GFP to be in the Golgi stack. BFA treatment confirms this, resulting in redistribution of VSVG-GFP to the ER (Figure 10, top 4 panels). At t=180min, knockdown cells (180min-siRNA) show that VSVG-GFP remains in the Golgi, and BFA treatment again results in the redistribution of the GFP signal to the ER (Figure 10). These data suggest that Rab34 is required for intra-Golgi transport of VSVG-GFP, not Golgi to TGN transport. As a control, cells were treated with the ceramide analog, PDMP. PDMP has been shown to block VSVG-GFP transport at the TGN as a result of defective protein ADP-ribosylation (De Matteis *et al.*, 1999). At t=180min, PDMP-treated cells also contain VSVG-GFP in the Golgi, but BFA treatment fails to redistribute the GFP signal to
the ER (Figure 10, bottom panels). The VSVG-GFP protein condenses into a discrete pocket in the juxtanuclear region, suggesting a TGN localization of VSVG-GFP in PDMP-treated HeLa cells, demonstrating the differential effect of BFA on Golgi and TGN-resident proteins.

Collectively, the above experiments show that Rab34 is required for intra-Golgi transport of VSVG-GFP in HeLa cells. This result places Rab34 upstream of PKD and PI4P in the secretory pathway.
Figure 11. Rab34 is required for intra-Golgi transport of VSVG-GFP, not exit from the TGN. HeLa cells transfected with siRNA directed against Rab34 or scrambled control were transfected with VSVG-GFP at 40°C. At t = 0, cells were treated with cycloheximide and shifted to the permissive temperature of 32°C. At t = 30 or 180 min, cells were treated for 30 min with BFA at 32°C, fixed, and viewed using a spinning disk confocal microscope. At t = 30 min, cells transfected with control siRNA (30 min-ctrl) or Rab34 siRNA (30 min-siRNA) had VSVG-GFP in the Golgi. After 30-min BFA treatment (+BFA), GFP signal redistributed to the ER. At t = 180 min, Rab34 siRNA-treated cells (180 min-siRNA) retained VSVG-GFP in the Golgi, and BFA treatment (+BFA) redistributed the GFP signal to the ER. As a control, cells treated with PDMP retained VSVG-GFP in the TGN at t = 180 min (180 min-PDMP), as BFA treatment (+BFA) resulted in the condensation of the GFP signal in the juxtanuclear region. Scale bar, 10 µm.
2.4.7 Rab34 is not involved in ER to Golgi Transport

While confocal microscopy suggested that Rab34 was not involved in the transport of proteins from the ER to the Golgi, we sought to examine this step of the secretory pathway biochemically using endogenous MHC class I protein as a reporter. HeLa cells transfected with either scrambled siRNA or siRNA targeting Rab34 were metabolically labeled with a pulse of $^{35}$S-methionine, and then chased with cold methionine for 0 to 60 minutes. Total MHC class I was immunoprecipitated from cell lysates, and subsequently digested with endoglycosidase H (endoH). EndoH cleaves N-linked oligosaccharides on proteins in the ER, but cannot cleave the high mannose oligosaccharides found on proteins that have progressed to the medial Golgi (Orlean et al., 1991). Thus, proteins in the ER are endoH sensitive, and proteins that have progressed to the Golgi are endoH resistant. This difference can be seen as a mobility shift on a polyacrylamide gel. As seen by the rates of acquisition of endoH resistance by endogenous MHC class I protein, depletion of endogenous Rab34 has no effect on ER to Golgi transport in HeLa cells (Figure 11). Taken with the VSVG-GFP secretion data, these results show that Rab34 specifically functions at a post-Golgi step of the secretory pathway. Further, since EndoH resistance is acquired in the medial Golgi, Rab34 must function at a transport step at or beyond the medial Golgi.
A

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B

% EndoH Resistant

Chase Time (min)
Figure 12. Rab34 is not involved in ER to Golgi transport. HeLa cells were transfected with either scrambled siRNA (control) or siRNA directed against Rab34 (siRNA). Cells were labeled with 35S-Methionine, and chased with cold methionine for the indicated times. Cells were lysed, and total MHC class I was immunoprecipitated and digested with endoH as indicated. Protein was then run on polyacrylamide gels. (A) Representative gel showing increase in endoH resistant (EndoHR) form of MHC I as compared to endoH sensitive (EndoHS), indicating progression of MHC I protein from the ER to the Golgi. (B) Rate of ER to Golgi transport of MHC I as shown by the acquisition of endoH resistance by MHC I protein (n=3). Results are shown +/- standard error.
2.5 Discussion

Previous reports have proposed various roles and subcellular localizations for Rab34 (Wang and Hong, 2002; Sun et al., 2003; Speight and Silverman, 2005). In the face of several published phenotypes, we sought to clarify the role of Rab34 first by re-evaluating the literature in our HeLa cell system, then by depleting HeLa cells of endogenous Rab34 using RNAi. Our findings limit Rab34 localization to the Golgi, and cast doubt upon its suggested role at the plasma membrane. The use of both fluorescence microscopy and immunoelectron microscopy have shown that wild-type GFP-Rab34 is localized to the Golgi stack, as well as to a population of cis-Golgi elements and intra-Golgi transport carriers. The report by Sun et al. (2003) led us to examine the potential role of Rab34 at membrane ruffles in HeLa cells. Our attempts to establish this role using quantitative colocalization with plasma membrane markers in HeLa cells were not successful. Clearly further work must be done before one can safely conclude that Rab34 is recruited to membrane ruffles in a manner that can be generalized to multiple cell types. We also found that Rab34 has no effect on fluid-phase uptake in HeLa cells. These results suggest that the findings of Sun et al. (2003) may represent a cell-specific phenomenon.

From the Golgi, our data demonstrate two roles for Rab34, which need not be completely independent of one another. First, we found that active Rab34 is capable of shifting lysosomes to the juxtanuclear region, which is consistent with published literature (Wang and Hong, 2002). Our finding with the greatest functional significance, however, is that Rab34 is required for the intra-Golgi transport of the model protein cargo, VSVG-GFP, as it traverses the secretory pathway. Using three separate methods – namely siRNA, dominant-negative Rab34, and rescue of the siRNA-mediated defect – we have shown that Rab34 is a novel member of the secretory pathway. HeLa cells depleted of Rab34 by RNAi or dominant-negative Rab34 expression
exhibited a marked decrease in transport of VSVG-GFP from the Golgi to the plasma membrane. The defect seen in siRNA-transfected cells can be rescued by expression of mouse Rab34, indicating that this phenomenon is specifically due to Rab34 depletion. This result establishes Rab34 as an essential player in a ubiquitous cellular function.

Both fluorescence microscopy of VSVG-GFP transport and rates of acquisition of endoH resistance of endogenous MHC showed that secretory transport from the ER to the medial Golgi remains intact in the absence of Rab34, since the medial Golgi is the site of complex N-glycosylation of transmembrane proteins (Orlean et al., 1991). This result, in combination with those described above, shows that Rab34 acts either within or immediately after the Golgi stack.

Since Rab34 is involved in intra-Golgi protein transport, one would expect that an effect would be seen on both the trafficking of VSVG and the trafficking of the M6PR, since this transport step is upstream of TGN sorting. Since no clear effect was seen on the steady-state distribution of the M6PR, two possibilities exist. One is that the effect on the M6PR is subtler than the effect on VSVG. Our data in Figure 8D may provide clues that while a gross change in M6PR distribution is not occurring, a smaller-scale change in M6PR trafficking may be present in HeLa cells transfected with Rab34 mutants. Further work involving dynamic measurements of M6PR trafficking to the endosomal system in the presence of Rab34 mutants and compartmental markers is required in order to elucidate a potential function for Rab34 in this pathway. A second possibility is that Rab34 exhibits cargo selectivity within the Golgi. While this may be an intriguing possibility, much more work needs to be done to attempt to define a mechanism by which this could occur.

While our data support the finding that Rab34 is capable of moving lysosomes toward the MTOC, the functional implications of lysosomal position remain unknown. From the Golgi, at
least two pathways for vesicle traffic depart – the classic secretory pathway to the plasma membrane, for which we have shown Rab34 to be necessary, and another pathway to the late endosome/lysosome. This second pathway is necessary for the delivery of acid hydrolases to the maturing lysosome (Ghosh et al., 2003). Since Rab34 appears to be involved in the transport of proteins through the Golgi along the secretory pathway, it is tempting to hypothesize that it is also involved in trafficking to the lysosome, and that increased Golgi-to-lysosome traffic by active Rab34 may be occurring while lysosomes shift toward the Golgi. However, as discussed above, dominant-negative and constitutively active Rab34 did not grossly affect the localization of the M6PR in HeLa cells, although a more subtle effect could not be ruled out. Thus, the significance of the phenotype characterized by Rab34 induced movement of lysosomes toward the MTOC remains to be determined.

While the mechanism by which Rab34 affects lysosomal position has been shown to require interaction with RILP, which links Rab34 to the dynein/dynactin microtubule motor system (Wang and Hong, 2002), the mechanism by which Rab34 effects secretion from the Golgi is less clear. To date, only two effectors have been identified for Rab34 – RILP, and hmunc13, which is a well-described PKC superfamily member that is involved in both vesicle priming and the induction of apoptosis at the Golgi in response to phorbol ester treatment (Augustin et al., 1999; Song et al., 1999). To determine the mechanism of Rab34 function in constitutive secretion, we first sought to define the precise site of action of Rab34 in the secretory pathway. BFA treatment revealed that Rab34 depletion arrests VSVG-GFP transport within the Golgi itself, not at the TGN. This result mechanistically separates the function of Rab34 from that of known mediators of TGN exit.
The exit of proteins from the TGN has been reported to involve protein kinase D (PKD), as well as Golgi-resident sphingolipids and PI4P (Rosenwald et al., 1992; Echard et al., 2000; Liljedahl et al., 2001; Hausser A, 2005). Dominant-negative PKD inhibits the fission of transport carriers from the TGN, and its expression results in extensive tubulation of the TGN, indicative of a release failure of these extending tubules from the TGN (Liljedahl et al., 2001). While it is known that Golgi-resident diacylglycerol is responsible for the recruitment of PKD to the Golgi, the molecular mechanism of PKD-mediated transport carrier fission has not been completely defined (Baron and Malhotra, 2002). It is known, however, that PKD can activate phosphatidylinositol 4-kinase III-β, resulting in an increase in TGN PI4P, which is in turn required for vesicle fission (Hausser A, 2005). It has also been shown that inhibition of de novo sphingolipid synthesis inhibits VSVG transport along the secretory pathway (Rosenwald et al., 1992). The importance of sphingolipids in this process is not known, but the Golgi is a key store of ceramide and sphingolipids (Rosenwald et al., 1992). Our work establishes that Rab34 functions upstream of these components of the secretory pathway.

Rab6A is involved in transport of VSVG through the Golgi itself (Echard et al., 2000). Interestingly, it is constitutively active Rab6A, not dominant-negative Rab6A, which inhibits secretion through the Golgi. This sets up an intriguing possibility whereby Rab6A and Rab34 may act in opposition to one another, since active Rab6A or loss of Rab34 inhibit secretion. It is possible that these two inputs could allow the cell to control constitutive secretion at the Golgi in response to various stimuli. Future experiments will seek to define the precise molecular events of Rab34-mediated transport, as well as defining cellular regulators of Rab34 activity.

Finally, it is interesting to examine our results in the light of the findings by Wang and Hong (2002) showing that Rab34 regulates lysosomal positioning from the Golgi. Their data
describe a pathway whereby Rab7-positive lysosomes migrate along microtubules to the peri-Golgi region via an association between Rab7, RILP, and Golgi-bound Rab34 (Wang and Hong, 2002). This region appears to be particularly active, housing the Golgi, TGN, recycling endosomes, lysosomes, and various other components of the endomembrane system. In addition, at this same location within the cell, our lab has previously shown that hmunc13 interacts with GTP-bound Rab34 via its second munc homology domain (Speight and Silverman, 2005).

Although it is premature to indulge in significant speculation at this point, it seems possible that a situation exists whereby regulated formation of a molecular platform occurs involving protein interactions between Rab34, Rab7, RILP, hmunc13 and probably as yet other unidentified proteins.
Chapter 3

3 Mesangial cell munc13 contributes to increased TGF-β secretion during hyperglycemia via a rab34-dependent pathway

3.1 Abstract

The mesangial cell plays a key role in the development and maintenance of diabetic nephropathy (DN). Hallmarks of the disease include cellular hypertrophy and renal fibrosis, both of which are influenced by dysregulated mesangial cell function, particularly aberrant secretion of cytokines and matrix components. The cytokine transforming growth factor-beta (TGF-β) is known to be important in many aspects of the pathology associated with DN, and several current treatments for DN decrease TGF-β levels in both human subjects and animal models of diabetes. Previous work has shown that the phorbol ester receptor, munc13 is upregulated in the diabetic kidney. We undertook a detailed study of the role of munc13 in DN within the context of its recently described interaction with rab34. Rab34 is a known member of the secretory pathway acting at the Golgi, and is required for secretion of a model protein. Hyperglycemic conditions resulted in upregulation of munc13 and a concurrent increase in the rate of protein secretion. The effect on secretion dynamics was inhibited when endogenous munc13 or rab34 were depleted, and could be mimicked by forced overexpression of munc13. The observed phenotype required the rab34-interacting domain of munc13, and was dependent on munc13 localization. Finally, knockdown of munc13 in mesangial cells decreased secretion of TGF-β, a critical molecule in the pathogenesis of DN. This work suggests that upregulation of munc13 during diabetes may contribute to the renal injury seen in DN via its interaction with rab34 in the secretory pathway.
3.2 Introduction

Diabetic nephropathy (DN) is the leading indication for dialysis in Canada, the USA, Europe, and Japan (Clark et al., 2000; Remuzzi et al., 2002). While the incidence of DN is increasing, the pathogenesis of the disease is not fully understood. Several important mediators of DN have been identified, including transforming growth factor beta (TGF-β), and protein kinase c (PKC), as well as extracellular matrix molecules such as collagen I and fibronectin (Reeves and Andreoli, 2000; Schena and Gesualdo, 2005; Noh and King, 2007).

Previous work in our lab identified hmunc13 (also known as ubiquitous munc13-2, or ubmunc13-2) as a protein that is upregulated in the renal cortex of rats with streptozotocin-induced diabetes (Song et al., 1998). This upregulation was seen in the cortical epithelium as well as in glomerular cells, and further work suggested that activated hmunc13 induced apoptosis in cultured renal cell lines (Song et al., 1999). Munc13s comprise a small family of C1 domain-containing proteins within the PKC super-family (Brose and Rosenmund, 2002). Munc13 proteins contain a single C1 domain, at least one C2 domain, and two munc13 homology domains, named MHD1 and MHD2 (Koch et al., 2000). The four family members, named munc13-1 through -4, have been studied to varying degrees – munc13-1 is expressed in the brain and pancreas, and is necessary for neurotransmitter vesicle priming and a process termed long-term potentiation, as well as for insulin secretion from pancreatic beta cells (Betz et al., 1998; Sheu et al., 2003). Munc13-3 is involved in the normal development of the visual cortex (Yang et al., 2002). Munc13-4, a distant relative lacking the C1 domain, is involved in degranulation of specific blood cell lineages, and is mutated in the genetic disorder familial hemophagocytic lymphohistiocytosis (Yamamoto et al., 2004). Munc13-2 has a ubiquitously expressed variant, and its role in the brain is also well studied (Rosenmund et al., 2002). Further to our work on
hmunc13 in DN, a recent study has found that a single nucleotide polymorphism (SNP) in the *UNC13B* locus is associated with the development of DN in patients with type 1 diabetes (Tregouet *et al.*, 2008). This SNP is completely associated with others at regulatory sites within the promoter region, meaning that changes in munc13-2 expression may be associated with the development of diabetic nephropathy (Tregouet *et al.*, 2008). Taken together, these data suggest that hmunc13 may play a crucial role in the pathogenesis of diabetic nephropathy.

Recently, our lab identified hmunc13 as an effector of the small GTPase, rab34 (Speight and Silverman, 2005). Hmunc13 binds rab34 in a GTP-specific manner via its MHD2 domain (Speight and Silverman, 2005). Rab34 is a member of the rab family of small GTPases, which are involved in virtually every aspect of vesicle transport – including vesicle budding, trafficking, targeting, and fusion (Zerial and McBride, 2001). Rabs are able to carry out these many functions by virtue of their compartmental distribution, and their interaction with a host of effector proteins, including enzymes, cytoskeletal proteins, SNAREs, and other molecules (Grosshans *et al.*, 2006). Previous work in our lab has shown that rab34 is required for constitutive secretion of the model viral protein, vesicular stomatitis virus glycoprotein (VSVG), working at the level of intra-Golgi transport (Goldenberg *et al.*, 2007).

It is within this context that we investigated the function of the hmunc13-rab34 interaction. In light of our work on rab34, we sought to examine a potential role for hmunc13 in the secretory pathway. Using a temperature-sensitive mutant of VSVG fused to GFP (VSVG-GFP), we were able to show that overexpression of hmunc13 increases the rate of VSVG-GFP transport to the cell surface in cultured rat mesangial cells (MC). Culture of MC in high glucose, which causes the upregulation of hmunc13, increases the rate of VSVG-GFP transport, and this increase can be inhibited by siRNA-mediated knockdown of endogenous munc13-2.
Knockdown of rab34 also decreased the rate of VSVG-GFP transport in MC. Mechanistically, studies in HeLa cells suggested that the effect of hmunc13 on VSVG-GFP transport requires the rab34-interacting MHD2 domain, and that sequestration of hmunc13 to the plasma membrane by treatment with the phorbol ester, 12-O-Tetradecanoylphorbol-13-acetate (TPA), abolished the hmunc13 effect. Perhaps most interestingly, knockdown of munc13-2 in rat MC decreased both basal and high glucose-stimulated secretion of TGF-β, placing munc13- and rab34-mediated secretion within the context of a clinically significant cellular process. To our knowledge, this is the first description of a role for hmunc13 in constitutive secretion, and is one that could have profound implications for the pathogenesis of DN.

3.3 Materials and Methods

**Antibodies and Reagents:** The following primary antibodies were used: TKG, a mouse monoclonal antibody raised against an extracellular epitope of VSVG, was a generous gift from Dr. Ira Mellman (Genentech, USA); mouse monoclonal anti-HA (Covance). Anti-mouse-Cy3 and anti-mouse-Cy2 (Jackson ImmunoResearch) were used as secondary antibodies for immunofluorescence. TPA, PDBu, and cycloheximide were from Sigma. VectaShield mounting medium was used for microscopy (Vector Labs).

**Plasmids:** The pEGFPdKA206K-N1-VSVG tsO45 vector encoding VSVG-GFP was a generous gift from Dr. J. Lippincott-Schwartz (NIH, USA). Plasmids encoding HA-tagged wild-type munc13, a mutant lacking the C1 domain (C1-less), and a mutant lacking the MHD2 domain (MHD2-less) have been described previously (Song et al., 1999; Speight and Silverman, 2005).

**Cell Culture and Transfection:** HeLa cells were grown in MEM plus 10% FBS, and maintained at 37°C in 5% CO₂. Primary rat mesangial cells (RMC) were a generous gift from Dr. Catherine Whiteside (Toronto, Canada), and were used between passages 13 and 20.
Isolation of RMC has been described previously (Hurst et al., 1995). RMC were cultured in low glucose DMEM plus 10% FBS, and maintained at 37°C in 5% CO₂. Plasmid DNA was transfected using FuGene 6 Reagent (Roche) according to manufacturer’s directions, using a DNA:FuGene ratio of 3:1.

**VSVG Secretion Assay and Surface Labeling:** Cells were plated on coverslips in 24 well plates overnight at 37°C. Transfections were performed, and cells were incubated a further 24 hours at 40°C. When VSVG-GFP and munc13 vectors were co-transfected, munc13 (wt, C1-less, or MHD2-less) was added in a 2:1 excess over VSVG-GFP in order to ensure co-transfection of munc13 constructs in all cells expressing VSVG-GFP. Cells were then treated with 50 µg/ml cycloheximide to halt protein synthesis, and were either washed in ice-cold PBS (t=0 min) or transferred to 32°C. For a 45 min chase, cells were either incubated at 32°C for 45min, or for 30min, followed by addition of either TPA or PDBu at 100 nM, and incubation at 32°C for a further 15 min (for a total of 45 min). Cells were then washed in ice-cold PBS, blocked in 10% milk, and treated with anti-VSVG antibody on ice. After further washes in PBS, cells were fixed in 3.7% paraformaldehyde, and quenched in 100 mM glycine. Cells were then treated with an anti-mouse-Cy3 antibody, washed, and mounted for microscopy. Slides were imaged using a spinning disk confocal microscope (Leica) and Volocity software (ImproVision). At least 10 cells were imaged per condition, and n≥3. Image analysis was performed using ImageJ (Altan-Bonnet et al.). Briefly, cells to be analyzed were selected, and mean pixel intensities were obtained. After subtracting background surface labeling at t=0 min, a ratio of surface to total VSVG-GFP staining (i.e. red versus green fluorescence) was calculated for each cell. The mean ratio was calculated for each condition, and results were expressed relative to VSVG-GFP alone at t=45 min.
**RMC Glucose Treatments:** For glucose response experiments using RMC, primary RMC were cultured in DMEM containing either 5.5 mM D-glucose (LG), 25 mM D-glucose (Yamamoto *et al.*), or 5.5 mM D-glucose plus 19.5 mM L-glucose as an osmotic control (OC). Cells were cultured as such for 72 hours, with a medium change at 48 hours. After 72 hours, cells were transfected as described, and incubated overnight at 40°C in LG, HG, or OC medium. VSV assay was performed the following day as described above.

**Real-Time RT-PCR:** Total RNA was isolated using the TRIzol Reagent (Invitrogen) and cDNA was synthesized from 1 µg of total RNA using the Omniscript RT (Qiagen) kit. Real-time PCR was then performed using ABI SYBR green on the ABI-PRISM 7900HT using the standard curve method. Munc13 primers were designed using ABI Primer Express Software and RPL32 was employed as a housekeeping gene for analysis of relative expression. Primers for rat munc13-2 were 5’- GGACGATGCATGGAAGGTGTAC -3’ and 5’- GGATGACAAACACGCAAAGTGC -3’. Primers for rat RPL32 were 5’- AAGAAACCAAGCACATGCTGCC -3’ and 5’- CTCAGCACAGTAAGATTTGTTGC -3’.

**siRNA Treatments:** Rat MC were transfected with Stealth siRNA (Invitrogen) 24 hours after plating. Transfections were performed in Opti-MEM (Gibco) using 20 pmol siRNA per well of a 12 well plate. Lipofectamine 2000 was used for transfections at a concentration of 2.5 µL per well. After 5 hours, medium was aspirated and replaced with normal DMEM (LG or HG) for further experiments. Transfection of VSVG-GFP, if required, was done 48 hours later as described above. siRNA sequences used were: munc13-2 5’- GGCCUGCUUGAACUCUCAUAUGAA -3’, and rab34 5’- GGAAGACCUGCUUUAAUAUAGGUU -3’.
**Munc13 Immunofluorescence:** HeLa or RMC were plated on coverslips in 24 well plates overnight. Cells were transfected with Munc13-HA (wt, C1-less, or MHD2-less), and the following day cells were treated with vehicle alone (DMSO), or with 100 nM TPA or PDBu for the indicated times. Cells were then washed in ice-cold PBS, fixed in 3.7% paraformaldehyde, and quenched with 100 mM glycine. Cells were then permeabilized in 0.2% Triton X-100, and blocked with 10% non-fat milk. Munc13-HA was labeled using anti-HA, and secondary antibody was added (anti-mouse-Cy2) following washing. Cells were washed again in PBS, and mounted on slides. Cells were imaged using a spinning disk confocal microscope (Leica) and Volocity software (ImproVision).

**TGF-β Secretion:** RMC were seeded in 24 well plates in either LG or HG medium. Cells were transfected with either control siRNA, or siRNA directed against munc13-2 as described above. The following day, medium was replaced with LG or HG medium containing 0.5% FBS. 48 hours later, supernatants were collected for TGF-β ELISA (R&D Systems). To assay total secreted TGF-β, samples were acidified to convert to active form according to manufacturer’s instructions. All samples were loaded in triplicate. Cells were lysed and assayed for total protein content using the DC protein assay (Bio-Rad), and results were expressed as pg TGF-β/mg protein in lysate.

### 3.4 Results

#### 3.4.1 Culture of RMC in high glucose increases the rate of VSVG-GFP secretion

Our lab initially identified munc13 as a protein that is upregulated in the renal cortex of rats with streptozotocin-induced diabetes, as well as in cultured human mesangial cells (Song et al., 1998, 1999). In the context of our work on rab34, we hypothesized that munc13 may
modulate the effect of Rab34 on the secretory pathway. Specifically, we sought to test whether
culture of RMC in conditions mimicking the hyperglycemic state could effect constitutive
secretion in a munc13-dependent manner. To test this hypothesis, we cultured primary RMC for
72 hours in medium containing 5.5 mM D-glucose (LG), 25 mM D-glucose (Yamamoto et al.) or
5.5 mM D-glucose plus 19.5 mM L-glucose as an osmotic control (OC). Medium was changed
after 48 hours to ensure that glucose levels remained stable throughout the test period. After 72
hours, cells were transfected with VSVG-GFP overnight at 40°C in the appropriate glucose-
containing medium. Following this incubation, cells were shifted to 32°C for 45 minutes in the
presence or absence of TPA for the final 15 minutes, and were surface-labeled with an antibody
raised against an extracellular epitope of VSVG-GFP. Strikingly, RMC cultured in 25 mM D-
glucose (Yamamoto et al.) exhibited a greater than 50% increase in the ratio of surface to total
VSVG-GFP as compared to LG or OC controls, indicating an increase in the rate of VSVG-GFP
transport to the plasma membrane (Figure 12).

3.4.2 The increased rate of VSVG-GFP secretion in HG-treated cells is due to upregulation of munc13

Previous work in our lab suggested that the increase in the rate of VSVG-GFP secretion
might be due to increased amounts of endogenous munc13 interacting with Rab34 at the Golgi.
While our lab has shown that high glucose upregulates munc13 expression in various renal cell
systems, we confirmed this result using real-time RT-PCR. RMC were cultured in LG, OC, or
HG medium as before, and total mRNA was extracted from the cells for RT-PCR analysis. As
seen in a representative experiment, HG medium caused an approximately 1.8-fold increase in
munc13 expression in RMC versus cells cultured in LG or OC conditions relative to the
housekeeping gene, RPL32 (Figure 13).
This result shows that munc13 is upregulated in our experimental conditions, but still does not directly indicate a role for munc13 in the secretory phenotype that was observed. To address this issue, we examined the rate of VSVG-GFP secretion in RMC cultured in LG medium in the presence or absence of exogenous munc13. RMC grown in LG medium were transfected with VSVG-GFP, with or without HA-tagged munc13 (HA-munc13). Munc13-transfected cells exhibited an over 3-fold increase in the rate of VSVG-GFP secretion as compared to untransfected cells (Figure 13B). This result, taken together with those above, suggests that upregulation of munc13 is responsible for the increase in VSVG-GFP secretion observed in RMC cultured in high glucose.

To further demonstrate that increased munc13 expression is responsible for the increase in VSVG-GFP secretion seen in HG-cultured RMC, we employed siRNA technology to knock down endogenous munc13 expression in RMC. When compared to cells transfected with control siRNA, cells transfected with munc13 siRNA displayed a modest decrease in the rate of VSVG-GFP secretion when cultured in LG medium (Figure 13C). When cultured in HG medium, munc13 knock down resulted in a dramatic decrease in the VSVG-GFP secretion rate – surface to total VSVG-GFP ratios for munc13 knock down cells were approximately 0.2 versus 1.4 in controls (Figure 13C). This result, along with the RT-PCR and munc13 overexpression data, strongly suggests that increased munc13 expression in RMC during exposure to high glucose is responsible for the increased rate of secretion of the model protein cargo, VSVG-GFP.
Figure 13. The rate of VSVG-GFP secretion is increased by high glucose. Primary RMC were incubated for 72 hours in 5.5 mM D-glucose (LG), 25 mM D-glucose (Yamamoto et al.), or 5.5 mM D-glucose plus 19.5 mM L-glucose (OC). Cells were then transfected with VSVG-GFP and surface transport of VSVG-GFP was assayed using our standard protocol. Note the increase in transport rate in cells treated with HG alone. The ratio of surface to total VSVG-GFP was calculated for 10 cells per condition, and was expressed relative to the ratio in cells expressing VSVG-GFP alone. Data are means of n=6 experiments +/- standard error. *, p<0.05, †, p<0.01.
A

B

C
Figure 14. The increased rate of VSVG-GFP secretion in HG is due to munc13 upregulation. 

A. Real time PCR data showing upregulation of munc13 in RMC cultured in high glucose. RMC were cultured in low glucose (LG), osmotic control (OC), or high glucose (Yamamoto et al.) as described in materials and methods. Gene specific primers were used to amplify munc13-2 from total cDNA. Results are expressed in arbitrary units as a function of the expression of the housekeeping gene, RPL32. Graph shows the results of a typical experiment. 

B. RMC were transfected with VSV-GFP plus or minus munc13, and incubated over night. VSVG-GFP protein was released from the ER for 45 minutes, in the presence or absence of TPA for the final 15 minutes as indicated. Cells were then fixed and surface VSVG-GFP was immunostained. Data are means of n=3 experiments, +/- SE. *, p<0.05.

C. RMC were transfected with either siRNA against munc13-2 or scrambled control siRNA (ctrl). Surface VSVG-GFP was labeled as per the standard protocol. Note the dramatic decrease in the rate of VSVG-GFP secretion in knockdown cells as compared to controls. Data are means of n=3 experiments, +/- SE. *, p<0.05, †, p<0.01.
3.4.3 **The increase in the rate of VSVG-GFP secretion occurs via a rab34-dependent pathway**

We have established that upregulation of munc13 in response to high glucose increases the secretion kinetics of VSVG-GFP. We next sought to determine the mechanism by which this occurs. Since the munc13 binding partner, rab34, is required for secretion of VSVG-GFP in HeLa cells, it was hypothesized that munc13 acts through rab34 to influence secretion rates in RMC. Rab34 has never been studied in RMC, so the role of rab34 in secretion in RMC was confirmed by monitoring the rate of VSVG-GFP secretion in cells transfected with siRNA against endogenous rab34. In cells cultured in LG medium, there was a modest block in VSVG-GFP secretion in rab34 knock down cells as compared to controls (Figure 14). In HG-cultured cells, rab34 knock down caused a near 50% decrease in the ratio of surface to total VSVG-GFP when compared to cells transfected with scrambled control siRNA (Figure 14). These data show that our previous results in HeLa cells can be generalized to the RMC system, and that VSVG-GFP secretion in RMC occurs, at least in part, via a rab34-dependent pathway.

To further investigate the role of the rab34-munc13 interaction in the secretory pathway, a culture model that lacked endogenous munc13 expression was desirable. Previous work had shown that HeLa cells do not normally express munc13, as tested using RT-PCR (unpublished results). Since we have thoroughly studied the rab34-mediated secretion of VSVG-GFP in HeLa cells, they are a useful system to exploit for these purposes. As expected, overexpression of munc13 in HeLa cells mimicked the result seen in HG-cultured RMC – the rate of VSVG-GFP secretion increased in a munc13-dependent fashion (Figure 14B). To determine the mechanism by which this occurs, HeLa cells were transfected with a mutant of munc13 lacking the rab34-interacting MHD2 domain (MHD2-less). While transfection of wild-type munc13 increased the rate of VSVG-GFP secretion, transfection of MHD2-less munc13 resulted in a modest decrease
in secretion of the model protein (Figure 14B). Since rab34 is the only known protein that binds munc13 via the MHD2 domain, this result suggests that the effect of munc13 overexpression on VSVG-GFP secretion occurs via its interaction with rab34.

To further demonstrate the importance of the rab34-munc13 interaction, we used phorbol esters to relocalize munc13 away from rab34. Rab34 is found at the Golgi, and munc13 is largely cytosolic, allowing the two proteins to contact one another at the cytosolic face of the Golgi stack. Short-term treatment (15 minutes) of cells with the phorabol ester 12-O-Tetradecanoylphorbol-13-acetate (TPA) results in the relocalization of munc13 to the plasma membrane. Conversely, 15 minute treatment with the short-chain phorbol ester, phorbol 12,13-dibutyrate (PDBu) does not relocalize munc13 at all (Figure 15). Further, this localization effect is the result of the direct interaction of the phorbol ester and munc13, since mutant munc13 lacking the C1 domain (C1less) does not translocate in response to TPA or PDBu treatment (Figure 15B). We exploited the differential effects of these two phorbol esters to investigate the role of the munc13-rab34 interaction on VSVG-GFP secretion. When TPA was added to cell cultures for the final 15 minutes of VSVG-GFP secretion in HeLa cells expressing munc13, there was a marked decrease in the rate of VSVG-GFP secretion, which was not seen in untransfected cells (Figure 15). This was not seen following similar treatment with PDBu, which does not result in munc13 relocalization (Figure 15). Taken together, these data suggest that the ability of munc13 to interact with rab34 is critical for its influence on protein secretion.
Figure 15. The effect of munc13 on VSVG-GFP secretion is dependent on rab34. A. RMC were transfected with either siRNA against rab34 or scrambled control siRNA (ctrl). Surface VSVG-GFP was labeled as per the standard protocol. Note the decrease in the rate of VSVG-GFP secretion in knockdown cells as compared to controls when cultured in HG medium. Data are means of n=3 experiments, +/- SE. *, p<0.05. B. HeLa cells were transfected with VSVG-GFP either alone (VSV 45min), or in combination with wild-type munc13 (Munc13 45min) or MHD2-less munc13 (MHD2-less 45min). Surface VSVG-GFP was labeled by the standard protocol. Note that while full-length munc13 increases the rate of VSVG-GFP transport, MHD2-less does not. Data are means of n=6 experiments, +/- SE. *, p<0.05.
Figure 16. Normal munc13 localization is required for it to exert its effect on VSVG-GFP secretion. **A.** HeLa cells transfected with either wild-type munc13, C1-less munc13, or MHD2-less munc13 were treated for 15 minutes with 100 nM TPA or vehicle (DMSO), and were fixed and stained with an anti-HA antibody. Note the C1 domain-dependent translocation of munc13 to the plasma membrane in the presence of TPA. **B.** HeLa cells transfected with wild-type munc13 were treated for 15 minutes with 100 nM PDBu, and were processed as in A. Note that PDBu does not relocalize munc13 to the plasma membrane, as seen with TPA. Scale bar = 20mm. **C.** HeLa cells were transfected with VSVG-GFP plus or minus wild-type munc13, and incubated over night. VSVG-GFP protein was released from the ER for 45 minutes, in the presence or absence of TPA or PDBu for the final 15 minutes as indicated. Cells were then fixed and surface VSVG-GFP was immunostained. Data are means of n=6 experiments, +/- SE. *, p<0.05, †, p<0.01, NS=not significant.
3.4.4 **Munc13-2 knockdown decreases TGF-β secretion**

Since we have determined that munc13 plays an important role in the secretion of a model protein, we wished to evaluate the potential effect of munc13 expression on the secretion of a physiologically important molecule. In DN, many pathological processes depend on the glucose-stimulated secretion of TGF-β by MC and other cell types (Ziyadeh, 2004). Because of this, we chose to assay the secretion of TGF-β by MC in response to high glucose. Using a commercially available ELISA, total TGF-β secretion (both active and latent forms) was assayed in cells transfected with either control siRNA, or siRNA directed against munc13-2. In agreement with the literature, culture of MC in high glucose stimulated TGF-β secretion by RMC by 10% (Figure 16). Strikingly, knockdown of munc13-2 resulted in a decrease in TGF-β secretion in cell culture in either LG or HG medium when compared to controls (Figure 16). This decrease was larger in cells cultured in HG – when cultured in LG, munc13-2 knockdown resulted in 80% of control TGF-β secretion, while knockdown of munc13-2 in HG-cultured cells resulted in 70% of control TGF-β secretion. These data show that our results using VSVG-GFP as a model protein cargo can be generalized to an endogenous molecule of clear clinical relevance.
Figure 17. Munc13 is involved in TGF-β secretion. RMC cultured in HG or LG medium were transfected with control siRNA, or siRNA targeting munc13-2. Cells were then serum-starved, and incubated a further 48 hours. Supernatants were collected, and were assayed for total TGF-β secreted, as described in Materials and Methods. Results are expressed as pg TGF-β in the supernatant normalized to mg cellular protein present in whole cell lysates. Data are means of n=2 experiments.
3.5 Discussion

Our lab has previously shown that rab34 is required for the intra-Golgi transport of the model cargo, VSVG-GFP, and that munc13 is an effector of rab34 (Speight and Silverman, 2005; Goldenberg et al., 2007). In light of these findings, we wished to examine the functional significance of the munc13-rab34 interaction in the context of known roles of each of these proteins. Since prior work identified that munc13 is upregulated in renal MC, we undertook experiments to investigate a role for munc13 in the secretory pathway in this cell system. We have demonstrated that culture of RMC in high glucose increases the rate of VSVG-GFP transport through the secretory pathway, and that this phenomenon occurs in the face of glucose-dependent upregulation of the munc13-2 gene. To address the munc13-specificity of this effect on protein secretion, we have shown that overexpression of munc13 in RMC mimics the effect of high glucose on VSVG-GFP secretion, and that siRNA-mediated knockdown of munc13 abolishes the effect of high glucose. Additionally, munc13 acts through a rab34-dependent pathway, since knockdown of rab34 in RMC reduces secretion of VSVG-GFP, and experiments carried out in HeLa cells have shown that the effect of munc13 on secretion relies on both the rab34-interacting MHD2 domain of munc13, and the subcellular localization of munc13. Taken together, these data allow us to hypothesize that a pathway exists linking high glucose, the increased transcription of munc13, its subsequent interaction with rab34, and increased protein transport through the secretory pathway. This is a pathway of great potential significance for DN. First, munc13 is a molecule that has been shown to be upregulated in the face of hyperglycemia (Song et al., 1998, 1999). In addition to its upregulation, munc13 is also activated in the diabetic state, since hyperglycemia is known to elevate levels of intracellular DAG via metabolic shunting into the polyol pathway (Whiteside and Dlugosz, 2002). Second, constitutive secretion is a system of critical importance in DN – dysregulation of extracellular matrix (ECM) protein
secretion, as well as regulators of matrix synthesis, is a hallmark of the diabetic kidney (reviewed in (Mason and Wahab, 2003). Our proposed model links the sensing of high glucose to the potentially increased secretion of various ECM components.

It is worth speculating about the precise mechanism by which munc13 and rab34 influence secretion at the Golgi. Recent work has resulted in a new model for Golgi function, whereby both Golgi-resident proteins and proteins traversing the secretory pathway dynamically partition between two microdomains within the Golgi membrane (Patterson et al., 2008). These microdomains are characterized by the presence of sphingolipids (SL), or glycerophospholipids (GL). Additionally, several studies have demonstrated the importance of DAG in Golgi function. The enzyme protein kinase D (PKD) is required for the scission of transport intermediates from the Golgi, and is recruited to the Golgi by locally produced DAG (Baron and Malhotra, 2002). Furthermore, a recent publication describes how the formation of tubules and vesicles at the Golgi requires DAG (Asp et al., 2008). All of these findings together suggest important roles for lipid organization within the Golgi membrane. Since munc13 is a C1 domain-containing protein with no catalytic activity, it is possible that munc13 at the Golgi membrane is involved in clustering DAG to specific sites. The interaction of rab34 and munc13 would then result in the local accumulation of rab34 at Golgi regions of high DAG concentration, which could be placing rab34 at its desired site of activity, or at a site where it will be brought into contact with other binding partners. Further work will seek to determine the validity of this model, and will examine the dynamics of rab34 and munc13 association.

TGF-β has emerged as a critically important player in the pathogenesis of DN. The hallmark fibrotic, hypertrophic renal injury of DN is largely accounted for by increased TGF-β expression and action in the diabetic kidney. Sustained production of TGF-β is known to result
in fibrosis, with the kidney being the most susceptible site (Border and Noble, 1994), and specific transgenic expression of TGF-β in the rat kidney has been shown to cause glomerulosclerosis (Isaka et al., 1993). Many of the mechanisms of TGF-β action during DN are known; TGF-β activity stimulates ECM accumulation via several routes – ECM protein production is increased in glomerular epithelia, MC, and renal fibroblasts (reviewed in (Isaka et al., 1993; Sakharova et al., 2001), production of ECM-degrading proteases is decreased (Wilson et al., 1993), and the production of ECM protease inhibitors is increased (Wilson et al., 1993). Additionally, hypertrophic changes in renal cell types can also be linked to increased TGF-β production; TGF-β influences cell proliferation and increases protein synthesis in several renal cell types, and treatment with antibody against TGF-β has been shown to reduce ECM production and renal hypertrophy in animal models (Sakharova et al., 2001).

Overproduction of TGF-β in diabetes is a result of several metabolic changes that occur in the course of the disease. Hyperglycemia itself has been shown to upregulate TGF-β expression via several pathways, including angiotensin II (AngII) production, and the formation of advanced glycation end-products (Rossner et al.) (Ziyadeh et al., 1994; Hoffman et al., 1998; Sakharova et al., 2001). Further, mechanical stress on the MC as a result of altered glomerular hemodynamics results in an increase in TGF-β production (Sakharova et al., 2001). Finally, proteinuria, which is itself a key factor in the progression of DN, causes an increase in NF-κB expression, which in turn increases production of TGF-β (Cohen et al., 2003).

Clinically, several drugs used in the treatment of DN have been shown to effect the expression of TGF-β. ACE inhibitor therapy is capable of reducing serum TGF-β levels in both animal models and human trials (Border and Noble, 1998; Sharma et al., 1999). It is worth noting that ACE inhibition alone cannot completely decrease TGF-β production to normal levels.
This points to the complex interplay between TGF-β signaling and the renin angiotensin system. Additionally, treatment with thiazolidinediones can also decrease TGF-β levels in animal models of diabetes (Ohtomo et al., 2007).

All of these factors clearly outline the pivotal role of TGF-β in DN. Future work will seek to identify the mechanism of glucose-mediated upregulation of munc13, and to evaluate the role of the munc13-rab34 pathway in animal models of diabetes. It would be an interesting result if munc13 expression were found to be TGF-β-responsive: this would set up a feedback loop whereby increased TGF-β secretion upregulates munc13, which then enhances the further secretion of TGF-β.
Chapter 4

4 Discussion And Future Directions

4.1 The subcellular localization of rab34

Our work to date has focused on two main areas: defining the cell biology of rab34, and elucidating the functional significance of the interaction between rab34 and munc13. Both of these paths of study have been carried out within the context of DN, and our goal of increasing our understanding of the pathogenesis of DN.

The role of rab34 was poorly defined at the outset of this project. Published reports placed rab34 at both the Golgi and at the plasma membrane, and suggested that rab34 was involved in the repositioning of lysosomes as well as plasma membrane ruffling and macropinocytosis (Wang and Hong, 2002; Sun et al., 2003). As a general rule, rab proteins have a specific subcellular localization, so these results were potentially at odds with one another. To clarify the existing literature, we chose the HeLa cell as an appropriate, generalizable, and simple model system in which to study rab34 function. In our hands, GFP-tagged rab34 was expressed at the Golgi, as seen in both fluorescence and cryoelectron microscopy. The distribution of rab34 within the Golgi was not immediately instructive as to its function – rab34 seemed to show no preference for specific subdomains of the Golgi stack, and it was also seen on transport intermediates in the close vicinity of the Golgi.

4.2 Potential roles for rab34 at the plasma membrane

To complete our localization studies, we re-investigated the hypothesis that rab34 was also found at plasma membrane ruffles. In live cells undergoing membrane ruffling, we used quantitative fluorescence microscopy to show that when normalized per unit area of membrane
rab34 is not significantly recruited to membrane ruffles. It is our supposition that previous data from Sun et al. (2003) may have represented an artifact of the morphological changes of a ruffling cell, or that they could have represented a cell-specific phenomenon. Interestingly, a recent report has again placed rab34 at the plasma membrane (Coyne et al., 2007). Rab34 was found to be expressed at tight junctions in polarized monolayers of intestinal Caco-2 cells, and expression of rab34 was found to be necessary for the entry of coxsackievirus into these cells via macropinocytosis (Coyne et al., 2007). This report also identifies Rab5 as a critically important rab in the process of occluding internalization (Coyne et al., 2007). It is tempting to speculate about fitting these data in with both ours and those of Sun et al. Again, it is possible that specific cell types are using rab34 for specific functions. Polarized cells are known to behave differently than non-polarized monolayers – non-polarized, non-confluent cells lack tight junctions altogether – and several molecular differences have been reported between polarized and non-polarized cells (Mostov et al., 2003). In fact, entire trafficking routes exist in polarized epithelia that do not in non-polarized systems – sorting between the apical and basolateral membrane domains, and transcytotic transport – suggesting the potential for different roles for the trafficking machinery in these two systems (Mostov et al., 2000). Future work should focus on defining the role of rab34 in both types of systems, and analyzing the physiological relevance of the data collected to various mammalian systems. For instance, one could envision a scenario whereby rab34 had specialized functions in a polarized epithelium or neuron that did not exist in non-polarized cells like the renal mesangial cell or fibroblast lineages.

4.3 The role of rab34 in lysosomal position and function

Once we had defined rab34 as a Golgi-resident protein in HeLa cells, we sought to determine the function of rab34 within the cell biology of the Golgi stack. To start, we revisited
the finding that rab34 was involved in the interorganellar relocalization of lysosomes toward the Golgi (Wang and Hong, 2002). These data were reproduced in our HeLa cell system. Two interesting questions arise from these findings – What is the precise molecular mechanism of this phenotype, and what is the functional significance of this finding? The answer to the first question is not clear. It is known that rab34 binds RILP, and that RILP binds to both rab7 on the lysosomal membrane and to microtubule motors within the dynein/dynactin system (Wang and Hong, 2002). It is also clear from the work of Wang and Hong that rab34 is exerting its influence on lysosomes without leaving the Golgi membrane. So how does this occur? One possible model is that RILP binds rab7, and begins to move lysosomes toward the minus end of microtubules via dynein motors. Once adjacent to the Golgi, RILP can then bind rab34, resulting in a tripartite complex between rab34, RILP, and rab7. Shifting the equilibrium of active rab34 – as was achieved by expressing constitutively active rab34 – provides more binding sites for RILP, as well as the capacity for a more stable rab34-RILP interaction, resulting in a mass action effect of shifting lysosomes toward the Golgi. The drawbacks of this model are that such a tripartite complex would certainly be beyond the resolution of light microscopy, so if this were the case, lysosomes (or at least RILP) should be seen to colocalize with rab34 at the Golgi, not to simply appose rab34.

To address the second question – what is the functional relevance of lysosomal repositioning – further work is required. Wang and Hong have shown that the repositioned lysosomes retain normal functionality (Wang and Hong, 2002). Additionally, caution must always be used when interpreting the results gathered using overexpression of a constitutively active protein. In this case, high levels of active rab34 were introduced to the cells, providing many ectopic binding sites for endogenous RILP. From these experiments alone, one cannot rule out the possibility that the observed phenotype is an artifact induced by forced expression of
active rab34. With this caveat in mind, it is still possible to speculate about a potential physiological role for the rab34 effect on lysosomal position. Transport between the Golgi and late endosome/lysosome follows a well-studied pathway. The M6PR transports sugar-containing acid hydrolases directly from the trans-Golgi to the developing lysosome, and then is recycled back to the trans-Golgi after delivering its cargo (Bonifacino and Rojas, 2006). The positioning of lysosomes near the Golgi could have an effect on the dynamics of this transport pathway. Our preliminary examination of this pathway revealed no gross changes in M6PR dynamics as a result of active or dominant-negative rab34 expression, but careful dissection of the pathway may reveal a more subtle involvement of rab34 in this system (Goldenberg et al., 2007).

A recent publication has revealed a correlation between mycobacterial infection and rab34 expression (Gutierrez et al., 2008). In this study, infection of a mouse macrophage cell line with Mycobacterium smegmatis resulted in transient upregulation of rab34 expression at both the mRNA and protein levels, and this upregulation was dependent upon NF-κB (Gutierrez et al., 2008). Infection of cells with M. avium did not cause the same rab34 changes, and inhibition of NF-κB signaling reduced lysosomal killing of M. smegmatis in infected cells (Gutierrez et al., 2008). M. smegmatis is non-pathogenic, and is routinely killed in the lysosomes of infected cells, while M. avium is not. These results suggest that rab34 upregulation by NF-κB – along with a host of other gene expression changes documented in this study – may be critically important for the killing of intracellular pathogens. If so, this would tend to support the model whereby the relocalization of lysosomes by rab34 is either functionally important for proper or enhanced lysosomal killing, or that maybe this phenotype is observed secondary to some other functional change due to rab34 activation, such as increased transport of lysosomal enzymes to the late endosome. Follow-up work from this study and the initial finding by Wang
and Hong could yield interesting and important information on the role of rab34 in lysosomal structure and function.

4.4 The role of rab34 in constitutive secretion

After establishing the localization of rab34 in our system, and re-evaluating the existing literature, we focused on determining the function of rab34 within the context of the Golgi itself. Our results have shown that rab34 is required for the secretion of a model protein, VSVG-GFP. Using dominant-negative rab34, siRNA-mediated knockdown of endogenous rab34, and rescue of the knockdown phenotype with rab34, we have established rab34 as a Golgi-resident member of the secretory pathway. Further mechanistic dissection of this phenotype revealed that rab34 works within the Golgi stack itself, and not in either the TGN, or the ER or ERGIC compartments, as evidenced by experiments using BFA and endoH resistance assays. Several lines of questioning stem from these results, many of which seek to further define the molecular mechanisms of rab34 function, and to place rab34 into the overall context of the secretory pathway and Golgi dynamics as a whole.

As described in Chapter 2, many key players in intra-Golgi transport and Golgi exit have already been defined. Critical roles have been assigned to PKD, PI4 kinase, and rab6a, as well as lipid signals from sphingolipids, DAG, and PI4P (Rosenwald et al., 1992; Echard et al., 2000; Liljedahl et al., 2001; Baron and Malhotra, 2002; Hausser A, 2005). Further, COPI coats are required for vesicle budding during intra-Golgi transport, and clathrin coat-mediated budding controls exit from the TGN (reviewed in (Bonifacino and Glick, 2004)). Like clathrin coats, COPI coats are multi-subunit complexes, with tight coordination with respect to assembly and disassembly, as well as lumenal domains involved in cargo selection (Bonifacino and Glick, 2004). The precise mechanisms governing both cargo selection and the determination of vesicle
budding sites are under intense investigation. The precise point at which rab34 fits into this puzzle is difficult to say using our current data. It is possible to speculate that it could play a role in coat protein recruitment, or that it could be involved in regulating the activity of Arf proteins, which are the activators of COPI coat assembly (Bannykh et al., 2005). Defining further protein-protein interactions for rab34, as well as additional studies looking specifically at cargo movement through the Golgi in the face of rab34 manipulation will be required to further define the role of rab34 in this process.

The Golgi stack is a site of massive transport movement and enzymatic flux. Lipid content turns over at a high rate, and vesicle traffic is constantly traversing the stack in both directions. Adding to the complexity of Golgi processes is the need to define molecules that are traversing the Golgi versus molecules that are Golgi resident. For many years, two competing models have dominated the debate surrounding intra-Golgi transport – cisternal maturation and vesicle transport (Pelham, 2001). The vesicle transport theory states that both anterograde and retrograde movement of cargoes through the Golgi occur by sequential budding and fusion of vesicles to the cisternae of the Golgi stack (Pelham, 2001). Cisternal maturation postulates that cargoes traversing the Golgi remain in the cisternae, and that each compartment matures over time from cis to trans – therefore, a picture of the Golgi stack represents a snapshot of transport through the organelle over time, with the cis-face containing newly synthesized cargo, and the trans-face containing older molecules awaiting export from the Golgi (Patterson et al., 2008). In this model, COPI-mediated vesicle transport serves to recycle Golgi-resident proteins to their preferred site, and does not participate in anterograde traffic. Currently, the cisternal maturation model has been the model of choice, largely due to both kinetic analysis and data suggesting that large cargoes like procollagen – which are too large to fit in transport vesicles – still traverse the Golgi at a predictable rate without ever leaving the cisternae (Bonfanti et al., 1998). More recent
work has used fluorescence microscopy to examine transport of VSVG-GFP, and a short pulse of VSVG-GFP is shown to move through the Golgi without ever leaving its cisterna – that is, only one cisterna contains VSVG-GFP at any given time (Mironov et al., 2001). While these studies do not rule out the use of vesicles for anterograde transport of Golgi cargoes, they certainly suggest that vesicular transport is not absolutely required.

Even more recently, the group of Jennifer Lippincott-Schwartz has put forward a new model for transport through the Golgi and retention of Golgi-resident proteins. This model involves rapid partitioning of proteins between a two-phase membrane system (Patterson et al., 2008). Using VSVG-GFP as a model, electron microscopy, inverse fluorescence recovery after photobleaching (iFRAP), and computer modeling were employed to observe transport kinetics within the Golgi in real time. In brief, the model describes two lipid domains within each of the seven Golgi cisternae: one is enriched in sphingolipids (SL) and contains proteins destined for export, and the other is enriched in glycerophospholipids (GPL) containing resident Golgi enzymes (Patterson et al., 2008). Both types of protein traverse both microdomains, but prefer one to the other. From cis to trans, the ratio of SL to GPL increases, suggesting that more exported proteins will eventually arrive at later cisternae. Additionally, resident proteins with different SL/GPL affinities will preferentially segregate to a given cisterna (Patterson et al., 2008). This model satisfies all observations of Golgi dynamics, and also unifies some other principles from the literature. For instance, protein effectors of various Golgi lipids, such as PI4P and PKD, could be recruited to various domains as a result of SL/GPL ratios (Patterson et al., 2008).

How does this all relate to rab34? First, while our EM studies showed no clear subcompartmental organization of rab34 in the Golgi, it is possible that rab34 preferentially
partitions to SL or GPL enriched areas – due to its role in transport through the Golgi, one would assume a preference for SL. Research on other rab proteins has shown a feedback loop involved in rab partitioning to specific membrane domains. Specifically, rab5 has been shown to segregate into a domain enriched for various protein and lipid components involved in its proper function (Zerial and McBride, 2001). As an example, active rab5 recruits PI3-Kinase, resulting in local production of PI3P. The combination of active rab5 and PI3P recruits the rab5 effector, EEA1 via its interaction with rab5 and with PI3P via its FYVE domain (Zerial and McBride, 2001). Several loops like this exist, resulting in the establishment of a functional rab5 domain. It is possible that a similar feedback system exists with rab34, whereby recruitment of rab34 to a specific lipid-enriched domain of the Golgi allows rab34 to enter an environment that is similarly enriched for its effectors and other partners. Interestingly, rab34 has been shown in our lab to bind the enzyme serine palmitoyltransferase (SPT) in a bacterial two-hybrid system (unpublished data). SPT catalyzes the rate-limiting step of de novo ceramide synthesis, and is a part of the sphingolipid synthesis pathway at the ER and Golgi (Hanada, 2003). Preliminary work carried out in our lab suggests that munc13 can increase the level of intracellular ceramide through SPT, and that this effect is dependent on the munc13 C1 domain, and the munc13 interaction with rab34 (Figure 17). It is tempting to speculate that a system could exist whereby recruitment of rab34 could lead to the recruitment of munc13 along with SPT, leading to an increase in local SL production, contributing to the SL/GPL gradients reported by Patterson et al. (2008).
Figure 18. Munc13 transfection increases ceramide levels via rab34 and SPT. HEK cells were transfected with either full length (hmunc13) or MHD2-less munc13, and treated as indicated with vehicle (DMSO), PDBu, or myriocin (a specific inhibitor of SPT). Ceramide levels were assayed, and normalized against total cellular protein. Note that PDBu treatment of munc13-expressing cells increases ceramide levels, and that the increase can be blocked by SPT inhibition. MHD2-less munc13 has no effect on ceramide levels. Data are means of n=2 experiments. This work was completed by Pam Speight.
4.5 The role of munc13 in constitutive secretion

Work in Chapter 3 defined a novel role for munc13 in constitutive secretion. Work in both HeLa cells and cultured rat MC showed that munc13 overexpression increased the rate of VSVG-GFP transport to the plasma membrane, and that this effect required the MHD2 domain of munc13. To date, the only known binding partner of the munc13 MHD2 domain is rab34, suggesting that the munc13-rab34 interaction is critical for inducing this phenotype. Additionally, relocalization of munc13 to the plasma membrane abrogates this effect, showing that the ability of munc13 to interact with rab34 at the Golgi may be required for its influence on secretion kinetics. The precise molecular mechanism of this phenomenon remains to be determined, but one can formulate a model based on current data. As outlined in the previous section, an interesting potential scenario exists whereby the interaction of munc13 and rab34 at the Golgi is involved in a feedback loop resulting in local production of sphingolipid species and the concomitant increase in secretion of proteins from the Golgi. Further work would be required to test this hypothesis, including detailed measurement of SL content in the Golgi in response to munc13 overexpression, and real-time imaging of protein movement through the Golgi to further define the site of action of munc13 on the secretory pathway. It is also possible that the effect of munc13 on secretion occurs through a previously undefined protein-protein or protein-lipid interaction. Munc13 is a large protein, with many potential interacting domains and no catalytic domain of its own. As an adaptor protein, munc13 could be scaffolding several molecules together to form a functional complex that is involved in protein trafficking. This proposed complex could be found using a proteomics approach and mass spectroscopy on proteins co-precipitating with munc13. As we learn more about secretion from the Golgi and about protein networks involving munc13, further mechanistic details of this pathway will be defined.
Based on the roles of rab34 and DAG in the secretory pathway, it is interesting to speculate on several potential models for the function of munc13 in the secretion at the Golgi. Clearly, DAG is a key molecule involved in the activation of C1 domain-containing proteins. It is known that vesicle budding mediated by PKD requires local DAG production at the Golgi in order for PKD to be recruited to the Golgi membrane (Baron and Malhotra, 2002). Additionally, the highest concentration of DAG in biological membranes is thought to be present in the Golgi itself (Gomez-Fernandez and Corbalan-Garcia, 2007). Finally, while DAG is required for vesicle budding via recruitment of PKD, DAG is also known to promote fusion of membranes, and as such DAG may also be involved in the fusion of transport carriers within the Golgi itself, or between the Golgi and the TGN (Gomez-Fernandez and Corbalan-Garcia, 2007). Within this context, we can attempt to place munc13 into the known pathways existing at the Golgi membrane. As stated previously, munc13 lacks any catalytic activity of its own; munc13 functions are therefore carried out via interaction with known binding partners at the Golgi, such as rab34 or SPT. One possibility is that recruitment of munc13 to the Golgi by DAG serves to concentrate DAG to specific membrane subdomains. This type of function for an adaptor protein has been described in other systems. For instance, the protein Annexin A2 has been shown to bind phosphatidylinositol 4,5-bisphosphate (PIP2) via its PH domain (Gerke and Moss, 2002). Interaction between the PH domain and PIP2 results in the clustering of PIP2 within biological membranes, creating localized regions of PIP2 enrichment that then influence various cellular functions, such as exocytosis via recruitment of other PIP2 binding proteins (Grishanin et al., 2004; Chasserot-Golaz et al., 2005). One intriguing possibility is that munc13 is recruited to the Golgi by DAG, and perhaps active rab34 as well, where it triggers the clustering of DAG to specific microdomains via its C1 domain. The clustering of DAG could then be involved in the recruitment of other proteins that are required for secretion, such as PKD, or DAG itself.
could then promote the fusion of membranes at sites of munc13-mediated DAG enrichment. This model would provide the mechanism by which munc13 influences secretion at the Golgi, and the interaction of active rab34 and munc13 would help explain our data showing modulation of secretion kinetics by munc13 overexpression in an MHD2 domain-dependent manner. In this model DAG and active rab34 recruit munc13 to the Golgi membrane, where clustering of DAG and potentially the recruitment of other DAG-binding proteins occurs. This cascade then results in an increase in membrane budding or fusion at specific sites. Experiments using *in vitro* membranes, or high-resolution fluorescence microscopy could be used to examine this hypothesis by searching for DAG clusters in the presence of munc13 or its C1 domain. When added to the Lippincott-Schwartz model of two-phase lipid partitioning at the Golgi membrane, it appears that sub-organellar clustering of specific lipids and lipid-binding proteins could be the driving force of trafficking through the Golgi stack.

### 4.6 Munc13 in diabetic nephropathy

DN is the most common indication for dialysis in the developed world (Clark *et al*., 2000; Remuzzi *et al*., 2002). With the overall number of patients with diabetes increasing at an alarming rate, the total burden of DN on the health care system will continue to expand. As such, developing effective treatment and screening tools for DN will be a critical challenge for medical research for years to come. Much is known about the molecular mechanism of the pathogenesis of DN – TGF-β, advanced glycation end-products (Rossner *et al*.), nitric oxide, the renin-angiotensin system, PKC activation, and dysregulated ECM homeostasis all play critical roles in the development and maintenance of diabetic renal injury (Friedman, 1999; Reeves and Andreoli, 2000; Whiteside and Dlugosz, 2002; Schena and Gesualdo, 2005; Noh and King, 2007; Cherney *et al*., 2008). Current treatment strategies are aimed at many of these mediators,
and further elucidation of the precise involvement of each of them will be required before a definitive treatment is identified.

Our lab has identified munc13 as a player in DN. Diabetic rat models, as well as cell culture models, indicate that munc13 is upregulated in response to hyperglycemia, and that this upregulation occurs along with the activation of munc13 by DAG and an increase in apoptosis in cells overexpressing munc13 (Song et al., 1998, 1999). Further work defined munc13 as an effector of rab34, and our most recent data suggested a role for munc13 in the regulation of protein secretion via the munc13-rab34 interaction. Most strikingly, rat MC cultured in hyperglycemic conditions upregulated endogenous munc13 expression, and the rate of protein secretion was increased in these cells. Forced downregulation of munc13 or rab34 using siRNA blocked this effect, showing that munc13 and rab34 were specifically required for this phenomenon. These data represent a first demonstration of the importance of munc13 and rab34 in a culture system of physiological importance in a significant medical condition. The precise role of this phenomenon in DN will be the subject of future investigations. However, our current data are consistent with a scenario whereby hyperglycemia induces munc13 expression, which occurs alongside increased TGF-β secretion. This results in increased expression of ECM molecules, which are now readily secreted into the extracellular space because of increased munc13 expression. If this scenario holds true, one could see that inhibition of munc13 could be of value in the treatment of DN. Furthermore, as stated above, rab34 expression is upregulated by NF-κB, which is also stimulated during diabetes. These factors could converge to upregulate both rab34 and munc13, leading to significant dysfunction of the secretory pathway.

During the time this work was being completed, a European group was independently completing a clinical study looking for SNPs that associated with the development of DN in
patients with type 1 diabetes (Tregouet et al., 2008). The only SNP found to have a significant association with the development of DN was found in the first intron in the \textit{UNC13B} gene locus. This SNP was associated with other SNPs found within regulatory regions in the locus, suggesting a potential association between munc13 expression and the development of DN. These data provide a potential link between our findings and the development of DN.

Increased secretion of various cellular products is a hallmark of diabetic nephropathy. As indicated above, secretion of fibronectin, collagen, TGF-\(\beta\), and other cytokines and ECM components is increased in the diabetic kidney. As such, targeting munc13 activation, or the munc13-rab34 interface could prove to be a significant therapeutic target in the treatment of DN. To test the efficacy of such a strategy, future directions will include testing \textit{in vivo} siRNA-mediated knockdown of munc13 in diabetic animal models, as well as functional analysis of the secretory pathway in MC isolated from munc13-2 knockout mice. Specifically, the development of a kidney-specific munc13-2 knockout animal would provide a powerful tool in the investigation of this hypothesis.

One line of current investigation in the treatment of DN is pharmacological inhibition of PKC-\(\beta\). Ruboxistaurin, a specific inhibitor of PKC-\(\beta\), is being investigated for the treatment of both diabetic nephropathy and retinopathy (Tuttle \textit{et al}., 2005; Tuttle \textit{et al}., 2007). In a one year, multi-centre pilot study, ruboxistaurin was shown to improve renal function in patients with DN that was unresponsive to ACE inhibitor therapy (Tuttle \textit{et al}., 2005). It is our hypothesis that current data regarding munc13 and DN may suggest that caution be taken in using PKC inhibition as a DN treatment. Our data show that munc13 is upregulated in the diabetic kidney, and that this upregulation comes along with physiological consequences on the secretory pathway and apoptosis. Munc13 and PKC isoforms are members of the PKC superfamily of C1
domain-containing proteins. Munc13 isoforms have been shown to bind phorbol esters and DAG with similar affinities to PKC isoforms, making them alternate effectors of DAG signaling (Ahmed et al., 1992). Furthermore, it is known that prolonged hyperglycemia increases intracellular DAG via sugar flux through the polyol pathway (Whiteside and Dlugosz, 2002).

What will the consequences be of munc13 activation in the face of PKC inhibition? It is clear that diabetes creates a highly favourable environment for munc13 activation – both DAG content and munc13 levels are increased – and that this would normally be joined by PKC activation. When PKC-β activation is specifically blocked, what is the effect of munc13 activation in this new context? One could envision a scenario whereby PKC and munc13 pathways act in opposition of one another – PKC signals for cell survival, and munc13 signals for apoptosis, among other phenomena (Song et al., 1999). Additionally, PKC and munc13 signaling are both important in normal brain function and neurotransmitter vesicle priming (Brose and Rosenmund, 2002). It is entirely possible that PKC inhibition will have significant off-target effects on neurotransmission in patients due to unopposed DAG signaling through munc13 in hippocampal neurons. Finally, the role of munc13 in insulin secretion has also been well documented (Sheu et al., 2003). It is also possible, therefore, that PKC inhibition will perturb munc13 signaling in the beta cell and lead to altered insulin secretion in patients taking ruboxistaurin. It is reasonable to suggest, therefore, that these types of off-target effects should be studied in depth prior to the large-scale adoption of PKC-β inhibition as a treatment for DN.
References


Appendices

Appendix A

Description of a PCR-based technique for DNA splicing and mutagenesis by producing 5' overhangs with run through stop DNA synthesis utilizing Ara-C

[Previously published as Ailenberg, M., Goldenberg, N.M., Silverman M. (2005). Description of a PCR-based technique for DNA splicing and mutagenesis by producing 5' overhangs with run through stop DNA synthesis utilizing Ara-C. BMC Biotechnology 5:23.]

My contribution to this paper included carrying out several of the experiments, as well as preparing several figures and the manuscript itself.

Abstract

Background: Splicing of DNA molecules is an important task in molecular biology that facilitates cloning, mutagenesis and creation of chimeric genes. Mutagenesis and DNA splicing techniques exist, some requiring restriction enzymes, and others utilize staggered reannealing approaches.

Results: A method for DNA splicing and mutagenesis without restriction enzymes is described. The method is based on mild template-dependent polymerization arrest with two molecules of cytosine arabinose (Ara-C) incorporated into PCR primers. Two rounds of PCR are employed: the first PCR produces 5' overhangs that are utilized for DNA splicing. The second PCR is based on polymerization running through the Ara-C molecules to produce the desired final product. To illustrate application of the run through stop mutagenesis and DNA splicing technique, we have carried out splicing of two segments of the human cofilin 1 gene and introduced a mutational deletion into the product.
**Conclusion:** We have demonstrated the utility of a new PCR-based method for carrying out DNA splicing and mutagenesis by incorporating Ara-C into the PCR primers.

**Background**

Splicing of DNA molecules is an important task in molecular biology that facilitates cloning, mutagenesis and the creation of chimeric genes. While the advent of restriction enzymes substantially advanced DNA splicing techniques, they cannot be applied universally, and their use is limited to enzyme-specific loci. Other techniques like site-directed mutagenesis by overlap extension [SOE; [1]], insertional mutagenesis with the megaprimer technique [2] and staggered reannealing [3,4] have further improved DNA mutagenesis and splicing. Each method offers advantages and inherent drawbacks. Another cloning approach involving the formation of 5' overhangs utilizes incorporation of nucleotide derivatives into PCR primers [5-7] that stall polymerization. These techniques are dependent on an established set of optimal conditions for strong polymerization arrest, including the correct choice of polymerase or the incorporation of three ribonucleotide derivatives in the primer [7]. Furthermore, chimeric DNA/RNA primers need to be removed and reverse-transcribed in order for the splicing to be completed. Although in the past we have successfully used the SOE technique for mutagenesis and splicing, we encountered difficulties while constructing larger genes. That led us to develop the staggered reannealing method [3,4]. This method proved to be useful as well, however, its efficiency declined as the gene to be mutagenized exceeded 1000 bp. Although these techniques allow splicing of any two DNA fragments without the need for restriction enzymes, their efficiency is inversely related to the length of the DNA fragments involved, since these techniques rely on the
successful melting and reannealing of DNA to create matching overhangs. We sought to offer an alternative approach to facilitate the splicing of any two DNA segments for mutagenesis and construction of chimeric genes. Our technique utilizes two rounds of PCR, and is based on moderate template-dependent polymerization arrest using cytosine arabinose (Ara-C). Ara-C is a nucleotide derivative (Fig A1) that is widely used in cancer therapeutics [8]. It is a competitive inhibitor of DNA polymerase and also affects polymerization initiation [9,10]. Ara-C exerts its therapeutic action on cellular DNA polymerase after phosphorylation by an endogenous kinase. Once phosphorylated, Ara-C facilitates inhibition of DNA replication in cancer cells. Sanger et al, in their search for polymerization terminating agents for use in sequencing techniques, found that while dideoxynucleotides were strong polymerase terminators, Ara-C only weakly halted polymerization [11]. Therefore, even today, dideoxynucleotides remain the terminators of choice in sequencing reactions. Previous studies have shown that while Ara-C could serve as a substrate for mammalian polymerases, it terminates polymerization by some prokaryotic polymerases [11]. Here we used Ara-C both as DNA polymerase inhibitor and template for DNA mutagenesis and splicing.
Figure A1: **Structure of cytidine and its derivatives.** The derivatives featured in this figure vary in their sugar substitutes. Note that in Cytosine Arabinose (Ara-C), the arabinose sugar contains hydroxyl groups in positions 3 and 5 in a similar orientation to native ribose, thus permitting reaction with other nucleotides in DNA synthesis. However, the hydroxyls in positions 2 and 3 are in the *trans* orientation. Comparing position 2 on the arabinose ring to that of 2-deoxyribose reveals that the hydrogen in 2-deoxyribose, that is in trans configuration to hydroxyl 3, is replaced by the
hydroxyl group found on arabinose. It should be emphasized that there are two types of polymerization arrest: a. Chain termination - the nucleotide is incorporated into the nascent DNA strand and synthesis is stalled because no new nucleotide is added. Dideoxy derivatives stall elongation after incorporation into the nascent DNA strand because they do not have hydroxyl in position 3. Arabinose nucleotides also belong to this group, but they offer only partial stalling [11]. b. Template-dependent termination - nucleotides already incorporated in the DNA (e.g. in primers) are able to stall polymerization when the polymerase reads the template. It is believed that due to stereo restraints, the polymerase falls off the template. The frequency of this event determines the efficiency of the stalling. Arabinose derivatives belong to this group. The property of template-dependent termination of Ara-C was utilized in this study to create 5' overhangs in the first PCR. However, since the template-dependent termination by Ara-C is moderate, it was utilized for the amplification in the second PCR.

Results and discussion

We were searching for a mild template-dependent polymerization terminator. The rationale for mild termination is as follows: Termination must be strong enough to create 5' overhangs in the first PCR reaction, but weak enough to allow the polymerase to continue through the modified nucleotid during the second round of PCR (Fig A2). For the reasons mentioned above, Ara-C was chosen for use in the present study. As proof of principal, a 20 bp deletion in the human coflin 1 gene was created. We tried to splice together two segments of the gene: one 5' (237 bp) and one 3' (309 bp) segment (Fig A2). Primers were designed with one or two Ara-C molecules replacing native deoxycytidine nucleotides. When two Ara-C molecules were incorporated into the primer (Hospital for Sick Children, Toronto, Canada), template-dependent termination can potentially occur before, after one, or after two Ara-C molecules. Therefore, to determine the termination location, we designed one side of the overhang to accommodate termination after two Ara-C molecules, and the other side of the overhang to accommodate termination after one Ara-C molecule (Fig A3). There were a total of 8 PCR reactions that included two Ara-C primers for each of the two segments, and the two polymerases (Taq and Pfu) for each set of primers. PCR products were gel-isolated. At this stage, gel-isolation is essential in order to remove any of the original plasmid that might serve as a template in the second PCR reaction. Alternatively, the original plasmid may be eliminated by digestion with DpnI, although this option is less recommended, since traces of undigested plasmid could affect the outcome of the
second PCR reaction. Corresponding segments to be spliced were combined (total of four tubes) and ligated. As indicated above, the rationale for this technique is that Ara-C is a mild polymerization terminator, and therefore it will produce a mixture of cohesive and blunt ends. Hence, the reaction is expected to both terminate (producing sticky ends essential for the splicing phase) and run through the Ara-C (producing blunt ends; this feature is essential to the second PCR reaction). Therefore, lowered concentration of ligase and reduced ligation time were used to optimize conditions to favor cohesive end ligation. The products of the ligase reaction were amplified by the second PCR with Taq or Pfu polymerases using the sense primer A, and the anti-sense primer B, which span the cofilin 1 gene. This PCR reaction produced the expected 552 bp product (blunt end ligation, is expected to produce an extra duplicated piece of DNA of 15 bp). The PCR products were either sequenced directly, or cloned into a plasmid and then sequenced. Based on sequencing results, we observed that incorporation of two Ara-C nucleotides into the PCR primers yielded the expected product. This suggests that the two molecules of Ara-C provided the desired mild termination to produce a product with 5' overhangs, but also allowed the polymerase to run through during the 2nd PCR. Furthermore, based on the design of the primers, the polymerization stalled both after the first and the second Ara-C molecule. Both 5 and 30 min incubations with DNA Ligase were sufficient to preferentially ligate the cohesive ends. This further suggests that two adjacent molecules of Ara-C produce 5' overhangs. Even though both 5 and 30 min ligations were successful in producing the desired product, it is not recommended to allow the reaction to proceed for a prolonged time, nor is it recommended to use high levels of ligase, since these conditions may facilitate blunt end ligation that may produce a mixture of the blunt and cohesive end products. Both Pfu and Taq polymerases were equally capable of producing termination products in the first PCR, while still running through the Ara-C in the second PCR. When one molecule of Ara-C was incorporated in
the PCR primers, no termination could be observed, as seen by the addition of a 15 bp segment in the PCR product indicative of blunt end ligation. Even ligation for 5 min in reduced concentration of ligase failed to produce cohesive end ligation when only one Ara-C was employed. The run through stop method utilizes a novel approach for DNA splicing and mutagenesis. While other mutagenesis techniques like SOE, megaprimer and staggered reannealing create matching overhangs after melting and reannealing, the run through stop method creates matching overhangs by polymerization arrest with Ara-C. We were motivated to design the Ara-C approach because we were not successful in creating gene mutations with the other techniques. Hence, the run through stop offers a good alternative to these techniques. It has been previously demonstrated that utilizing abasic or RNA nucleotides like tetrahydrofuran derivative or 2-o- methyl ribonucleotide in PCR primers produced 5' overhangs that facilitated cloning of DNA fragments into plasmids [5-7]. These approaches were dependent on strong polymerization termination by the nucleotides. Our technique established the conditions for mild termination of DNA polymerization with two Ara-C molecules. This enables us to use the Ara-C-containing primers in two steps of PCR for DNA splicing and mutagenesis. Although in the present study we used relatively short segments of DNA for proof of principal (~500 bp of the human cofilin gene), this technique, unlike the staggered reannealing technique, is not limited to short DNA fragments. Since both rounds of PCR in the present study are based on conventional PCR, the length limit of the DNA fragments to be mutagenized is that of the PCR technique.
Figure A2: **Schematic representation of run through stop DNA mutagenesis and splicing technique with Ara-C.** In this example two pieces of DNA are to be spliced (5’ and 3’ DNA segments) and mutated with an insertion of additional DNA. The 5’ segment is amplified using PCR primers A (sense) and Ara-C2-A (anti-sense). Primer Ara-C2-A is designed to contain hybrid DNA with two adjacent molecules of Ara-C to stall polymerisation and produce a 5’ overhang. Mutational addition is also incorporated into this primer. (Note that in this paper we created a mutational deletion in the human coflin 1 gene, but here for illustration purpose, we describe a mutational addition). The 3’ segment is amplified using PCR primers Ara-C2-B (sense) and B (anti-sense). Primer Ara-C2-B contains overlapping sequence with primer Ara-C2-A, and 2 molecules of Ara-C are incorporated to stall polymerization and produce a 5’ overhang that is complementary to the overhang in Ara-C2-A. Both Ara-C primers are phosphorylated for downstream ligation. Since two adjacent Ara-C molecules produce moderate termination, PCR products contain a mixture of 5’ overhang and blunt end DNA. Each PCR product is gel-isolated and subjected to short ligation, where cohesive end ligation is predominant. A portion of the ligation reaction is then subjected to a second PCR reaction, using
primers A and B that span the entire mutated chimeric DNA. As mentioned above, 2 Ara-C molecules are moderate polymerization terminators. This assures that at the first round of the second PCR, the polymerase will run through the Ara-C in the template and incorporate native dGMP, that will ensure in turn proper polymerization in the next rounds and a product that will contain the native dCMP. For cloning purposes of the final PCR product, primers A and B can include restriction sites (as used in this study). Alternatively, by using Taq polymerase in the second PCR reaction, the product can be cloned into TA cloning plasmids. Another alternative is to design primers A and B to contain at least 2 molecules of Ara-C to produce 5’ overhangs to match cloning plasmids.

Figure A3: **Ara-C primer assignment.** Shown is the double-stranded DNA segment of human cofilin 1 gene that was used for mutagenesis. Capital letters and arrows represent primers containing Ara-C molecules. Lower case letters represent deleted nucleotides, achieved with primer Ara-C2-A (broken line). Xs in primers denote Ara-C molecules that replace the original deoxycytidine molecules. Note that the 5’ end of primer Ara-C2-A was designed to produce an overhang, that restricts ligation to the 3’ segment of the PCR product (see also Fig 2) only if termination occurred after the first Ara-C molecule. The 5’ segment of primer Ara-C2-B was designed to produce an overhang that restricts ligation to the 5’ segment of the PCR product (see Fig 2) only if termination occurred after two Ara-C molecules. Additionally, two primers containing only one Ara-C molecule insertion were synthesized (not shown).
Conclusion

The run through stop method can be summarized in four steps: 1. Amplify two segments of DNA to be spliced using PCR, with phosphorylated primers containing two adjacent molecules of Arabinose nucleotide with overlapping sequence. 2. Gel-isolate the two DNA products, combine and ligate. 3. Amplify the spliced product with flanking primers using PCR. 4. Clone the product into a plasmid.

Methods

First PCR

For the first PCR, 4 primers were designed: Primers A and B flanking the human cofilin 1 gene (Fig 2) and two primers containing Ara-C molecules (Figs 2 and 3). Primer A- 5'-
ATActgcagATGGCCGCTGGTGTGGCTGTCTGTG-3' -sense primer of human cofilin 1. Lower case letters represent Pst I sequence and bold letters represent Ala to Ser mutagenesis for downstream usage. Primer Ara-C2-A-5'
GGCATAcgcgGAGGCAGTCXAAAGGTGGCGTAGGGATCG- 3'-anti-sense primer that contains two Ara-C molecules (XX) and designed to delete a 20 bp segment from the human cofilin 1 gene (Fig 3). Primer Ara-C2-B-5'- ACTGCCCAGTTGCTGCXCTCTGATGCAACCTATGAG- 3'-sense primer that contains two Ara-C molecules (Fig 3). Additionally, two primers containing only one Ara-C molecule insertion were synthesized. Primer B-5'
CAActgcagGGCTGCAGATGCTCCAGGCAGG-3'-anti-sense primer of the 3' end of human
cofilin 1 gene. Lower case letters represent the sequence for the Xho I gene. In the first PCR, Primer A was used with primer Ara-C2-A, and Primer Ara-C2-B was used with primer B. In the second PCR, primer A was used with primer B (see also Fig 2). Ara-C primers were phosphorylated for 30 min at room temperature using T4 polynucleotide kinase (Invitrogen, Burlington, ON), followed by inactivation at 65°C for 10 min, and used for PCR with no further purification. PCR was performed with corresponding primers (see above and Fig 2, 3) using 1 U Pfu polymerase (Stratagene, La Jolla, CA) or 1 U of Taq polymerase (Sigma, Oakville, ON), and plasmid pOTB7 containing the human cofilin 1 gene as template (ATCC, Manassas, VA). PCR conditions were as follows: heating to 94°C for 5 min; 40 cycles of: 94°C, 55°C and 72°C each for 30 seconds; final elongation for 7 min. PCR products were gel-isolated using Min-Elute Plasmid Purification Kits (Qiagen, Mississauga, ON). Corresponding segments to be spliced were combined (total of four tubes) and ligated for 30 min with 400 U, or five min with 200 U of T4 ligase (NEB, Pickering, ON) followed by inactivation for 10 min at 65°C.

**Second PCR**

Two µl of the ligase reaction were amplified by the second PCR with Taq or Pfu polymerase using the sense primer A, and the anti-sense primer B. Conditions for the second PCR were similar to those of the first PCR. The PCR products were purified (Qiagen). Alternatively, the PCR products were subjected to double digestion with PstI and Xhol followed by ligation into plasmid pcDNA3.1Zeo+ (Invitrogen). One µl of ligation reaction was used to transform 20 µl competent cells (DH5α; Invitrogen), using a short procedure: competent cells were incubated for 5 min on ice, and heat-shocked by immediate plating on pre-warmed (37°C) agar plates. Plasmids were prepared using Fast Plasmid Mini Kit (Eppendorf, Mississauga, ON), and sequenced using the T7 primer.
**DNA sequencing**

The products of PCR, as well as the products that were cloned into plasmid pcDNA3.1Zeo+ were sequenced in both directions, utilizing primers A and B, or primer T7, respectively (Hospital for Sick Children).

**Authors' contributions**

MA conceived and designed the study, performed the experiments and drafted the manuscript. NMG carried out some of the experiments, participated in critical evaluation and drafted the manuscript. MS provided general guidance, coordination and funding for the study, and drafted the manuscript.

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**References**


Appendix B

The Inositol Phosphatase MTMR4 Is A Novel Target of The Ubiquitin Ligase NEDD4

[Accepted manuscript, Biochemical Journal, Pamela J. Plant, Judy Correa, Neil Goldenberg, James Bain and Jane Batt]

My contribution to this paper was the microscopy work for Figure B2.

Abstract

The inositol phosphatase, MTMR4, was identified as a novel interactor of the ubiquitin ligase Nedd4. hMTMR4 and Nedd4 co-immunoprecipitated and co-localized to late endosomes. The PY-motif of hMTMR4 binds to WW domains of hNedd4. MTMR4 expression was decreased in atrophying muscle while Nedd4 expression was increased and hMTMR4 was ubiquitinated by hNedd4, suggesting this novel interaction may underlie the biological process of muscle breakdown.
Introduction:

Cellular ubiquitination is a vital process regulating protein stability, internalization and endosomal trafficking [1-3]. It is a multi-enzyme cascade whereby ubiquitin moieties are selectively transferred onto target molecules which, in many cases, tags them for degradation by the 26S proteosome. Specificity, in large part, is determined by the HECT and RING type ubiquitin ligases that interact with their substrates via specific protein-protein interaction domains. Nedd4 is a ubiquitously expressed HECT domain containing E3 ubiquitin-protein ligase that has been shown to regulate the stability and/or localization of several membrane proteins through interactions of its WW domain with substrate PY motifs [4-9]. Nedd4 has previously been shown to increase in models of skeletal muscle atrophy [10-12] and has been shown to target Notch1 in skeletal muscle [11], although the mechanisms by which Nedd4 contributes to the biological process of muscle breakdown are unknown. We sought to identify novel protein substrates of Nedd4 in muscle. Using a bioinformatics approach, we identified a PY motif-containing protein expressed in skeletal muscle, myotubularin related protein 4 (MTMR4), that belongs to the family of tyrosine/dual specificity phosphatases (PTP/DSP) [13]. Myotubularins are specific 3’-phosphatases for phosphoinositol 3-monophosphate (PI3P) and phosphatidylinositol 3,5-bisphosphate (PI3,5P2). It has been proposed that myotubularins regulate endosome trafficking by dephosphorylation of PI3P [14]. Interestingly, MTMR4 is the only family member possessing a PY motif [15] and having a unique distribution to endosomes [14], the major site of substrate lipid accumulation. In this study we found that MTMR4 and Nedd4 co-immunoprecipitate, interact via a WW domain-PY motif interaction and co-localize in
cells to the PI3P-rich late/recycling endosomes. Using a rat model of denervation atrophy we have confirmed the increase in Nedd4 expression in atrophying muscle (versus control muscle) is coincident with a decrease of MTMR4 in atrophying muscle. We demonstrate that Nedd4 ubiquitinates MTMR4 and mutation of the MTMR4 PY motif inhibits Nedd4 binding and MTMR4 ubiquitination, suggesting that MTMR4 is a *bona fide* Nedd4 target. We propose that MTMR4 may play an important role in the progression of Nedd4 mediated skeletal muscle atrophy.

**Materials and Methods**

*Cell Culture*

All cells were cultured at 37°C in 5% CO2. For biochemical characterization of the Nedd4/MTMR4 interaction, plasmids expressing wild-type (human) hNedd4 (accession number NM006154), catalytically inactive hNedd4 C-S and human MTMR4 (accession number NM004687), a gift from Dr. Joe Zhang (Vanderbilt-Ingram Cancer Center, Nashville, Tennessee), either untagged or HA-tagged, wild-type or PY motif mutant (whereby the second invariant proline of the XPPXY motif was mutated to alanine using Quik Change mutagenesis kit; Stratagene), were transfected into Phoenix 293 or HeLa cells grown in DMEM, 10% FBS, Pen/Strep using standard CaCl2 transfection. 48 hours post-transfection, cells were harvested and lysed in lysis buffer (150 mM NaCl, 50 mM HEPES, 1% Triton X-100, 10% glycerol, 1 mM MgCl2, 1 mM EGTA) vortexed for 10 sec. and incubated on ice for 10 min. Lysate was then cleared by centrifugation at 12,000 xg for 10 min. and the protein concentrations in the supernatant were quantitated using Pierce BCA protein assay.

*Immunoprecipitation and Western blotting*
For co-immunoprecipitation of MTMR4 and Nedd4, equal amounts of lysate from hMTMR4 or HA- hMTMR4 wild-type or PY mutant transfected or untransfected HeLa cells were incubated with 2µg anti-MTMR4 or anti-Nedd4 antibodies for 1 hour on ice. 50 ul of Protein-A sepharose beads (50% slurry) was then added to the lysate and incubated for 1 hr. at 40°C while spinning. Beads were then precipitated by centrifugation (10,000xg 1 min. 40C) and washed 2x in high salt HNTG (500 mM NaCl, 20 mM HEPES pH 7.5, 10% glycerol, 0.1% Triton X-100) and 3x in low salt HNTG (same with 150 mM NaCl). The beads were mixed with 45 µl 1x Sample Buffer and boiled at 95°C for 5 min to elute proteins. Protein eluate was resolved on 8% SDS-PAGE and transferred onto Protran 0.2 µm nitrocellulose (PerkinElmer, Boston, USA). Western blotting was performed using the following primary antibodies: polyclonal anti-Nedd4 WW2 antibodies (Upstate Biotechnology, Lake Placid, NY) at a 1:1000 dilution; polyclonal anti-MTMR4 antibodies (Abgent, San Diego, CA.), 1:200 dilution; monoclonal anti-α-tubulin antibodies (Sigma-Aldrich, St Louis, MO), 1:10,000 dilution; monoclonal anti-HA antibodies (Covance, Berkeley, CA), 1:1,000 dilution; monoclonal anti-ubiquitin antibodies (Covance), 1:1000 dilution and polyclonal anti-HPRT antibodies (Abcam, Cambridge, MA), 1:1000 dilution. Protein bands were detected with Horse Radish Peroxidase-linked goat anti-rabbit or anti-mouse secondary antibodies (Cell Signaling Technology, Inc., Beverly, MA.) used at a 1:20,000 dilution.

**Immunostaining**

HeLa cells grown in MEM were plated on coverslips. After 24 hours, cells were transfected as indicated using FuGene 6 reagent (Hoffmann-LaRoche Ltd, Mississauga, ON). After a further 24 hours, cells were fixed in 3.7% paraformaldehyde, permeabilized in 0.2% Triton X-100, and blocked in 10% milk in PBS. Primary antibodies were incubated for 1 hour at room temperature,
and then were washed in PBS. Secondary antibodies conjugated to fluorophores were added for 45 minutes, washed and coverslips were mounted on slides in Vectashield mounting medium (Vector Labs Canada, Burlington, ON). Images were acquired using a spinning-disc confocal microscope (Zeiss Canada Ltd, Toronto, ON) and Volocity software (Improvision, Perkin Elmer, Waltham, MA). Primary antibodies used were: mouse anti-GM130 (BD Biosciences, Mississauga, ON), mouse anti-LAMP-1 (Developmental Studies Hybridoma Bank, University of Iowa), mouse anti-Transferrin Receptor (Zymed, San Francisco, CA), and mouse anti-HA (Covance). Secondary antibodies used were anti-mouse-Cy3 and anti-mouse-Cy2 (Jackson ImmunoResearch, West Grove, PA). In addition to HA-tagged MTMR4, some cells were transfected with either GFP-tagged Rab5, GFP-tagged Rab7, PX-mCherry (generous gifts from Dr. Sergio Grinstein, Hospital for Sick Children, Toronto) and/or GFP-hNedd4. For quantitation of co-localization, images were analyzed with the Volocity software (Improvision) co-localization feature that calculates measurement statistics including the Manders correlation coefficient.

In vitro binding assays

Human Nedd4-1 WW domains in pQE-30 (Qiagen) were a gift from Daniela Rotin (Hospital for Sick Children, Toronto). The WW domains were cloned with the following boundaries: WW I 638-760 bp; WW II 1112-1231 bp; WW III 1318-1425 bp; WW IV 1487-1606 bp. 6x His-tagged proteins were produced in M15 [pREP4] bacteria and purified following manufacturers instructions (Qiagen). GST fusion proteins of hMTMR4 were produced by PCR of the PY-motif containing region (2965-3081 bp) and a proline-rich region (1972-2091 bp), TA cloning into pCR2.1-TOPO (Invitrogen), subsequent cloning into pGEX 5X1 and expression in BL21 (DE3) pLys S bacteria according to manufacturers protocol (Promega). GST fusion proteins were
bound on glutathione agarose beads (50% slurry) by incubating 1 hr at 40C. Proteins were eluted with 30 mM reduced glutathione by incubating 1 hr at 40C. Eluted GST proteins and bound 6x-His proteins were quantitated and equal amounts of GST-PY (PY motif) and GST-Pro (proline-rich region) hMTMR4 fusion proteins were incubated with equal amounts of 6x-His bound proteins for 2 hr at 40C. The beads and bound proteins were washed 3x with high salt HNTG (as above) and 2x with low salt HNTG. Beads were then mixed with 20 µl 1x sample buffer and boiled at 950C for 5 min. to elute proteins. Protein eluate was resolved on 15% SDS-PAGE and transferred onto Protran 0.2 um nitrocellulose and subjected to Western blotting with anti-Penta His (Qiagen) and anti-GST antibodies (Sigma).

Experimental denervation model and muscle preparation

The model of muscle breakdown/atrophy was the gastrocnemius muscle denervation model previously described [16, 17]. Briefly, the right tibial nerve of eighteen male Lewis rats (200g each) was transected under inhalational Halothane anaesthesia completely denervating the gastrocnemius muscle. The contralateral leg served as an internal control in each animal. Rats were maintained under conditions of routine care for 30 days after which they were sacrificed and the gastrocnemius muscles were harvested from the operated experimental limb and non-operated control limb. After rapid, atraumatic dissection, the muscle was snap frozen in liquid nitrogen. Half of the muscle was used for total RNA isolation and the other half was used to extract soluble and insoluble cellular protein. Total protein was extracted by homogenizing (Polytron PT 1200E, Kinematica, Lucerne, Switzerland) the muscle in Muscle Lysis Buffer (5 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM EGTA, 1mM β-mercaptoethanol, 1% glycerol, PMSF (1 mM), leupeptin, aprotinin (10 µg/ml each) for 3x 30sec. and homogenates were centrifuged at 1600xg for 10 min at 40C. The supernatant (soluble fraction) was centrifuged
further for 10 min at 40°C, 10,000xg. The pellet (insoluble fraction) was washed with phosphate buffered saline (PBS), resuspended, dissolved by sonication in 200 µl muscle lysis buffer containing 0.5% sodium dodecyl sulfate (SDS) and further centrifuged at 10,000xg at 40°C for 10 min. and the supernatant designated as the insoluble fraction. All fractions were quantitated using Pierce (Rockford, IL) BCA Protein Assay Kit and normalized for equal loading. 25 µg of the muscle lysate was separated on 8% SDS-PAGE and Western blotting was performed using anti-MTMR4, anti-Nedd4 and anti-HPRT antibodies (Abcam Inc.). The chemiluminescent signal was acquired using a CCD camera (BioRad Fluor-S Max) and the total signal quantified using Quantity One software (Biorad Laboratories, Hercules, CA) with volume analysis.

Statistical Analyses

Continuous data are reported as a mean and standard deviation and were compared using ANOVA, followed by Tukey post-analysis to compare multiple means if p < 0.05. Statistical significance was assumed if p < 0.05.

Results

Myotubularin related protein 4 (MTMR4) is a PY motif containing protein expressed in muscle that interacts with Nedd4. As Nedd4 over-expression was correlated with the progression of atrophic processes in muscle cells, we sought potential substrates in muscle that may be targeted by Nedd4 to elicit its effects. To this end, we took a bioinformatics approach to elucidate PY-motif containing proteins that were expressed in skeletal muscle. Nedd4 consists of an N terminal C2 domain, 3 or 4 WW domains and the ubiquitin ligase HECT domain and in many cases, engages in interactions with its substrates via a WW domain-PY motif interaction [4-9]. Searching a SIDNET MGC clone database (http://www.sickkids.ca/sidnet/) for PY motif containing proteins that were expressed in skeletal muscle limited potential substrates of Nedd4...
to several proteins, including myotubularin related protein 4 (MTMR4) which belongs to a family of lipid tyrosine phosphatases with specificity towards PI3P and PI(3,5)P2 [14] 15. MTMR4 possesses several protein-protein and protein-lipid interaction domains (Figure B1A) including the FYVE domain, shown to mediate its localization to endosomes through its interaction with PI3P and PI3,5P2 [14] and is the only family member possessing a PY motif. The MTMR4 PY-motif (VPPLY) at the C-terminus (positions 1000-1003) is conserved across mammalian species (data not shown), adhering to the canonical L/XPPXY consensus sequence for WW domain type I binding. To verify that Nedd4 and MTMR4 can interact, cDNA for human MTMR4 (hMTMR4) was transfected into 293 cells and lysate from these cells was subjected to immunoprecipitation with anti-MTMR4 antibodies. Immunoprecipitates were separated on SDS-PAGE and probed with anti-MTMR4 anti-Nedd4 antibodies to determine if the two proteins interacted and anti-tubulin as control. Immunoprecipitated transfected hMTMR4 interacted with endogenous Nedd4 but no band was detected in untransfected lysate (Figure B1B). Similarly, cell lysate transfected with HA-tagged hMTMR4 wild-type and PY-motif mutant was immunoprecipitated with anti-Nedd4 antibodies, and subjected to Western blotting with anti-Nedd4, -HA and –tubulin antibodies. Endogenous Nedd4 was able to immunoprecipitate transfected wild-type hMTMR4 but not the MTMR4 PY mutant suggesting that the endogenous association of the two proteins is likely mediated through the WW-domain PY-motif interaction (Figure B1C).
Figure B1: Myotubularin related protein 4 (MTMR4) is a PY motif containing protein expressed in muscle that interacts with Nedd4. (A) Schematic of human MTMR4, modified from [33]. Protein domains predicted with SMART (http://smart.embl-heidelberg.de/) and PFSCAN (http://hits.isb-sib.ch/cgi-bin/PFSCAN) include GRAM (Glucosyltransferase, Rab-like GTPase Activator of Myotubularins) domain, RID (Rac-Induced recruitment domain), PTP/DSP (phosphotyrosine phosphatase/dual-specificity phosphatase) domain, SID (SET-interacting domain), PY motif (XPPXY), CC (coiled-coil) domain and FYVE (Fab1p, Y01B, Vac1p and EEA1) domain. (B) Western blot of hMTMR4, Nedd4 and tubulin from transfected (MTM tx) or untransfected (untx) 293 lysate immunoprecipitated with anti-MTMR4 antibodies (MTM I.P.). Equal amounts of whole lysate were run as control. (C) Lysates (lysate) and immunoprecipitates using anti-Nedd4 antibodies (Nedd4 I.P.) or anti-p70 S6 kinase antibodies (p70 S6 I.P.)
**Nedd4 and MTMR4 co-localize in cells:** Previous studies have found MTMR4 having unique distribution to endocytic compartments [14]. We examined co-localization of transfected hMTMR4 and hNedd4 in HeLa cells. In a representative image, the two proteins show a striking overlap with a punctate distribution (Figure B2A). Examination and counting of cells in 14 fields of view at 63x magnification showed that Nedd4 and MTMR4 co-localized in 72% of cells that were co-expressing both transfected proteins. Quantitative analysis of co-localization yielded high Manders correlation coefficients, indicative of significant overlap of the Nedd4 and MTMR4 signal \([r(20) = 0.93, p<0.05]\). To verify that these punctae are endosomal in nature, we examined the co-localization of hMTMR4 in HeLa cells with several markers of early, late and recycling endosomes as well as Golgi and lysosomal markers (Figure B2B). No co-localization was observed of hMTMR4 and the early endosomal marker Rab5 \([r(13) = 0.19]\), nor was there significant overlap in MTMR4 signal with the lysosomal marker LAMP \([r(9) = 0.16]\) or the Golgi marker GM130 \([r(8) = 0.09]\) (data not shown). There was significant overlap between MTMR4 and Rab7/Transferrin (Figure B2B) indicative of localization to late and recycling
endosomes, an impression that was validated quantitatively, $r(10) = 0.81$, $p<0.05$ and $r(13) = 0.78$, $p<0.05$, respectively. Since PI3P is a major lipid substrate of MTMR4, we co-transfected hMTMR4, GFP-hNedd4 and PXmCherry (the PX domain of NADPH oxidase binds to PI3P with high specificity [19]) to determine localization. hMTMR4 and hNedd4 co-localized specifically to areas of PX (PI3P) staining (Figure B2C) consistent with endosomal localization, $r(10) = 0.85$, $p<0.05$ and $r(8) = 0.91$, $p<0.05$, respectively.
Figure B2: MTMR4 and Nedd4 co-localize to late endosomes/recycling endosomes in living cells. (A) Immunofluorescence of HeLa cells transfected with HA-tagged hMTMR4 (Klein et al.) and GFP-hNedd4 (green). Overlay shows merging of the two images. (B) Co-localization of HA-hMTMR4 with markers of late endosomes (Rab7) and recycling endosomes (Transferrin Receptor). HA-hMTMR4 does not co-localize with markers of early endosomes (Rab5) (C). Triple labeling of HeLa cells transfected with HA-hMTMR4 (blue), GFP-Nedd4 (green) and PX-mCherry (Klein et al.) to represent PI3P localization. Arrowheads point to examples of co-localization. Mander's co-localization coefficients, r (degrees of freedom), are shown for each set of co-staining. Scale bars represent 11 µm.
**Nedd4 and MTMR4 interact via a WW domain-PY motif interaction:** To verify that the interaction between the two proteins was mediated by a WW domain-PY motif interaction, we expressed 6x His-tagged versions of the four WW domains of human Nedd4 and incubated equal amounts of the fusion proteins (on Ni2+ beads) with GST fusion proteins of regions of hMTMR4 encompassing the PY motif (GST-PY) and a proline rich region N-terminal to the PY motif (GST-Pro) as a control. We found that the GST-PY consistently bound to WW III and IV of hNedd4 (n=3) but GST-Pro did not bind to any of the 6x His-WW fusion proteins (Figure B3), verifying that the interaction between hMTMR4 and hNedd4 is mediated through a PY-WW domain interaction. This finding with MTMR4 is consistent with other substrates of Nedd4, as hNedd4 WW IV corresponds to the high affinity WW III in rat, which was found to bind to the PY-motif of a subunit of the epithelial sodium channel, β-ENaC[20]. Mutation or deletion of the ENaC PY motif obliterates Nedd4 binding, resulting in in Liddle’s Syndrome [20].
Figure B3: MTMR4 and Nedd4 binding is mediated through a PY-motif WW-domain interaction. GST fusion proteins of the area encompassing the PY motif of hMTMR4 (GST-hMTMR4 PY) and of a non-PY containing proline rich region of hMTMR4 (GST- hMTMR4 PRO) as control were generated in bacteria as described in Materials and Methods. Soluble 0.3 µg of GST-PY and GST-Pro were incubated with 0.5 ug His-tagged hNedd4-WWI, -WWII, -WWIII, -WWIV immobilized on Ni2+ beads (His-hNedd4 WW domain I-IV) or His beads alone (His). Following thorough washes of the beads, bound proteins were eluted with sample buffer, separated on SDS-PAGE and Western blotting was performed with anti-GST and anti-His antibodies.
Nedd4 protein is increased in muscle atrophy: As we, and others, have shown that Nedd4 expression increases upon denervation induced atrophy, we hypothesized that any potential targets of Nedd4-mediated ubiquitination (and subsequent degradation) would be decreased. To determine if MTMR4 protein levels are affected by denervation induced atrophy, we performed Western blot analysis of protein lysate from experimental (denervated atrophied muscle) and control (contralateral innervated rat muscle) with anti-MTMR4 antibodies. We found that the antibody recognized several bands of different size (data not shown); many were differentially expressed in experimental versus control limbs. 293 cell lysate, transfected with hMTMR4 cDNA was run alongside the control and experimental insoluble muscle lysates to confirm the identity of MTMR4 in these fractions. A faint band (approximately 160 kDa) was recognized by the anti-MTMR4 antibodies in the control lysate, corresponding to what has been reported in previous studies with hMTMR4 [15] (Figure B4A). The intensity of the band decreased in the experimental (denervated) lysate versus control, whereas Nedd4 expression increased in the denervated muscle lysates versus control. The expression of HPRT remained unchanged. Quantitation of the blots with CCD acquisition of the chemilluminescent signal shows that MTMR4 decreases approximately 90% with a 3-fold increase in Nedd4 expression (Figure B4B). These data suggest that decreased levels of MTMR4 protein correlate with increased levels of Nedd4 protein, supporting the notion of MTMR4 being a target of Nedd4-mediated degradation.
Figure B4: MTMR4 and Nedd4 are differentially regulated in atrophied muscle. (A) Representative Western blot of MTMR4, Nedd4 and HPRT (as loading control) from gastrocnemius muscle insoluble protein fractions from denervated (Den) and contralateral control (Con) limbs. MTM tx is positive control lysate from MTMR4 transfected or untransfected (untx) 293 cells. (B) Bar graph of quantitation of the chemiluminescent signal from Western blots performed using BioRad Fluor-S Max acquisition system and Quantity One software. Each bar represents 8 muscles (mean+SE). P-value < 0.05 denote statistically significant differences between mean values.
MTMR4 ubiquitination is dependent on an intact PY motif: MTMR4 co-precipitates with Nedd4 and has a differential expression pattern in muscle compared to Nedd4, so we hypothesized that it is a potential substrate for ubiquitination and degradation by Nedd4. Conjugation of ubiquitin moieties tags proteins for degradation by the proteosome, so we rationalized that if MTMR4 was a bona fide target for degradation by Nedd4, then Nedd4 would ubiquitinate MTMR4. Mutation of the MTMR4 PY motif prevents Nedd4 binding and thus would spare the mutant MTMR4 from ubiquitination and subsequent degradation. Cellular lysates from HeLa cells transfected with HA-tagged hMTMR4, HA-tagged PY mutant hMTMR4 (both transfected together with hNedd4) or untransfected lysate were subjected to immunoprecipitation with anti-MTMR4 antibodies (Figure B5). Immunoprecipitates were separated on SDS-PAGE and probed with anti-ubiquitin and anti-HA antibodies to detect the expression of the protein. Ubiquitinated MTMR4 (seen as a high molecular weight smear) was detected in immunoprecipitates only when wild-type MTMR4 was transfected, but not in immunoprecipitates from the PY mutant transfected or untransfected lysate (Fig. B5). These data suggest that the MTMR4 PY motif is necessary for Nedd4 interaction and subsequent ubiquitination.
Figure B5: MTMR4 ubiquitination is dependent on an intact PY motif. (A) HeLa cell lysate transfected with HA tagged-hMTMR4 or HA tagged-hMTMR4 PY motif mutant, together with hNedd4 or untransfected lysate (untx) were subjected to immunoprecipitation with anti-MTMR4 antibodies. Beads were washed and separated by SDS-PAGE with total lysate and then subjected to Western blotting with anti-ubiquitin antibodies to detect ubiquitinated MTMR4 and anti-HA antibodies to detect transfected hMTMR4. Aliquots of whole cell lysate were subjected to immunoblotting with anti-HA and anti-Nedd4 antibodies. Blots were stripped and re-probed with anti-α tubulin antibodies as a loading control. Ubiquitinated MTMR4 is seen as a high molecular weight smear (top left panel) in only the hMTMR4 transfected lysate.
Discussion

In this report, we describe a novel interaction between the ubiquitin ligase Nedd4 and the dual specificity phosphatase MTMR4, a regulator of phosphoinositide metabolism and thus, of endosomal function. While this interaction has not been previously identified, it is not unexpected, as previous studies have shown an association of Nedd4 with phosphoinositides and an involvement in endocytosis. The N-terminal C2 lipid-binding domain of Nedd4 has been shown to mediate Ca2+-dependent phospholipid binding and membrane association [21] in addition to mediating its own protein-protein interactions that serve to localize it to apical rafts [22]. The Nedd4 C2 domain displayed no preferences towards lipid composition in an in vitro binding assay but was shown to bind phosphoinositides, and this binding was partially augmented in the presence of Ca2+. In addition, the yeast Nedd4 homologue, Rsp5, has a well-defined role in endocytosis; the C2 domain of Rsp5 localizes it to the plasma membrane, where Rsp5 directly binds membrane phosphotidylinositolphosphates and directs ubiquitination of endosomal cargo [23-25]. Little is known of the regulation and physiologic role of MTMR4 but studies show a unique distribution to endosomal structures, the major site of substrate lipid (PI3P) accumulation [14]. In this report, we show MTMR4 co-localized with the late endosomal marker, Rab7 in addition to the Transferrin Receptor, a marker for recycling endosomes. In addition, MTMR4 and Nedd4 both localize to areas of PI3P staining, all suggesting that MTMR4 and Nedd4 are co-localized to late endosomes. Phosphatase inactive forms of MTMR4 or over expression of WT MTMR4 were shown to inhibit EGF receptor degradation [14]. This effect was abrogated if the MTMR4 FYVE-domain was mutated, and was recapitulated by the expression of the FYVE domain alone at very high levels, promoting the notion that this family member mediates endosomal trafficking by engaging in specific interactions between its FYVE
domain and endosomal phosphoinositides, thus affecting the balance of PI3P synthesis and degradation. Mutations in other MTM family members are associated with muscle dysfunction. This is not unexpected since muscle is the predominant location of MTM expression. In humans, MTM1 mutations are associated with a myotubular myopathy (XLMTM), which is a congenital myopathy associated with major hypotonia at birth [26, 27]. The histopathology of skeletal muscle reveals small rounded fibres with central nuclei. Mtm1 knockout mice recapitulate the histological signs of XLMTM and show a progressive myopathy starting at a few weeks after birth, while muscle histology appears normal at birth [26]. Adenoviral mediated replacement of MTM1 reverses the myopathy completely, restoring muscle mass and contractility [28]. This suggests that the defect in the muscle fibres is due to a defect in structural maintenance rather than an impairment of myogenesis, as was previously hypothesized. Furthermore, knockdown of C. elegans MTMR3, the closest relative of MTMR4 and the only other family member to contain a FYVE domain, results in severe impairment of body movement revealing a critical role for MTMR3 in maintaining muscle function [29]. In this current study, the finding that increased levels of Nedd4 in atrophied muscle correlate with a decrease in the levels of MTMR4, supports the notion of MTM family regulation of muscle structure and function.

The role of the Nedd4/MTMR4 interaction in the physiologic progression of muscle atrophy can be speculated upon. Phosphoinositides (PIs) are important membrane lipids that regulate cell growth and survival, cell division, and membrane trafficking through recruiting and activating effector proteins and enzymes that contain PI binding domains, to specific membrane micro domains [30]. The MTM substrate PI(3)P, which is highly enriched in early endosomes and internal vesicles of multi-vesicular endosomes late in the endosome pathway, plays a major role in endocytic trafficking [30, 31]. Depletion of PI(3)P results in inefficient sorting and delayed trafficking of a number of proteins (such as internalized growth factor receptors) through
the endocytic pathways to the late endosome and lysosome. A main function of the other MTM substrate, PI(3,5)P2 is to catalyze the budding of vesicles from the late endosome as yeast mutations in Fab1p/PIKfyve kinase, (the enzyme that phosphorylates PI(3)P to PI(3,5)P2) results in enlarged, poorly acidified vacuoles and late endosomes [32]. Thus, decreased levels of MTM expression, or alternatively MTM loss of function mutants, likely result in cellular dysfunction because of inadequate dephosphorylation of PI(3)P and PI(3,5)P2 and dys-regulation of the lipid signaling within the endocytic pathway. Experimental models examining the status of endosomal trafficking and receptor recycling in the absence of MTMR4 and /or mutants that are defective in its ubiquitination can address the contribution of Nedd4 to these processes and the participation of endosomal trafficking to the progression of muscle atrophy.

In conclusion, we have identified a novel Nedd4 target in muscle, the inositol phosphatase MTMR4, which further implicates a role for Nedd4 regulating endosomal signalling and distinguishes a candidate for future studies examining the physiological role of Nedd4 action in atrophying muscle.

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