WNK FAMILY KINASES AND THE REGULATION OF ION FLUX

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DEDICATION

Dedicated to Big Chick and his daughter Mary.

WNK FAMILY KINASES AND THE REGULATION OF ION FLUX

by

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The four mammalian examples of the with no K (lysine) or WNK family of serine/threonine protein kinases have an unusual arrangement of their catalytic lysines. WNK1 and WNK4 have been genetically linked to a rare type of hypertension. Studies demonstrate that these kinases regulate the activity of a diverse group of proteins that *in toto* suggest WNKs are involved in ionic homeostasis by means of constitutively regulated endocytosis of ion transporters and channels. The mechanism is reliant on an amino terminal fragment of WNK that does not include the kinase domain. How WNK1 exerts this effect, however, remains unclear. This dissertation will detail the examination of the amino terminal fragment of WNK1, its interaction with predicted molecular adaptor proteins, and how these interactions may modulate the activation of the serum- and glucocorticoid-inducible kinase 1, SGK1, leading to the internalization of the epithelial Na^+ channel, ENaC via the ubiquitin ligase neuronal precursor cell- expressed, developmentally downregulated protein 4-2, or Nedd4-2.

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PRIOR PUBLICATIONS

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CHAPTER I

Introduction

Perhaps the single most fundamental precept in nature is that of conservatism and evolutionary success of the structure of a protein can be inferred by its conservation. Of the more than 500 currently known members of the human kinome (http://kinase.com/kinbase/), all but four feature a strictly structurally conserved location for their catalytic lysine. The four comprise an aberrant family dubbed With No K (lysine) because in WNKs, that catalytic lysine has been shifted towards the amino terminus by 17 residues while a cysteine (C_{250}) occupies the site that is otherwise invariant amongst more than 99% of kinases.

If we accept for the moment that structure begets function, WNKs 1-4 must have an unique role to have not only been selected for but refined, ostensibly by gene duplication (owing to the greater than 85% identity between their kinase domains), four such oddities as these. Although the kinase domain is highly conserved within the WNK family, two observations about the remaining portions of the protein deserve discussion. First, other than the kinase domain, there are *no* conserved domains and considering that in WNK1 the kinase domain accounts for only ~ 15% of the protein, the question becomes why does such a massive (234 kDa) protein subsume such a small functional unit? In the case of WNK1, the answer may lie in the abundance of motifs implicated in protein interactions. Second, outside of the kinase domains homology and especially identity is very low. While the kinase domains of WNK family members may have similar functions, divergence of primary sequence outside of the kinase domain suggests that each isoform interacts with a discrete set of proteins.

The importance of WNKs is evinced by the fact that homozygous disruption of WNK1 is lethal by day 13 of gestation in mice (Zambrowicz et al., 2003). The same gene-trap study was used to show that heterozygous mice are hypotensive. The design of the gene trap, however, did not affect the expression of the first exon of WNK1 and the first exon does not include any of the kinase domain, a fact that will become a critical piece of support for latter discussion. As yet a *bona fide* substrate remains elusive. Data from our laboratory suggests that the Oxidative and Stress Responsive kinase, OSR1, may be a WNK target *in vivo* as it is highly phosphorylated by WNK1 *in vitro* (Anselmo et al., 2006).

Our laboratory was the first to identify, clone and characterize WNK1, the largest of the four vertebrate WNKs. Yeast two-hybrid studies using the first 555 residues of the protein identified two interacting proteins: the Serum- and Glucocorticoid-inducible Kinase (SGK1) and Munc18c. The former is a highly volatile protein that has been linked to the regulation of many ion channels and transporters (reviewed in Pearce 2003). The function of Munc18c remains enigmatic; however studies demonstrate that Munc18c binds to the synaptosomal associated *or* soluble N-ethylmaleimide-sensitive factor attachment factor receptor (SNARE) protein syntaxin 4a, and that when so bound, syntaxin remains in a closed conformation, unable to bind to synaptobrevin and thus preventing the docking of vesicles that would comprise the population known as the readily releasable pool.

Our work was aided by the positional cloning study that genetically linked WNKs 1 and 4 to a rare type of hypertension known by a panoply of titles such as Pseudohypoaldosteronism Type II, Familial Hyperkalemic Hypertension, or Gordon Syndrome (Wilson et al., 2001). PHAII, the term used most often, comprises the following

pathologies: hyperkalemia - high serum potassium; hypervolemia arising from salt retention and concomitant water retention; and tubular acidosis (Mansfield et al., 1997). Mutations linked to this disease occur in the first intron of WNK1, leading to its over-expression, and in the coding regions of WNK4, C terminal to the kinase domain, thought to lead to loss of function of the protein as a whole - i.e. no data exist to determine if kinase activity is impacted by the WNK4 mutations.

The mitogen-activated protein kinase (MAPK) network

Our laboratory identified WNK1 as a consequence of its relationship to MAPK cascades. Nested PCR screens looking for upstream activators of the MAPK ERK2 produced WNK1 1-555 (Xu et al., 2001).

Single-celled organisms require the ability to sense and respond to their environment. Prokaryotes have rudimentary signaling networks that allow for the transduction of information about such vital external matters as the presence of nutrients or toxins. For instance, *E. coli* have membrane proteins OmpF and OmpC that respond to the osmolarity of their environment. The former, larger protein is expressed more highly in low osmotic conditions, and that sensory input is transduced by the histidine kinase EnvZ to the transcriptional regulator OmpR (Stock et al., 1990).

The evolution of multicellular organisms necessitated a more sophisticated signaling network to respond to the micro environments encountered by specialized cells. The MAPK signaling network is one such cascade that has been the subject of intense interest due to its multiple roles in such functions as cell cycle arrest (Margadant et al., 2007), growth (Qiu et

al., 2006), stress response (Cadalbert et al., 2005), and survival (Friedman and Perrimon, 2006).

MAPKs are serine/threonine protein kinases with a conserved general structure. The amino-terminal domain is comprised mostly of β strands with two α helices, while the carboxy-terminal domain contains mostly α helices with 4 β strands (Wang et al., 1997). Substrate binds at the interface of these domains which can be occluded by a disordered stretch of amino acids referred to as the activation loop. Kinase activity is enhanced by phosphorylation of the activation loop, a process effected by upstream MAP2Ks. Substrate binding is preceded by binding of ATP, and the catalytic lysine (K_{52} in extracellular signal-related kinase 2/ERK2), which coordinates the transfer of the γ phosphoryl group from ATP to the substrate, is highly conserved.

The canonical MAPK pathway and its related pathways are represented in Figure 1. ERK2 is a MAPK which is doubly phosphorylated by the dual-specificity kinase MEK1/2 (MAP/ERK kinase) on a tyrosine and a threonine residue. MEKs are themselves activated by their upstream effectors the MAP3Ks or MEKKs. Once activated, MAPKs become localized to sites of action including the nucleus (Whitehurst et al., 2002).

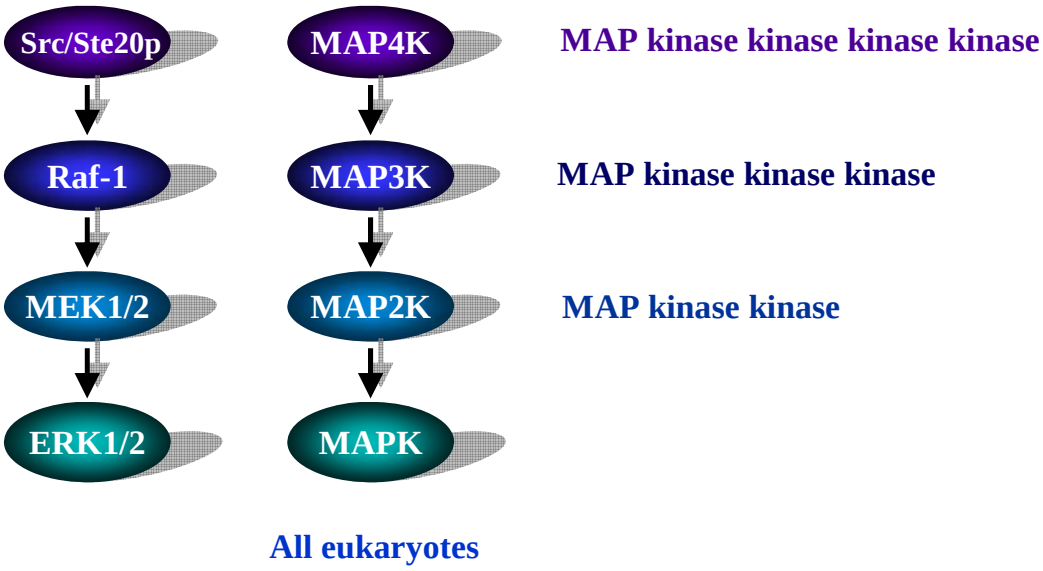

Figure 1. Scheme of the MAPK signaling pathway. The right-hand column denotes nomenclature of the general pathway and corresponding proteins of a typical MAPK cascade are in the left-hand column. This signaling network is present in all eukaryotic organisms.

WNK kinases

WNK1 was the first family member to be identified and cloned. In 1993, our laboratory began looking for upstream activators of ERK2 using a nested PCR strategy with primers for the kinase domain of MEK. A fragment containing the first 555 residues of WNK1 was one of the hits pulled from this screen.

Discovery and characterization

WNK1 is the largest member of four (Fig. 2) in vertebrates and is present only in metazoan organisms. *Drosophila melanogaster* and *Caenorhabditis elegans* have one homolog while *Arabidopsis thalania* has as many as nine.

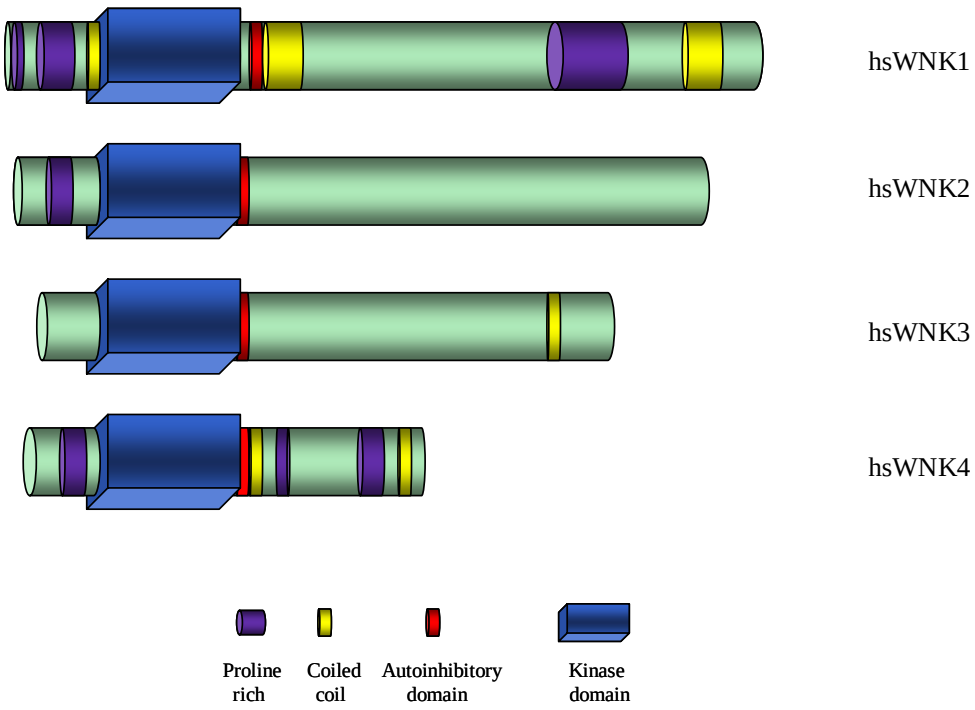

Figure 2. Representative drawings of the human WNK family members (to scale).

In mammals, the family can be broadly described as having an amino terminal kinase domain as their only identifiable functional structure. Identity between the kinase domains is high (>85%), as is common in MAPKs, but the striking difference is, again, the location of the lysine responsible for catalyzing the transfer of the γ phosphoryl group from ATP to the protein.

The structure of the kinase domain was solved (Min et al., 2004) which confirmed our findings that the catalytic lysine was K_{233} in subdomain I while a cysteine (C_{250}) resides in the position typical of all other known kinases in subdomain II. When this arrangement was mutated to recreate the typical structure, kinase activity was lost but, perhaps more

interestingly, when the atypical WNK structure was recreated in the canonical MAPK, ERK2, kinase activity was retained.

At this point, we can only speculate about the reason that such a unique arrangement can function irrespective of its host protein. Analysis of the human kinome (Manning et al., 2002) places the WNK family as a discrete branch between the sterile 20 (STE20) family and the tyrosine kinase like (TKL) family suggesting that WNK structure evolved from the typical kinase structure and not *vice versa*. STE20 is a yeast protein which would suggest that whatever the evolutionary pressure that selected this unusual catalytic arrangement, it occurred after and perhaps as a result of the evolution of multicellular organisms.

Beyond the kinase domain, there are no other identified conserved domains and we are left to examine motifs. WNK1 is rich in proline (> 10%) and there are 24 PxxP motifs, putative interactors with Src homology 3 (SH3) domains. Additionally, there are several coiled-coil domains which are thought to be important in oligomerization. Indeed gel filtration analysis demonstrates that WNK1 is a tetramer (Lenertz et al., 2005) but whether or not oligomerization is dependent on the coiled-coil regions has not been examined. Finally, two conserved phenylalanines, F_{524} and F_{526}, have been shown to act as key determinants in an autoinhibitory motif that inhibits the kinase function of WNK1, both in *cis* and in *trans* (Xu et al., 2002).

Examination of the expression of the message and the protein has only begun which should not be surprising, given that the first paper about WNK1 was published a scant six years ago. Nonetheless, the RNA message of WNK1 is nearly ubiquitous including tissues such as brain, kidney, muscle, and lung (Xu et al. 2000). Protein expression patterns are

similar for tissues as well as a variety of tissue culture cells (Lenertz et al., 2005). Expression patterns of the other three mammalian WNKs have not been thoroughly investigated. However, UNIGENE analysis of human expressed sequence tags (ESTs) suggests that while WNK1 is expressed in all tissues analyzed (save tonsil, skin and esophagus), the expression of WNK2 is less prevalent than WNK1, WNK3 is lesser still and WNK4 is expressed least of all (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene). Interestingly, all four genes are expressed in kidney, colon and brain, and WNKs 1, 2 and 4 are expressed in the lung. While such *in silico* data should be interpreted with caution, the suggestion that WNKs are generally expressed in absorptive epithelia is worth remembering.

Because WNK kinases are so large, it should not be surprising that splice variants arise. The WNK1 gene comprises 28 exons (Wilson 2001; O'reilly et al., 2003) and two distinct mRNA species have been identified in kidney (Xu et al., 2002). The first has already been discussed and is known as the long form (L WNK1) while the second occurs as a result of the inclusion of an alternate first exon 4a (Xu et al., 2002). This abbreviated species lacks the N terminal fragment preceding the kinase domain (1-220) as well as much of the kinase domain itself resulting in a shortened protein referred to as the kidney specific form (KS WNK1). Investigation suggests that KS WNK1 is the predominant message expressed in kidney (O'Reilly et al., 2003).

A consensus has not been reached as to the subcellular localization of WNK kinase proteins. Initial reports using immunofluorescence suggested that WNK1 was expressed in a diffuse cytosolic pattern while WNK4 colocalized with tight junctions in the kidney (Wilson et al., 2001). Recent work by a colleague, Lisa Lenertz, has demonstrated that WNK1

localizes predominantly to discrete puncta on structures resembling endosomes (personal communication). At this time, no data exist on the subcellular localization of WNK2 or WNK3.

Examination of the kinase function of WNK1 demonstrated that full functionality requires autophosphorylation (Xu et al., 2002). WNK1 has also been shown to be responsive to osmotic stress. When a variety of cell lines are exposed to hypertonic or hypotonic levels of such osmolytes as sorbitol, sucrose, NaCl and mannitol, WNK kinase activity increases substantially while growth factors, disruptors of the cytoskeleton, and hormones such as aldosterone and dexamethasone have little to no effect (Xu et al, 2000; Lenertz et al., 2005).

Genetics of WNK kinases

WNK1, WNK2, WNK3, and WNK4 are located on chromosomes 12, 9, X, and 17 respectively (Kahle et al., 2005). In accordance with our functional data, WNK1 and WNK4 were genetically linked to a type of hypertension known as pseudohypoaldosteronism type II (PHAII) (Wilson et al., 2003). A mutation in the first intron of WNK1 that leads to its over-expression and several mutations in the coding sequence of WNK4 in the coiled-coil region that lead to a putative loss of function were identified by positional cloning. PHAII, also known as Gordon syndrome or Familial Hyperkalemic Hypertension is characterized by sodium and potassium retention with concomitant hypervolemia and tubular acidosis.

Genetic analyses are accumulating to implicate the WNKs in the maintenance of ion balance. Comparison of an Israeli family, eight members with and eight members without a Q565E mutation in WNK4, showed affected family members were hypertensive and hyperkalemic and were 6-7 fold more responsive to thiazide diuretics (Mayan et al., 2002).

The importance of mutations in the acidic cluster of WNK4 is further bolstered by the discovery of another PHAII-inducing mutation, D564H (Golbang et al., 2005). Another analysis of a French pedigree compared 23 unaffected relatives to 17 affected subjects. Of those affected patients, 9 of 10 with large deletions in the first WNK1 intron were hypertensive and hyperkalemic (Achard et al., 2003). Moreover, the importance of single nucleotide polymorphisms in WNK1 was suggested as they relate to ambulatory blood pressure. In a White European sample of 996 individuals from 250 families, WNK1 polymorphisms (more than 100) were positively correlated to increased blood pressure with the highest being $\chi^2 = 3.39$, $p = 0.065$ (Tobin et al., 2005).

The physiology of hypertension

The kidney functions to filter the blood, removing waste products for excretion while maintaining the delicate isotonic balance necessary for proper nutrition and cellular volume. On a microstructural level, the kidney is composed of millions of functional units termed nephrons (Fig. 3). Blood is filtered across
a venous evagination known as the glomerulus. Solutes are absorbed into the lumen of the Bowman's capsule, which ensheathes the glomerulus. While these filtrates travel from the proximal to the distal portions of the nephron, highly active primary cells reclaim biologically useful solutes such as Na^+, K^+, Cl^-,
glucose and water, thus concentrating the waste products into urine, which then passes from the collecting duct into the ureter for collection in and elimination from the bladder. It is this process that allows the organism to adapt to drastic fluctuations in the availability of ions

necessary for life and derangement of this process has long been known to have profound impacts on the health of the organism.

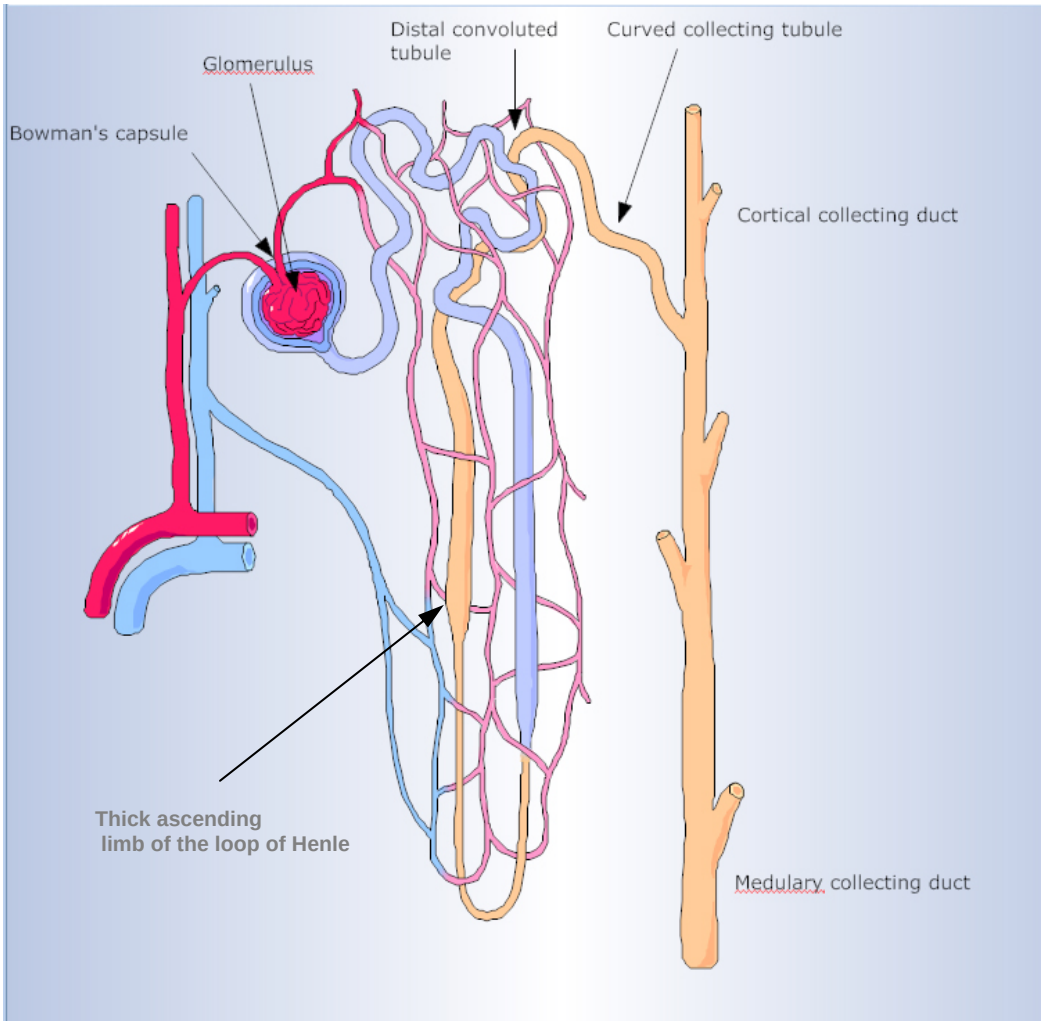

Figure 3. Structure of the nephron. Filtrates travel from proximal (Glomerulus) to distal (Medullary collecting duct) a path which also traverses from the cortex to the medulla. The aldosterone-sensitive distal nephron is comprised of the distal convoluted tubule through the medullary collecting duct. Figure adapted from SmartDraw™ free trial.

Although not unique to the kidney, the luminal face of the epithelia of the nephron is studded with membrane proteins that regulate the uptake of solvents and solute for reclamation (Fig. 4). The proximal portion of the nephron can be thought of as the site of gross regulation while the distal portion is responsible for fine tuning this process in response to the metabolic needs of the organism and/or the availability of these products in the diet. The distal portion, also known as the Aldosterone-sensitive distal nephron (ASDN) is comprised of the distal convoluted tubule, the curved collecting tubule and the medullary and cortical collecting ducts. Membrane proteins that populate the ASDN are the renal outer-medullary K^+ channel (ROMK), the epithelial Na^+ channel (ENaC), the sodium potassium chloride cotransporter 2 (NKCC2), and the sodium chloride cotransporter (NCCT). ROMK is found on the luminal side of the ASDN epithelia and is responsible for the uptake of K^+, which is then transported across the baso-lateral surface by the sodium potassium ATPase. ENaC is another apical ion channel that reclaims Na^+. NCCT is also an apical protein responsible for the transport of Na^+ and Cl^- and NKCC reclaims Na^+, K^+ and Cl^-.

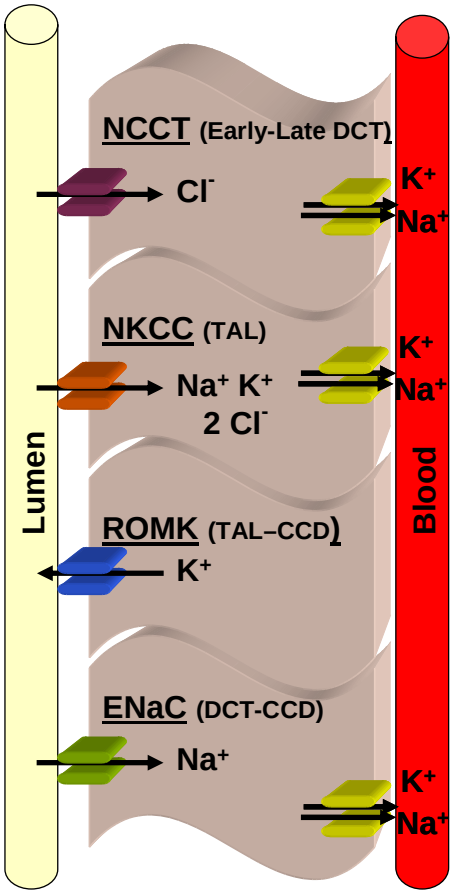

Figure 4. Activities of renal transporters and channels. Thick ascending Loop of Henle (TAL), Distal convoluted tubule (DCT), Cortical collecting duct (CCD).

It should be immediately apparent that retention of excess solutes requires a requisite retention of solvent, water, and that this increases the volume of the blood. Increased volume contained in the finite space of the venous system increases resistance and thus, increases blood pressure. However, this is only the most obvious explanation in an intricate system that subsumes genetics, hormonal regulation and diet.

More than 60 million people in the United States have high blood pressure as assessed by the clinical parameters of systolic pressures > 140 and/or diastolic pressures of > 90. Such elevated blood pressure has been well-documented in the etiology of diseases such as atherosclerosis, heart attack and stroke (Thom et al., 2006). Many studies have shown that ion channels and transporters that populate the apical and basolateral membranes of the epithelia of the nephron are often culprits in hypertension. For example, mutations in NCCT are linked to Gitelman's syndrome, a type of *hypo*tension that is the mirror image of PHAII – i.e. salt wasting, hypokalemia and tubular alkalosis.

Gitelman's patients are responsive to the diuretics of the thiazide class (benzothiadiazines), and the development of pharmacological therapeutics can provide insight about the mechanisms underlying the disregulation of kidney function. Broadly, diuretics increase urine volume by a variety of methods depending upon the targeted tissue. Thiazide decreases sodium uptake by inhibiting NCCT, also known as the thiazide-sensitive channel (TSC). Loop diuretics, such as bumetanide or furosemide, affect NKCC. Both thiazide and loop diuretics increase potassium excretion, but the latter can often produce hypercalciuria and excessively rapid reduction in blood volume. Hypercalciuria occurs as a result of the dependence of calcium transport upon that of sodium by means of an as yet

undefined sodium/calcium transporter/channel. In fact, it is still unclear whether renal calcium reclamation is active or passive, but calcium-transporting proteins such as transient receptor potential family members TRPV5 and TRPV6 are potential candidates (reviewed in Hoenderop et al., 2005).

Amiloride, at micromolar levels and below, inhibits ENaC (also known as the amiloride-sensitive channel), as does the aldosterone antagonist spironolactone, which acts by reducing ENaC synthesis. Both amiloride and spironolactone reduce potassium excretion. Administration of amiloride or spironolactone to most PHAII patients would be a poor choice in that they would exacerbate hyperkalemia, although there is potentially a sub-set of patients where spironolactone is efficacious (Xie, J. et al., 2006). Moreover, thiazide or loop diuretics do not affect ENaC, which has been shown to be the major regulator of sodium in the distal nephron (Schild 2004). Again, the complex intercalation of transporter and channel function complicates pharmacological therapy.

Proteins that interact with or are affected by WNK kinases

In the short time since the first paper on WNK was published, a number of interacting proteins have been identified. Among them are such proteins as ROMK, ENaC, the vesicular protein sorting defect protein 4a (VPS4a), synaptotagmin 2 (Syt2), NKCC2, NCCT, the serum- and glucocorticoid-inducible kinase 1 (SGK1) and others. Table 1 outlines the some of the ion transporters and channels that have been shown to be affected by WNK kinases. Presently, there are no data on WNK2 interacting proteins.

WNK1 and	**Interacting domain**	**Kinase activity required**	**Effect on channel activity**	**Reference**
ENaC	1-220 * KS	No No	Increase Increase	Xu et al., 2005a, b Fejes-Toth et al., 2004
ROMK	1-491 L 502-1100	Yes Yes No ($D_{368}A$)	Decrease Decrease Decrease	Lazrak et al., 2006 Wade et al., 2006 Cope et al., 2006
NCCT	1-555	Yes	Counters WNK4	Yang et al., 2005
NKCC1	1855-1960*	Possibly	Increases	Anselmo et al., 2006

WNK3 and	**Interacting domain**	**Kinase activity required**	**Effect on channel activity**	**Reference**
ROMK	Full length	No	Decrease	Leng et al., 2006
KCC	Full length	Yes	Increase	de los Heros et al., 2006
NCCT	Full length	Yes	Increase	Rinehart et al., 2005
NKCC1/2	Full length Full length	Yes	Increase	Rinehart et al., 2005 Kahle et al.,

WNK4 and	**Interacting domain**	**Kinase activity required**	**Effect on channel activity**	**Reference**
ROMK	Full length 400-1221	No No	None Decrease	Yamauchi et al., 2005
NCCT	1-620 Full length Last 222 Full length	Yes - No Yes	Decrease Decrease Decrease Decrease	Golbang et al., 2006 Cai et al., 2006 Yang et al., 2005 Wilson et al., 2003
NKCC1	Full length	-	Decreases	Kahle et al., 2004
CFEX	Full length	-	Decreases	Kahle et al., 2004

Table 1. Data on WNK effects on various transporters. Where data agree, only the most recent study is cited unless the data refine the model. Where data conflict, multiple studies are cited. Acronyms not defined in text: CFEX - Cl(-)/base exchanger; KCC – potassium chloride cotransporter; KS – kidney specific; L – long form; a "-" denotes not investigated; and Interacting Domains marked by an asterisk denote an indirect interaction.

Interactions between WNKs

It is becoming evident that WNKs do not only influence the function of other proteins. There is significant crosstalk within the WNK family. One such example was demonstrated by expressing WNK4 and NCCT in *Xenopus* oocytes. WNK4 co-expression lead to the reduction in plasma membrane residence of NCCT. However, when WNK1 was co-expressed with both WNK4 and NCCT, this effect was reversed (Yang et al., 2003). It was further shown that the effect of WNK1 was kinase-dependent while the effect of WNK4 was kinase-independent (Yang et al., 2005). In both WNK1 and WNK4, the effects were dependent upon more than the kinase domains alone.

Biochemically, recent data show that WNKs can be substrates of one another. To whit, WNK1 phosphorylates both WNK2 and WNK4 while WNK4 phosphorylates WNK1 (Lenertz et al., 2005). Thus, WNK-dependent effects must be considered in the context of inter- and intra-WNK regulation.

General hypothesis

In the years following the discovery of WNK1, many proteins that directly or indirectly manage ionic balance at the cellular and organismal level have been functionally tied to the WNK family. While many of the proteins are ion transporters or channels, an equal share of directly interacting proteins are tied to constitutively regulated endocytosis at either a mechanistic (Syt2, Munc18c, Vps4a) or a signaling level (SGK, OSR, and phosphatidylinositol-3-kinase (PI3K)). Given the convergence of these data, the general hypothesis underlying all three projects described in this dissertation is that WNK kinases are involved in the regulation of ionic homeostasis. Chapters 2 and 3 will describe my first and

second projects, respectively. The fourth chapter will describe my final project upon which my dissertation defense will be based.

CHAPTER II

Introduction

Ion flux across absorptive epithelia is regulated by an array of membrane proteins that form a pore restrictive to specific ions (*e.g.* ENaC) or that transport ions in an energy dependent fashion (*e.g.* Na^+ K^+ ATPase). Ion transport is further regulated by the open probability and the number of these proteins. Channels and transporters have been shown to be regulated by gating. There are obvious examples of gating by ligand (*e.g.* ionotropic glutamate receptors such as alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid or AMPA) and by voltage (*e.g.* the voltage gated potassium channel, Kv), but there are also intriguing examples of open-probability gating by the very transport machinery itself. For instance, ENaC Na^+-current in *Xenopus* oocytes has been shown to be reduced by the over-expression of Syntaxin 1A (Condliffe et al., 2003). Nonetheless, the preponderance of research has been focused on mechanisms that control the time that an ion transporter or channel spends residing on the plasma membrane as a means of controlling the activity of that protein.

Endocytosis

Constitutively regulated endocytosis is a paradigm that attempts to explain membrane protein activity as a function of time in plasma membrane residence. Cellular fractionation has been used to characterize the life-cycle of the dopamine transporter (DAT). This method has shown that DAT is present in transferrin-positive vesicles discrete from small, secretory vesicles and large dense-core vesicles in PC-12 cells (Melikian and Buckley, 1999; Loder and Melikan, 2003). Similar fractionation has revealed that γ-amino-butyric acid transporter

1 (GAT1) and Rab11 (a recycling endosome marker) co-localize in primary neurons (Deken et al., 2003). This means of regulation is not limited to neuronal tissue as the activity of aquaporin-2 is regulated by endocytosis and phosphorylation (Lu H et al., 2004).

The processes that deliver and retrieve plasma membrane proteins involve the fusion and fission of small vesicles. The archetypal model of vesicle docking and fusion is centered on a group of proteins known collectively as the SNARE complex. Target (T) SNARE proteins are so named because they reside on the destination membrane (e.g. syntaxin1A and SNAP25), while the vesicular (V) SNARE protein (e.g. synaptobrevin) is so named because it resides on the outer face of synaptic or secretory vesicles. Synaptobrevin complexes with the T SNARES and forms a heterotrimeric complex that is fusion competent. Vesicles so docked are referred to as the readily-releasable pool. The literature is unresolved as to how the fusion process is effected, however there is general consensus that this fusion process is calcium-dependent and that the calcium sensor is the protein synaptotagmin. Synaptotagmin is also implicated in endocytosis. It has been shown to bind to the clathrin-mediated endocytosis adaptor protein 2 (AP2) and expression of a synaptotagmin lacking the second C2 domain which binds AP2 severely disrupts clathrin-mediated endocytosis (von Poser et al., 2000).

There are at least 11 isoforms of synaptotagmin in mammals, and they demonstrate varied expression patterns as well as differing structural domains. The most widely characterized isoform is synaptotagmin 1, which is expressed mostly in the brain. However, ubiquitously expressed synaptotagmin 2 (Syt2) shares the highest homology with synaptotagmin 1 at 62%. All isoforms have a transmembrane domain and all but one isoform

contain at least one example of the structural domain known as C2, first discovered on protein kinase C and shown to bind phospholipids in a calcium-dependent fashion (Newton and Johnson, 1998). This domain is comprised of a sandwich of beta sheets joined by flexible linker regions (Fig. 5). At one end of the C2 domain, there are conserved

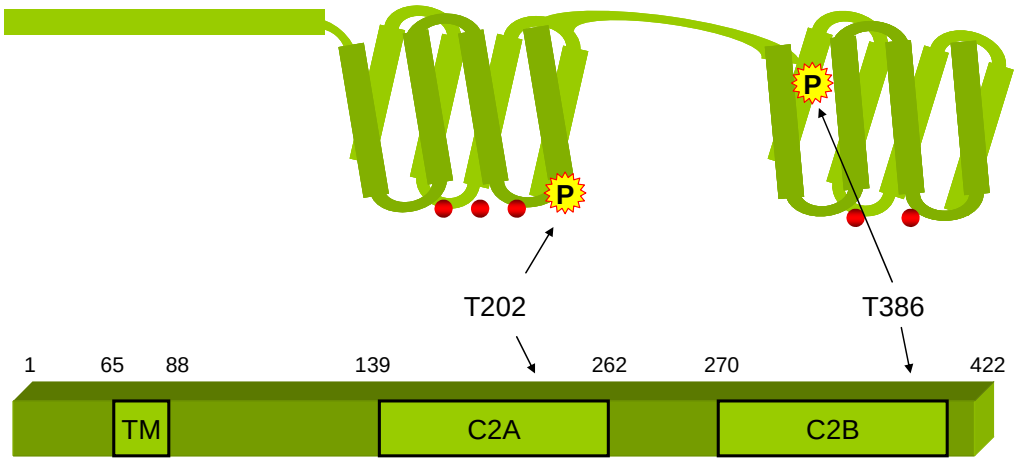

Figure 5. Synaptotagmin 2. Representative structure of Syt2 based upon homology to the solved structure of Syt1. The two C2 domains form β sandwiches and at one end they bind Ca^{2+} ions, here represented by red balls. P = rough location of phosphorylation sites predicted by Lee et al., 2004.

acidic residues that bind calcium atoms, 3 by the C2A and 2 by the C2B. One popular model suggests that these acidic residues can bind to the negatively-charged phospholipid-enriched plasma membrane by coordinating calcium atoms. Synaptotagmin is the most abundant protein on the surface of the readily-releasable pool of vesicles and there is wide agreement that it functions as the calcium switch, but the mechanism is unclear.

The mammalian homolog of *C. elegans* uncoordinated locomotion (Munc) family members are proteins with an enigmatic function implicated in the endocytic pathway. Sec1p (the yeast homologue of Munc18a) as well as Munc18b and Munc18c are referred to as SM (Sec/Munc) proteins and have been linked to the functionality of all known SNARE

complexes (Latham et al., 2006). Munc18c is expressed ubiquitously and is the sole Munc protein that binds to syntaxin 4 (Oh and Thurmond, 2006). The transmembrane segments of 5-8 syntaxins have been shown to line the fusion pores that immediately precede exocytosis (Han et al., 2004). It was once thought that Munc18a only bound to monomeric syntaxin prompting the theory that Munc18a was a negative regulator of vesicle fusion (Zhang et al., 2000). Insulin-dependent Glut4 upregulation was enhanced in Munc18c null mice (Kanda et al., 2005) and Munc18c null mice have reduced insulin secretion that can be overcome by overexpression of syntaxin 4 (Spurlin et al., 2003). However, recent data on Munc18c conflict with this model in that it binds preferentially to binary and ternary SNARE complexes seemingly facilitating complex formation (Latham et al., 2006).

Rationale

Following the identification of WNK1, the first yeast two-hybrid screen using WNK1 residues 1-555 (J. English) yielded Munc18c as an interacting protein. Another yeast two-hybrid screen using the WNK1 residues 1-491 (B.H. Lee) revealed an interaction with synaptotagmin 2.

Work in our laboratory by a colleague, Byung-Hoon Lee, demonstrated that WNK1 binds to synaptotagmin 2 and phosphorylates it on at least two sites, one in each of the C2 domains of Syt2 (Lee et al., 2004). Lee further demonstrated that WNK1 phosphorylation of Syt2 at residue T202 right-shifted the calcium-dependent affinity of synaptotagmin 2 for phospholipid vesicles. This suggested that the phosphorylation of Syt2 by WNK1 may be involved in the regulation of endocytosis or exocytosis of ion channels and transporters on the plasma membrane.

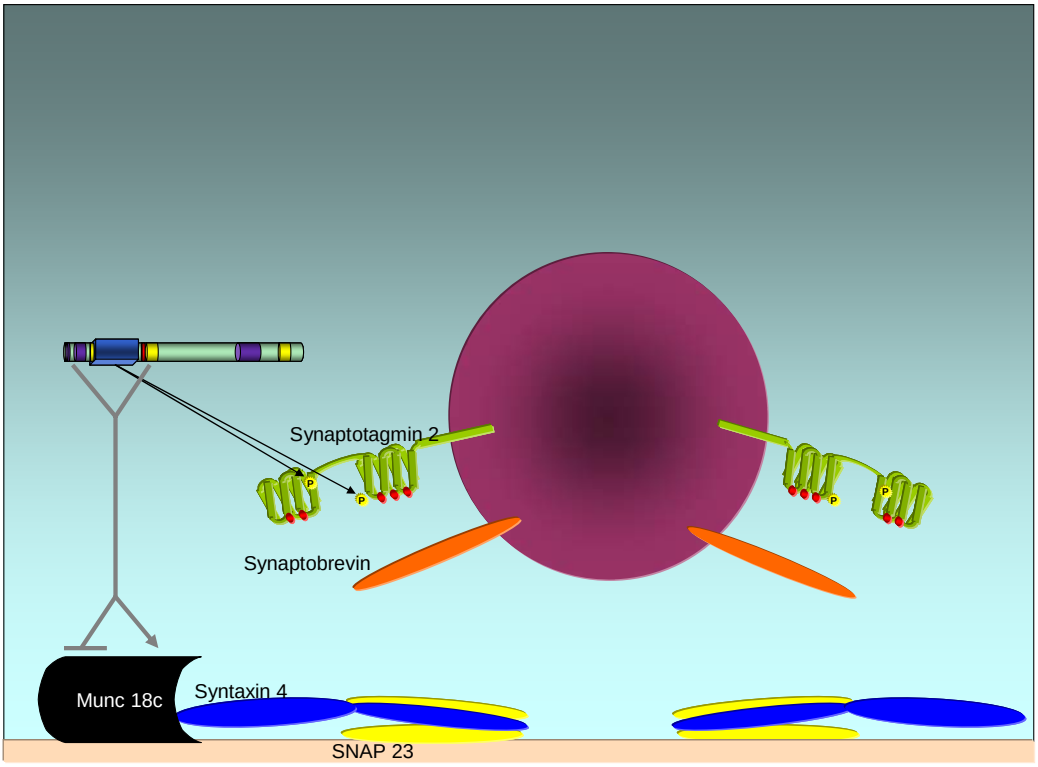

Figure 6. Proposed scheme involving WNK1 in the delivery of vesicles to the plasma membrane. WNK1 binds to and phosphorylates Syt2 and binds to Munc18c.

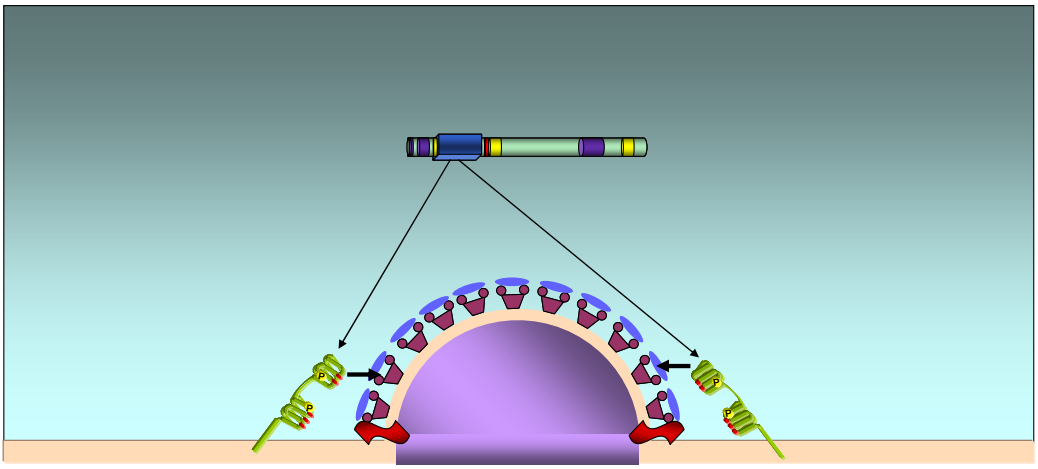

Figure 7. Proposed scheme involving WNK1 in the recovery of membrane proteins by endocytosis. WNK1 binds and phosphorylates Syt2. WNK1 phosphorylation of Syt2 could affect the Syt2/AP2 interaction. Dynamin and clathrin are also implicated.

Synaptotagmin has been shown to be vital to the efficacy of endocytosis as chromaffin cells isolated from Syt1 null mice lose the ability to secrete catecholamine (Nagy et al., 2006). One model suggests that endocytosis is dependent upon the C2B domain of Syt as the linker that binds to the μ2 subunit of AP2 (Grass et al., 2004). If this is true, WNK1-phosphorylated Syt2 might be either less likely to bind to phospholipids at the interior face of the plasma membrane or may bind less well to adaptin. Either or both of these possibilities would reduce the ability of cells to downregulate their surface ion channels or transporters by endocytosis. Therefore I hypothesized that WNK1-phosphorylated Syt2 would reduce endocytosis generally and transporter and channel endocytosis specifically. The effect of Munc18c would be more difficult to hypothesize, owing to the division of data between two conflicting theories. Nonetheless, two methods described presently would be employed to determine the function of Munc18c.

Methods

Protein purification. cDNAs of mouse Syt2 141-422 and 81-422 were subcloned into pRSET and Munc18c in pGEX was a gift from Csiro Labs. Plasmids were transformed into chemically competent BL21 E. coli bacteria via standard protocols. A single colony was grown in 1000 ml Luria-Bertani Broth (LB) at 37°C, shaking at 250 rpm until OD595 of 0.6-0.8 was reached. Culture was induced with 50μM isopropyl β-D-1-thiogalactopyranoside for 4 hours. Bacteria were centrifuged in a Beckman J6B with a TS4.2 rotor at 4°C and 2700 x g for 15 minutes.

The cell pellet was lysed in 30 ml of lysis buffer (50 mM Tris pH 8, 200 mM NaCl, 10 mM imidazole, 1% Triton X-100) and sonicated (40% amplitude, 1 second pulses, 3 cycles of

10 pulses each). The lysate was clarified by centrifugation (4 ° C /18,000 x g/ 30 minutes) and soluble proteins were batch bound to Ni^{2+} NTA resin (4 ° C/rocking/1 hour). Resin was transferred to a column,washed with 50 bed volumes of buffer (50 mM Tris pH 7.4, 200 mM NaCl, 10 mM imidazole), eluted with 10 ml of elution buffer (50 mM Tris, 200 mM NaCl, 300 mM imidazole, 1 mM dithiothreitol (DTT)) and dialyzed overnight in elution buffer without imidazole plus 10% glycerol. The GST-tagged protein was purified in essentially the same manner except for the omission of imidazole in all buffers and the addition of 5.0 mM reduced glutathione to the elution buffer. Cleavage of the tags was effected on the affinity column before elution by adding thrombin (0.03 U/μg protein) and 2.5 mM $CaCl_2$. FPLC purification was per manufacturer's directions for 5/5 Mono Q, 5/5 Mono S, and 10/30 Sephadex75. Protein concentration was assayed by the Bradford method and purity was assessed by SDS-PAGE and Coomassie stain.

Tissue culture. Ins1 cells were grown in RPMI 1640 medium (Sigma) containing 5.5 mM glucose, 10% fetal bovine serum, 10 mM Hepes, pH 7.4, 10.2 mM L-glutamine, 50 mM sodium pyruvate, 2.5 mM β-mercaptoethanol, streptomycin (0.1 mg/ml), and penicillin (100 units/ml) at 37 °C in 10% CO_2. A431 cells were grown in Dulbecco's modified Eagle's medium with 4 mM L-glutamine and pen/strep, 90%; fetal bovine serum, 10%, at 37° C in 5% CO_2.

Analytical ultracentrifugation. Protein OD_{280} for all samples was between 0.4 and 0.6. Concentrations of CaCl2 (10μM to 10 mM) and EGTA (0.1 to 1.0 mM) were used. Calibration spins of 3000 rpm resulted in loss of signal due to protein precipitation.

Endocytosis assay. This process has been previously described (Schmid et al., 1991). Briefly, human A431 cells (10-20 x 10^6) were serum-starved for 1 hour, washed 3x in KSHM buffer (20 mM HEPES, pH 7.4, 85 mM sucrose, 100 mM potassium acetate, 1 mM magnesium acetate) and floated in liquid nitrogen. Cells were then scraped off of the dish using a rubber policeman into 10 ml KSHM buffer and cell opening was assessed by Trypan blue exclusion. Cells were washed with buffer and pelleted by centrifugation (4°C/800 x g/3 minutes) three times. Cells were resuspended to a density of 10^7 cells/ml in KSHM buffer plus 0.8% BSA and 8μg/ml of biotinylated transferrin (BSST), left for 15 minutes at 4°C, aliquotted into tubes for 37° C time points, and rat brain cytosol and an ATP regenerating system was added. BSST that was not internalized was subject to reductive cleavage of the disulfide bond (BSST) between biotin and transferrin with the reducing agent sodium 2-mercaptoethanesulfonate (MesNa - 50 mM). Reduction was stopped with iodoacetic acid (0.5 M). Cells were then lysed and added to the ELISA plates prebound with anti transferrin. After washing the plates, avidin conjugated to horse radish peroxidase was added. After developing (51 mM Na_2HPO_4, 27 mM citric acid, 5 mg ortho-phenylenediamine in 12.5 ml total volume) for 2-5 minutes, reactions were stopped with HCl and OD_{490} was read.

Antibody production. Munc18c protein or GST-tagged Munc18c was coupled to hemocyanin from Limulus polyphemus with glutaraldehyde and used to immunize rabbits at 2- to 4-wk intervals. Serum was tested for immunoreactivity to immunizing protein.

Sucrose gradient. Rat Ins1 cells (10-20 x 10^6) were trypsinized, washed 3 times in ice-cold PBS and resuspended into 2 ml lysis buffer (0.32 M sucrose, 4 mM HEPES pH 7.4, 2 mg/ml pepstatin A, 100μM phenylmethanesulfonyl fluoride). Cells were then passed through a

Balch cell cracker with a 0.1551" ball bearing 20 times and opening was assessed by Trypan blue exclusion. Cracked cells were layered on a 10 ml continuous sucrose gradient (0.4 – 2.0 M) and centrifuged in an SW-41 rotor (4°C/110,000 x g or 26,500 rpm on an SW41 rotor/20 hours). Fractions of 0.5 ml were collected from the bottom to the top using a Beckman Fraction Recovery System. Proteins were resolved by SDS-PAGE and immunoblot with Syt2 (U6129), WNK1 (Q256), and Munc18c (U6130) antibodies.

<u>In vitro kinase assay</u>: GST-tagged WNK1 1-491 or WNK4 1-444 was combined with GST-tagged Munc18c or myelin basic protein in 30 μl reactions containing 10 mM HEPES, pH 8, 10 mM $MgCl_2$, 1 mM benzamidine, 1 mM DTT, 50 μM ATP containing 1 cpm/fmol [γ-^{32}P] ATP. Reactions were incubated at 30° C for 30 minutes and stopped by the addition of 7.5 μl of 5x SDS sample loading buffer and boiling for 2 minutes. Proteins were resolved by SDS-PAGE and autoradiography.

Results

Conformational changes in synaptotagmin 2

One proposed mechanism by which Syt can effect calcium-dependent endocytosis is that the linker region connecting the two C2 domains can hinge resulting in a mechanical constriction (Garcia et al., 2000). This mechanism suggests that one C2 domain would bind the phospholipids in the plasma membrane on opposite sides of the SNARE complex so that the linker region would span the SNARE complex and connect to the two anchored C2 domains. Calcium binding would then cause the C2 domains, and the membrane they bind, to be drawn into closer apposition. One obvious method to test this was to measure the calcium-dependent conformational changes in recombinant protein by sedimentation velocity

in an analytical ultracentrifuge. If conformational changes were evident, then the impact of WNK1-dependent phosphorylation of Syt2 could be examined.

Three attempts were made with two different protein preparations of Syt2 but each time millimolar to micromolar calcium was added to the protein, the protein would precipitate within minutes. Apparently, recombinant Syt2 co-purifies with endogenous phosphates (J. Rizo, personal communication) so that added calcium would precipitate the Syt2 rather efficiently ($Ca_3(PO4)_2$). Further purification of the protein by FPLC using ion exchange (Mono Q and Mono S) and size exclusion (Sephadex 75) did nothing to prevent the precipitation upon addition of calcium. Cleavage of the His_6 tag and different portions of Syt2 had no effect either. In an attempt to control the amount of calcium, EGTA was added before titrating in calcium. However, the addition of EGTA also caused the protein to precipitate.

Synaptotagmin 2 and endocytosis

Another method employed to assess the impact of WNK1 phosphorylation of Syt2 on its function was the endocytosis assay developed by Schmid and Smythe (1991). This assay uses partially permeabilized cells whose cytosol is washed away and replaced with rat brain cytosol, an energy regeneration system, and any protein of interest to examine the effect of that protein upon endocytosis via an ELISA-based read out (Appendix A and Methods). This assay could be useful in determining the impact of WNK1-phosphorylated Syt2 on endocytosis.

This experiment was performed once on an analytical level and four times to test the principle. The first proof of principle run was performed using mono-ferric transferrin

because the articles detailing the methods were not specific. The next four runs were performed using di-ferric transferrin for which the transferrin receptor has an order of magnitude greater affinity. The last three proof of principle runs compared control and experimental conditions of endocytosis (restrictive and permissive temperature or with and without cytosol). The expectation was that addition of cytosol would increase endocytosis which is an energy-dependent process. Similarly, increasing the temperature from an

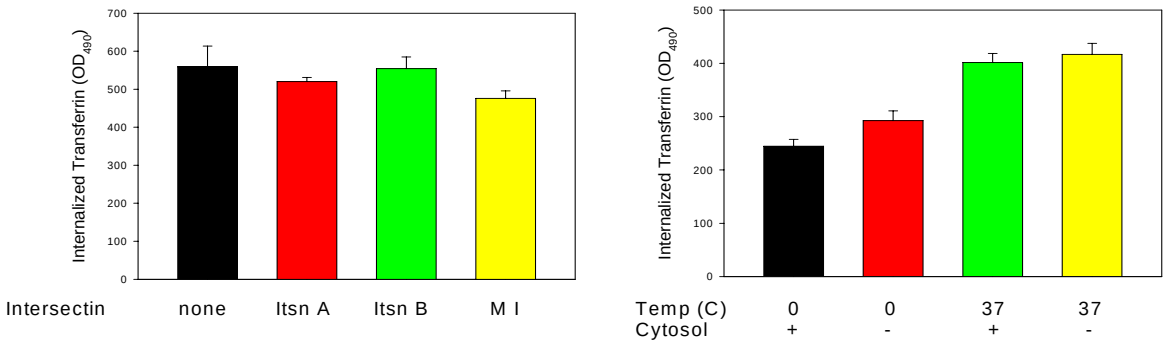

Figure 8. Endocytosis assay. A. Effect of SH3 domains (intersectin and myosin I) on endocytosis. **B.** Proof of principle. Endocytosis as a function of temperature with and without cytosol.

endocytosis-restrictive temperature (4ºC) to an endocytosis-permissive temperature (37ºC) was expected to increase endocytosis. In both cases, temperature and cytosol failed to elicit an effect that would rise above the background (Fig. 8a). As a last resort, another run comparing the effect of the SH3A and SH3B domains of intersectin (Itsn) was performed. The SH3A domain of Itsn inhibits endocytosis in this same assay (Simpson et al., 1999). Figure 8B shows that while there may be an effect, as before, it was not sufficient to overcome the poor signal to noise ratio.

-Co-localization of WNK1, Syt2, and Munc18c

Evidence in our laboratory suggested that WNK1 and Syt2 co-localize by immunofluorescence microscopy (Lee et al., 2004). Therefore, sucrose-gradient centrifugation was employed to support these data. The rat pancreatic β-cell line, Ins1, was used to determine if WNK1 is located on the same structures as Syt2.

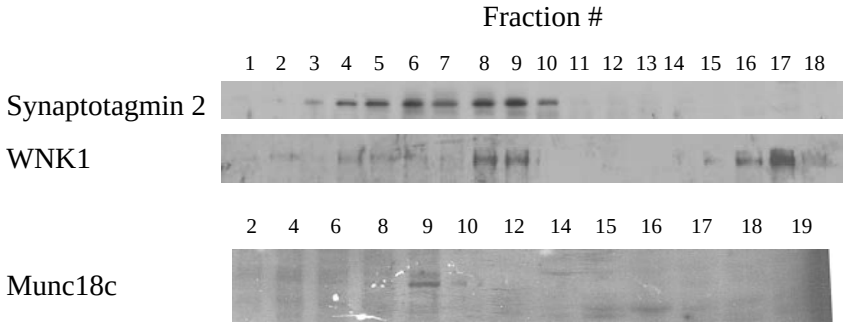

Figure 9. Co-migration of endogenous Syt2, Munc18c, and WNK1. Sucrose-gradient density centrifugation of Ins1 cells. Left to right is heaviest to lightest fraction.

Figure 9 shows that the heavier fractions contain a bi-modal distribution of Syt2 and that a small portion co-migrates with Syt2 in the heavier peak, approximately 25% of WNK1 co-migrates with the Syt2 in the lighter peak while the rest of WNK1 is ostensibly soluble. Interestingly, Munc18c co-migrates with WNK1 and Syt2 in the lighter peak. This could suggest that those vesicles containing WNK1, Syt2, and Munc18c are a distinct population, relative to those heavier vesicles. Additional evidence has been gathered to suggest the presence of WNK1 on several vesicle types (Fig.10 and Lenertz et al., in preparation). The presence of these proteins *en suite* supports the hypothesis that they interact, however failing a functional test such as the endocytosis assay, these data cannot stand alone.

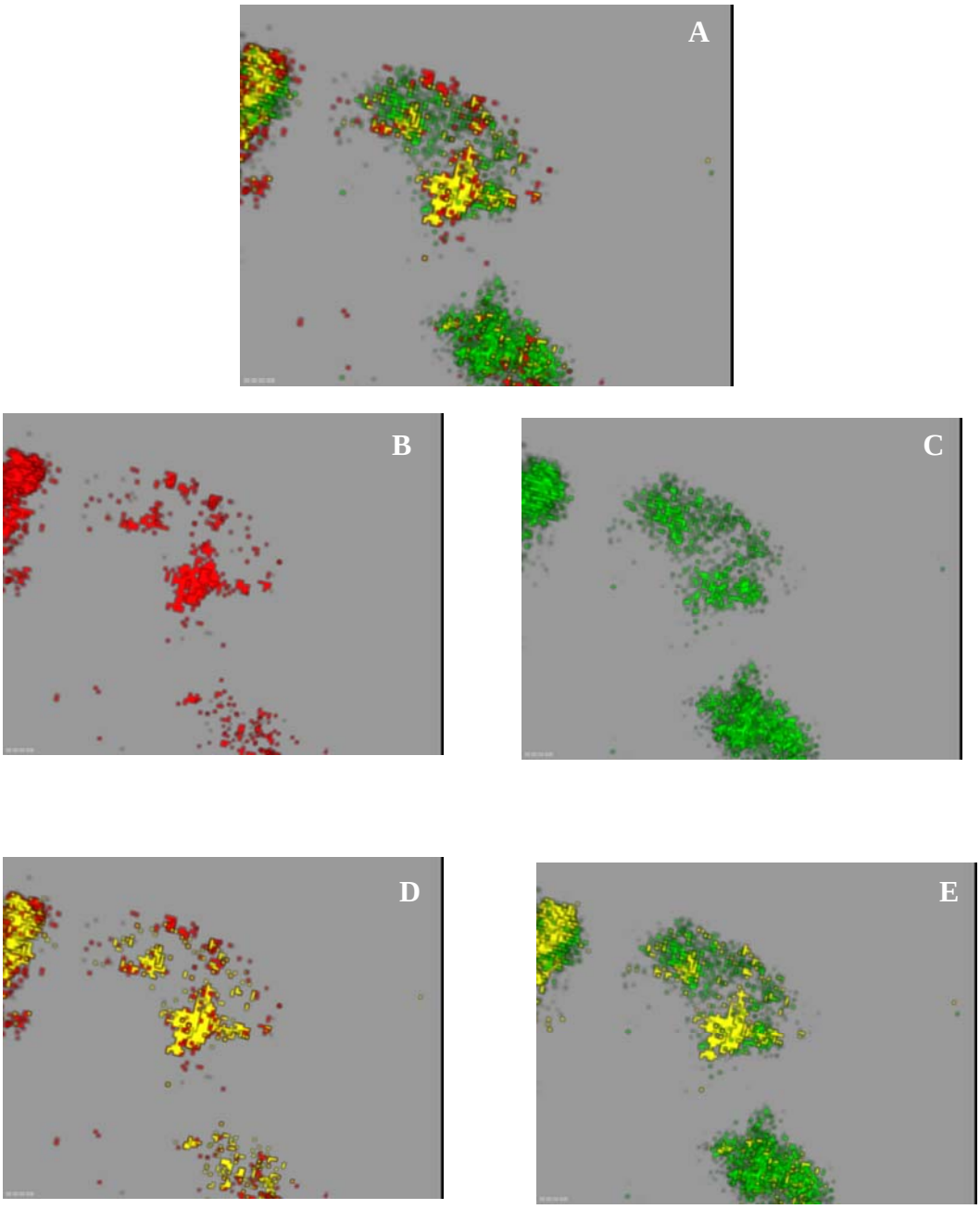

Figure 10. WNK1 co-localizes with insulin-secretory vesicles. Three-dimensional reconstruction (by K. Luby-Phelps) of Z stacks from immunofluorescent micrographs (by B.H.Lee) using Amara Software. Insulin (green) and WNK1 (red) localize to specific subset of vesicles. Computer-generated channel reduction allows the visualization of co-localization (yellow) in **D** and **E** by isolating WNK/red channel (**B**) and insulin/green channel (**C**).

Munc18c is not a WNK1 substrate

Finally, in my hands, Munc18c is not a WNK1 substrate. Two kinase assays using recombinant Munc18c and WNK1 1-491 or Munc18c and WNK4 1-444 showed no increased incorporation of ^{32}P-phosphate in Munc18c (Fig. 11).

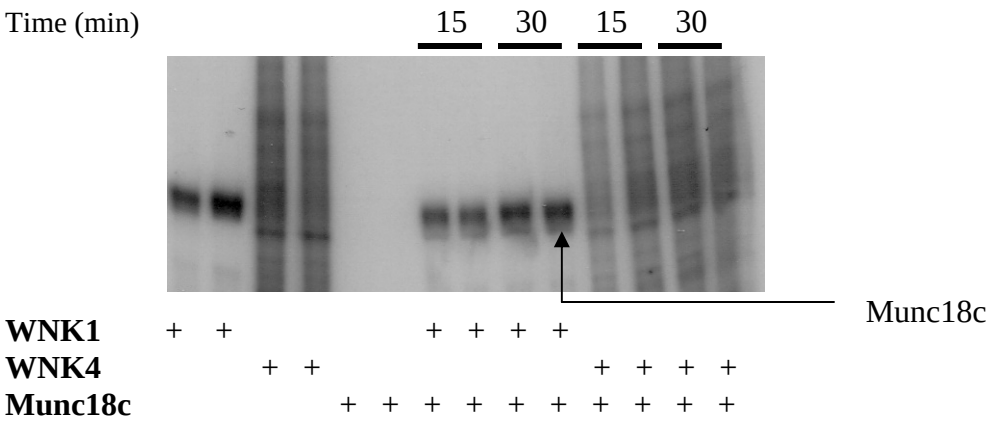

Figure 11. Munc 18c is not a WNK1 or WNK4 substrate. *In vitro* kinase assay shows limited Munc18c phosphorylation.

In contrast, autophosphorylation of both WNK1 and WNK4, essential for the activity of both kinases, was evident (Fig. 11). There is evidence from the laboratory of a collaborator that WNK1 phosphorylates Munc18c (D. Thurmond). However, while some basal level of phosphorylation may be evident in my assays, I am unwilling to suggest that Munc18c is a *bona fide* substrate for either WNK1 or WNK4. In fact, glucose-induced insulin secretion has been shown to be dependent upon tyrosine phosphorylation of Munc18c by an as yet unidentified kinase, not serine/threonine phosphorylation (Oh and Thurmond, 2006).

However, in our collaboration with Dr. Thurmond, we have information that Munc18c and WNK1 binding is essential for glucose-dependent insulin secretion.

Discussion

The role synaptotagmin plays in vesicular trafficking is becoming more clear. Munc18c is implicated as well, albeit the research on this protein is only beginning. What remains elusive is what role WNK1 plays with both of these proven interactors. Lack of a robust means of assessing the role of WNK1 is, at this writing, the key limitation preventing further research.

This project was abandoned for several reasons. First, there was evidence from parallel research by another colleague, Bing-e Xu, that the activation caused by WNK1 leading to increased ion absorption may be independent of its kinase function. Second, the scrape-load endocytosis assay, upon which this project would depend, was never sufficiently sensitive to prove useful. Because analysis of Syt2 function by sedimentation velocity was not possible with recombinant protein, there seemed to be no facile means of examining the hypothesis that WNK1 phosphorylation of Syt2 altered endocytic regulation of ion-trafficking membrane proteins.

A more thorough discussion will be presented in Chapter 5.

CHAPTER III

Introduction

Background

Synaptic transmission of neural impulses is a tightly orchestrated event that relies upon spatial and temporal coordination of receptors and their neurotransmitter ligands. Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system. Upon stimulation, vesicles containing glutamate are exocytosed from the pre-synaptic neuron releasing glutamate into the synapse where it binds to and activates glutamate receptors on the post-synaptic neuron, thus propagating the signal. Excess neurotransmitter must be cleared from the synaptic cleft because prolonged exposure to glutamate will damage the post-synaptic neuron by a process known as excitotoxicity.

Transporter	Other names	Expression	K_D (μM) Asp/Glu	Inhibitors IC50 (μM) e.g. DHK
EAAT1	Glast SLC1A3	Broad	60/48	3000
EAAT2	Glt-1 SLC1A2	Broad	54/97	23
EAAT3	EAAC SLC1A1	Broad	47/62	3000
EAAT4	- SLC1A6	Broad	-	-
EAAT5	- SLC1A7	Retinal	-	> 500

Table 2. Glutamate/Aspartate transporter basics. The excitatory amino acid transporters are known by their solute carrier nomenclature as well as other names. Glast – glutamate/aspartate transporter; EAAC – excitatory amino acid carrier; DHK – dihydrokainate; "-" – no reported data.

Such clearance is effected by a family of high-affinity amine transporters, the excitatory amino acid transporters (EAATs). There are five such mammalian transporters and their various nomenclature and expression patterns are outlined in Table 2.

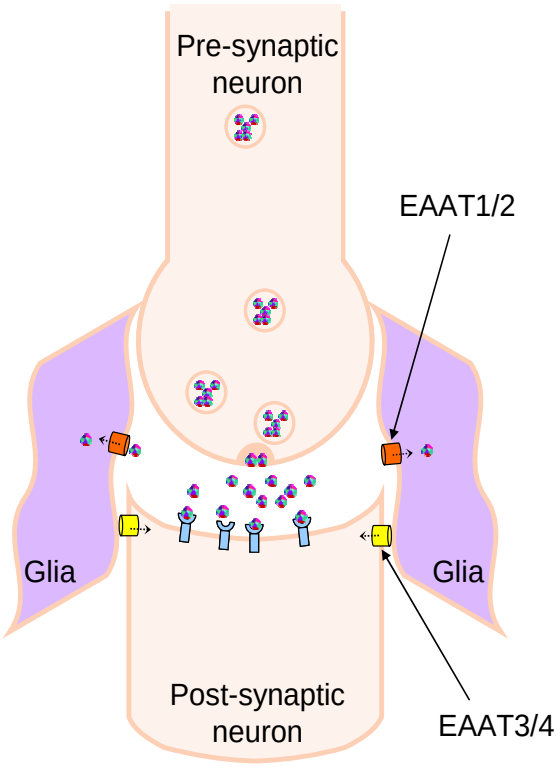

Figure 12. Model of a synapse. Pre-synaptic cells secrete glutamate into the synaptic cleft where it binds and activates iGluR and mGluR. Excess glutamate is cleared from the synapse by the actions of EAATs 1-4.

In order to concentrate and contain the signal, synaptic clefts are ensheathed by glial cells (Fig.12). EAAT1 and EAAT2 are primarily expressed in glia while EAAT3 and EAAT4 are expressed in the post-synaptic neurons (O'Shea et al., 2002). There is 65% identity between EAATs 1 and 2 (Fig. 13). Despite an enormous amount of data on the function of the EAATs, their structure is unresolved and as such, the mechanics of transport remain elusive. Secondary structure predictions and labeling suggest that EAAT2 has at least 7 transmembrane segments with cytosolic amino and carboxy termini (Fig.14). The topology of the region between the sixth transmembrane segment and the carboxy terminus is poorly understood, and models suggest that this portion is comprised of re-entrant or partially penetrant helices (Seal et al., 2000) and the oligomerization of multiple monomers is predicted to range from 3

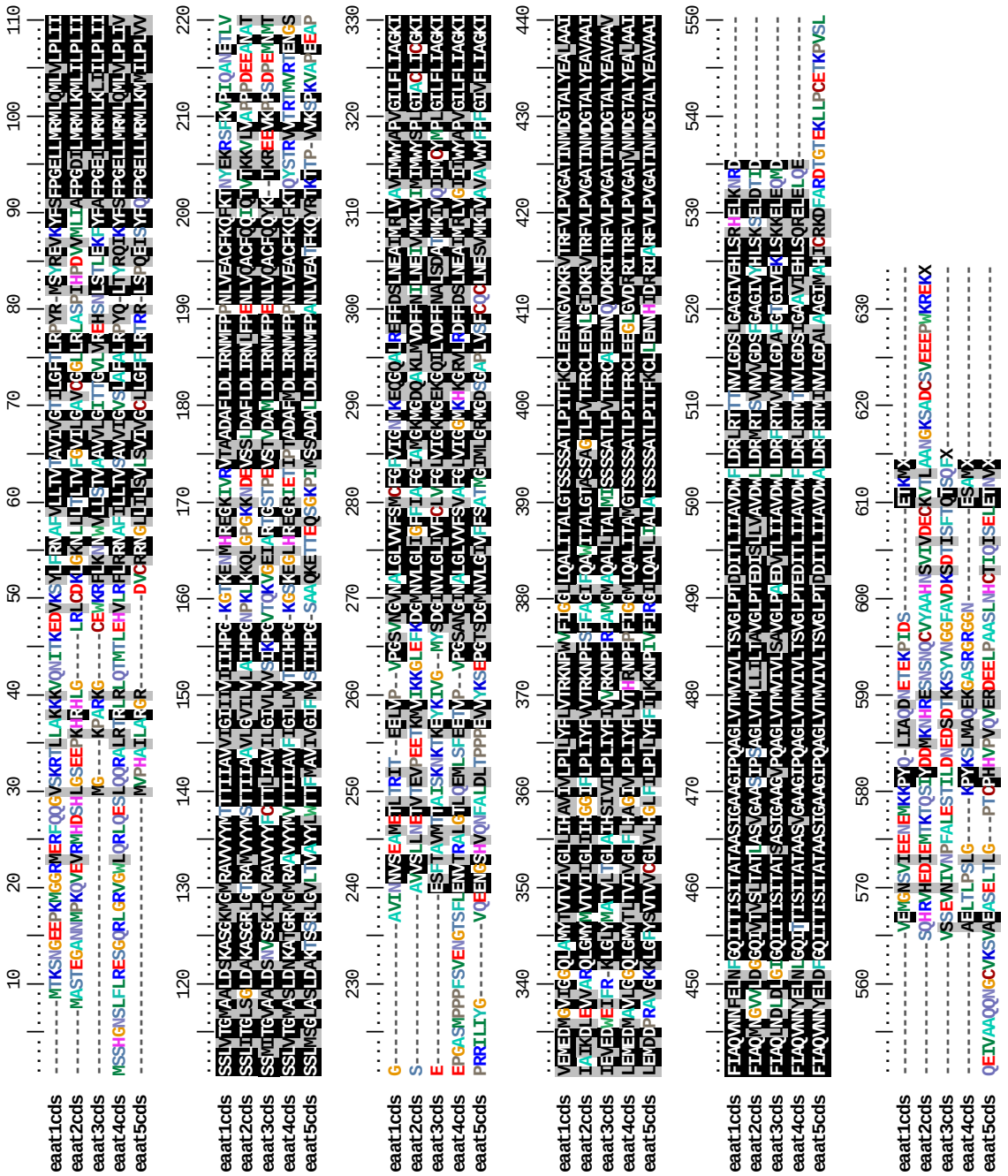

Figure 13. Alignment of EAAT family members. Human EAAT1-5 protein sequence alignment. High homology coincides with the predicted transmembrane regions.

to 5 but is likewise unresolved. What is known from heterologous expression systems is that one molecule of glutamate transported is accompanied by one hydrogen, three sodiums, and an uncoupled

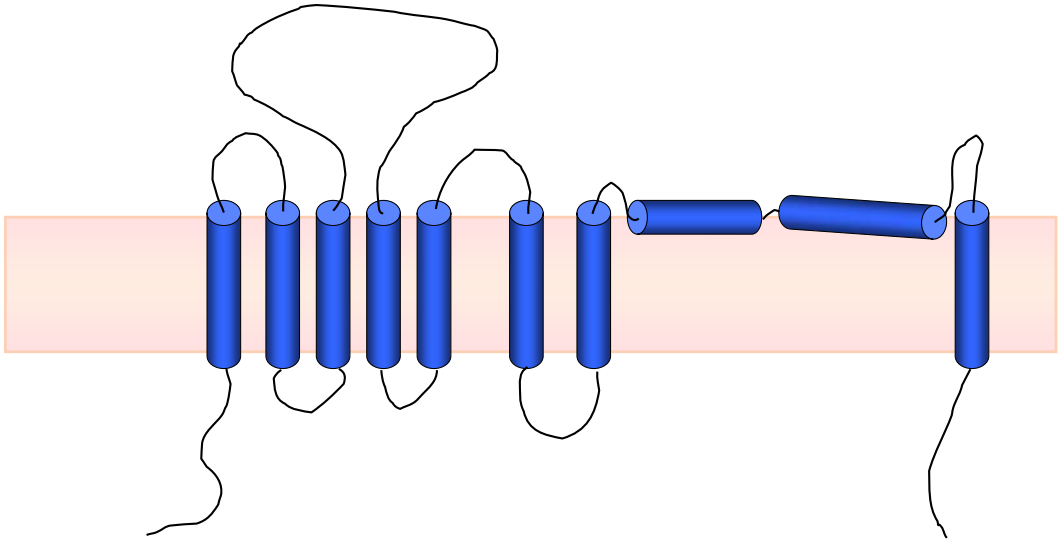

Figure 14. Proposed topology of EAAT1. Based upon hydropathy and models (Seal et al., 2000). The first 6 transmembrane spans are generally accepted while the topology of the carboxy-terminal portion of the protein remains unresolved.

transport of chloride; transport of glutamate against its concentration gradient is driven by the co-transport of sodium (Bridges and Esslinger, 2005). The EAATs are capable of transporting both L and D enantiomers of aspartate but can only transport the metabolically active L enantiomer of glutamate (Arriza et al., 1994). It is this fact that allowed researchers to use the metabolically inert D-aspartate to study EAAT function. Moreover, a vast array of transport inhibitors with EAAT-type specificity are the result of more than three decades of research on these transporters (e.g. in Table 2; reviewed in Bridges and Esslinger, 2005).

Exocytosis

Clearly, regulation of the membrane localization of these transporters must be tightly regulated. In fact, defects in this regulation are suggested causative agents in such diseases as Alzheimer's (Zoia et al., 2005), amyotrophic lateral sclerosis (Bristol and Rothstein., 1996; Vanoni et al., 2004), ischemic damage (Camacho and Massieu, 2006), Parkinson's (Plaitakis and Shashidharan, 2000), and multiple sclerosis (Vallejo-Illarramedi et al., 2006). Conversely, EAAT2 expression level was reportedly inversely proportional to gliomal proliferation (de Groot et al., 2005). This laundry list of illnesses makes obvious the need for investigating the regulation of these transporters.

The study of exocytosis has mostly focused on the secretion of soluble factors such as neurotransmitters and hormones. However, examination of exocytosis for non-secretory purposes is gaining attention. Translocation of membrane proteins from subcellular locations to the plasma membrane in response to extracellular cues is ultimately accomplished by exocytosis. For example, the renal water channel, aquaporin 2, is translocated to the apical surface of primary and cultured cells in the nephron when stimulated by vasopressin (Marples et al., 1995). Misregulation of aquaporin 2 results in diabetes insipidus (Brown, 2003); tetanus toxin, which cleaves the V SNARE synaptobrevin, blocks this transport, a fact that implicates the SNARE exocytic machinery (Lorenz et al., 2003).

Endocytosis

Endocytosis is a process whereby cells recover membrane proteins as well as membrane itself. This process has been divided into two mechanisms: clathrin-dependent and clathrin-independent. In the former, proteins to be internalized bind to the μ2 subunit of

adaptor-protein 2 (AP2) by tyrosine motifs on their cytosolic tails. This recruits clathrin. The best characterized example of this regulatory process is the transferrin receptor but ion channels such as ENaC have been shown to be regulated by endocytosis as well (Shimkets et al., 1997). In ENaC regulation, two motifs are essential for endocytosis: first a PPxY motif that binds to the ubiquitin ligase neuronal precursor cell-expressed developmentally downregulated protein 4-2 (Nedd4-2); and second a YxxL motif that binds to AP-2 (Rotin et al., 2001).

Signaling of internalization

Ample evidence exists that transporters in general and EAATs specifically are internalized by a clathrin-dependent mechanism. One laboratory has linked a well-known internalization signaling pathway to the entire family of EAATs (Boehmer et al., 2003; Boehmer et al., 2004a; Boehmer et al., 2004b; Schniepp et al., 2004; Boehmer et al., 2006). Two of the proteins from this pathway have been linked to the regulation of ENaC: the serum and glucocorticoid inducible kinase 1 (SGK1) and Nedd4-2. Their roles in the regulation of ENaC will be discussed in the following chapter but here, their purported regulation of EAATs will be examined.

SGK

SGK is a member of the AGC superfamily of serine/threonine protein kinases which includes protein kinase A, B (also known as Akt and to which SGK1 is structurally similar), and C. There are three SGK genes in mammals, SGK1-3. There is evidence of homologs in

-yeast, flies and worms. They are characterized by a variable amino terminus and a carboxy terminal catalytic domain (Fig. 15). The activation loop of SGK1 occludes the substrate binding pocket until activated by the phosphoinositide-dependent kinase (PDK) at T_{256}. Serine$_{422}$ is essential to the activity of SGK1. Phosphorylation of S_{422} recruits a domain of PDK1 also known as the PDK interacting fragment (PIF). In SGK1, the catalytic residue is K_{127} and its mutation ablates activity. SGK1 is a highly unstable protein with a half-life of approximately 30 minutes (Brickley et al., 2002; Bogusz et al., 2006). Its transcriptional regulation is responsive to

Figure 15. Architecture of SGK1. SGK1 (to scale) is comprised of a variable N-terminus and a C-terminal kinase domain. The hydrophobic domain is carboxy-terminal relative to the kinase domain.

many growth factors, serum, and has been shown to be upregulated in response to ischemic injury (Nishida et al., 2004) and excitotoxicity (Hollister et al., 1997).

Nedd4-2

Nedd4-2 is a substrate of SGK1. Nedd4-2 is phosphorylated on S_{444} and S_{338} by SGK1. Ubiquitin ligases such as Nedd4-2 are often thought to be arbiters of destruction as poly-ubiquitinated proteins are proteasomally degraded. However, Nedd4-2-ubiquitination of ENaC leads to its internalization but whether or not ENaC is degraded or recycled remains

unresolved. Thus, Nedd4-2 is a pivotal player in the regulation of membrane protein function.

Nedd4-2 belongs to the E3 family of ubiquitin ligases because its catalytic activity is directed by a HECT domain (homologous to E6-AP C-terminal ligase). Structurally it is comprised of an amino-terminal C2 domain, 4 WW domains (conserved di-tryptophan residues), and a carboxy-terminal HECT domain (Fig. 16). The WW domains of Nedd4-2 bind to PY (PPxY) on its substrates (Henry et al., 2003) and SGK1 phosphorylation of Nedd4-2 at S_{444} creates a 14-3-3 binding site which prevents Nedd4-2 from binding its

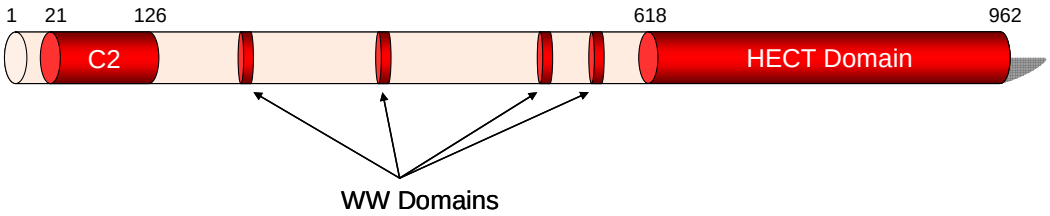

Figure 16. Architecture of Nedd4-2. Nedd4-2 (to scale) is comprised of an N-terminal C2 domain, 4 WW domains, and a C-terminal HECT domain.

substrate (Nagaki et al., 2006). UNIGENE expressed sequence tag analysis of Nedd4-2 demonstrates broad tissue expression.

Rationale

Florian Lang has implicated SGK and Nedd4-2 in the regulation of all five EAATs (Boehmer et al., 2003; Boehmer et al., 2004a; Boehmer et al., 2004b; Schniepp et al., 2004; Boehmer et al., 2006) to varying degrees. Expression of cRNAs in *Xenopus* oocytes was used to show that SGK1 increases the activity of EAATs 1, 2, 3, 4, and 5 and that Nedd-2

decreases EAAT1. Moreover, this laboratory showed that insulin-like growth factor (IGF 1) increased plasma membrane residence of EAAT1 in a PI3K-dependent manner (Boehmer et al., 2003). Our laboratory demonstrated that WNK1, by an IGF 1/PI3K-mediated pathway, was capable of activating SGK1 which then phosphorylated Nedd4-2. The target of Nedd4-2 here was ENaC, however, given the similarity of the signaling cascade, I attempted to relate it to the regulation of the EAATs as outlined in figure 17.

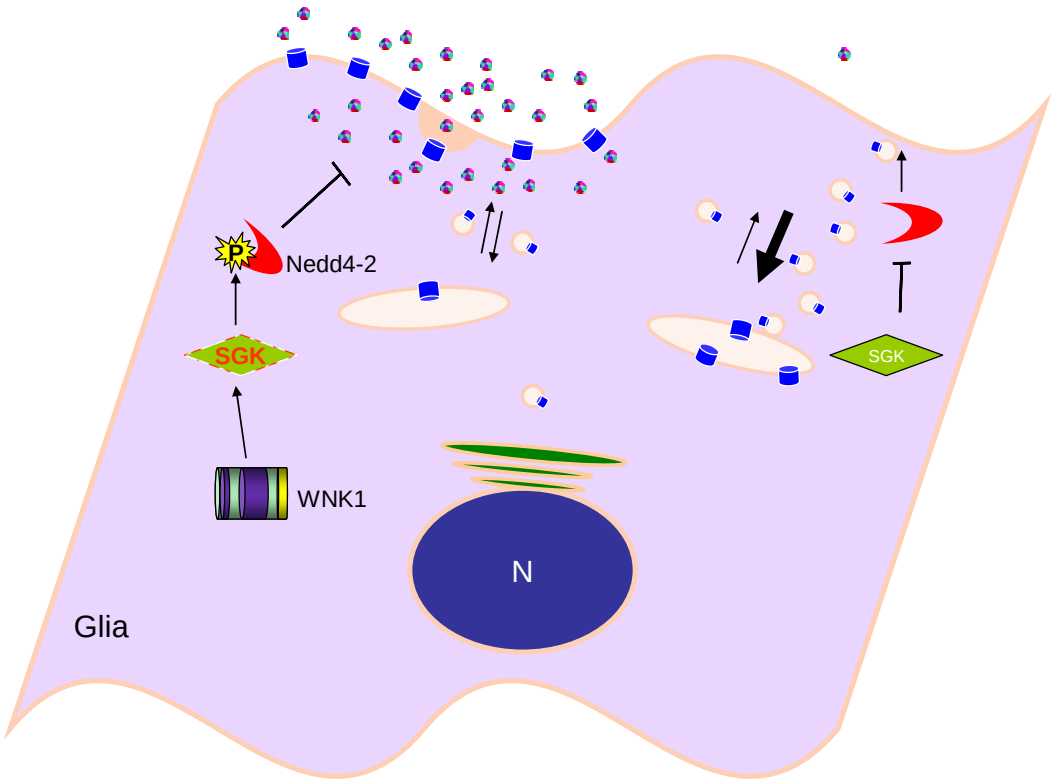

Figure 17. Proposed scheme for the WNK1 regulation of EAAT. WNK1 activates SGK1 which then phosphorylates Nedd4-2 which is then sequestered from EAAT1 (left side of cell) while the absence of WNK1 leads to reduced uptake of glutamate, ostensibly by a reduction in plasma membrane resident EAAT1 (right side of cell).

Methods

Constructs. cDNA for EAAT1 was purchased from OpenBiosource and subcloned into pCMV5 and CFP:pCMV5. eYFP $A_{206}K$ was a gift from A. Gilman and was subcloned into pCMV5:EAAT1. Fluorescent proteins were fused to the amino-termini of EAAT1. Rabbit polyclonal antibodies to EAAT1, 2, 3, and 4 were obtained from Alpha Diagnostics Incorporated and mouse monoclonal anti-EAAT1 was obtained from Santa Cruz. D-aspartate was purchased from Fluka and ^{3}H-2,3-D-aspartate was purchased from Amersham Biosciences. Methyl-β–cyclodextrin was from Sigma.

Cell culture and transfection. Rat C6 glioma cells were a gift from E. Ross and were cultured in Ham's F12K containing 2 mM L-glutamine, 1000U/ml of both penicillin and streptomycin, (82.5%), horse serum (15%,), and fetal bovine serum (2.5%) at 5% CO_2. Human SH-SY5Y neuroblastoma cells were grown in a 1:1 mixture of Eagle's Minimum Essential Medium and Ham's F12 Medium, 2 mM L-glutamine, 1 mM sodium pyruvate, and fetal bovine serum (10%) at 5% CO_2. Hamster CHO cells were grown in DMEM, 2 mM L-glutamine, 1000U/ml of both penicillin and streptomycin, and fetal bovine serum (10%) at 5% CO_2. WNK1 gene-trap MEFs were obtained from A. Anselmo from animals obtained from Chou-Long Huang and cultured in DMEM at low oxygen conditions as described by the Shay/Wright laboratory. Cells were transfected by FuGene6 (Roche) or nucleofection (Amaxa) as indicated and according to instructions of the manufacturers.

Microscopy. All images were taken using a Zeiss Axiovert 200M fluorescence microscope.

2,3-^{3}H D-aspartate uptake assay. Indicated cells were washed 3 times with room temperature mPBS (phosphate-buffered saline containing 1 mM $MgCl_2$, 0.9 mM $CaCl_2$, and

5.6 mM D-glucose) and incubated with mPBS containing a 100:900 nM ratio of ^{3}H D-aspartate to D-aspartate at indicated temperature and time. The labeling mixture was removed and cells were washed 3 times with ice-cold mPBS. Cells were lysed and uptake of ^{3}H D-aspartate was measured by liquid scintillation counting. Background was determined by using excess (1 mM) D-aspartate to determine trapped (non-specific) radiolabeled signal.

Protein purification. cDNAs for EAAT1 residues 1-49, 462-549, and 503-549 were subcloned into pMal (New England Biolabs). Plasmids were transformed into chemically competent BL21 E. coli bacteria via standard protocol. A single colony was grown in 1000 ml Luria-Bertani Broth (LB) at 37°C shaking at 250 rpm until OD595 of 0.6-0.8 was reached. The culture was induced with 50μM isopropyl β-D-1-thiogalactopyranoside for 4 hours. Bacteria were pelleted in a Beckman J6B centrifuge with a TS4.2 rotor at 4°C and 2700 x g for 15 minutes. The pellet was lysed in 30 ml of lysis buffer (50 mM Tris pH 8, 150 mM NaCl, 1% Triton X-100) and sonicated (40% amplitude, 1 second pulses, 3 cycles of 10 pulses each). The lysate was clarified by centrifugation (4°C /18,000 x g/ 30 minutes) and batch bound to amylose resin (New England Biolabs; 4°C/rocking/1 hour). Resin was transferred to a column,washed with 50 bed volumes of buffer (50 mM Tris pH 7.4, 200 mM NaCl, 1 mM DTT). Immobilized protein was then used for analysis. Protein concentration and purity were assessed by SDS-PAGE and silver stain (Novex).

Results

EAAT1 localization in live cells.

Observing the trafficking of EAAT1 in live cells would be a powerful means of addressing the impact of WNK1 on its behavior. To accomplish this, fluorescently tagged EAAT1 constructs were created by fusing cyan and enhanced yellow fluorescent to the amino terminus of EAAT1. The CFP-tagged construct, when expressed in Chinese Hamster ovary (CHO) cells, localizes to the plasma membrane and discrete cytosolic puncta (Fig. 18). However, in addition to this, the protein also localized to perinuclear structures. This localization was most probably the endoplasmic

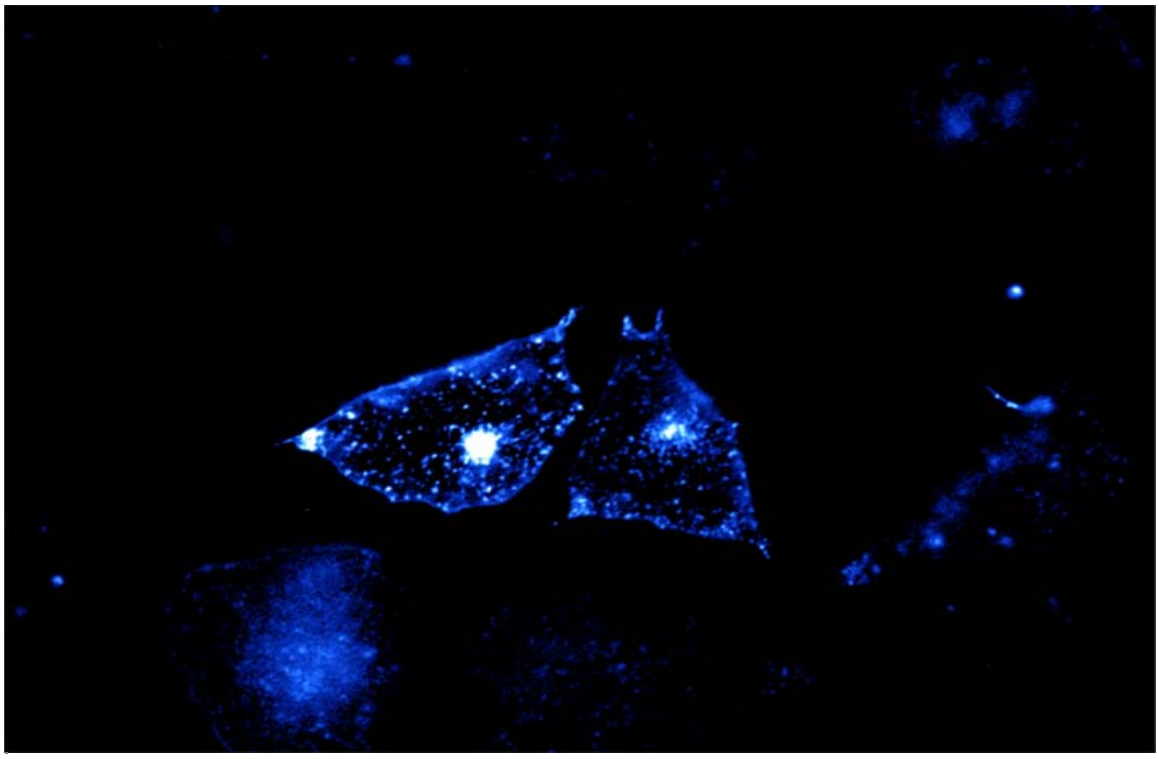

Figure 18. Localization of EAAT1. CFP:EAAT1 expressed in CHO cells localizes to cytosolic puncta, plasma membrane and the perinuclear region.

reticulum. A concern was that this pattern might represent gross overproduction of protein or aggregates of mis-folded protein. Examination of populations of transfected cells as well as titration of DNA amount used in transfection had no effect on this phenomenon. Because fluorescent proteins are known to be weakly dimeric (Tsien, 1998), a construct fusing EAAT1 to eYFP $A_{206}K$ (oligomerization incompetent) was created. This new construct was expressed in human epithelial kidney 293 cells rather than CHO cells because of the dearth of information on the hamster genome. Moreover, CHO cells were very difficult to transfect at high efficiency and the uptake assay would likely require high transfection efficiency. In other words, the fact that all cells tested exhibited some basal level of D-aspartate transport (discussed presently) would require that transfection efficiency was sufficiently high to overcome this background enabling the measurement of subtle effects. The expression pattern of eYFP:EAAT1 in 293 cells was better than that of CFP:EAAT1 in CHO cells in that the perinuclear staining was lessened. However, the membrane localization was less dramatic (Fig. 19). One explanation that was learned several

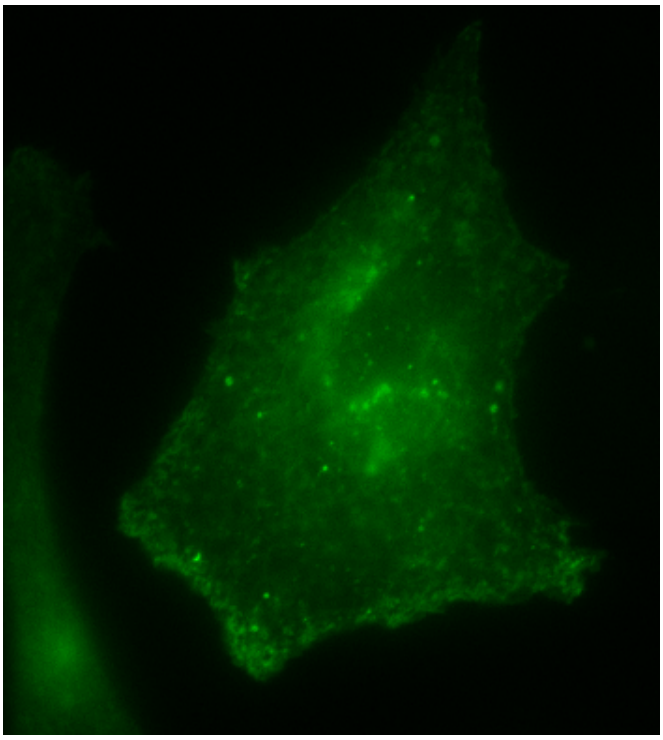

Figure 19. Expression of eYFP:EAAT1 in 293 cells. Membrane localization was less pronounced and background was higher.

months after moving to the final project (discussed in Chapter 4) was the use of $Ca_3(PO4)_2$-based transfection. Kate Luby-Phelps mentioned to a colleague that $Ca_3(PO4)_2$ transfection leads to a grainy background and this was most certainly the case.

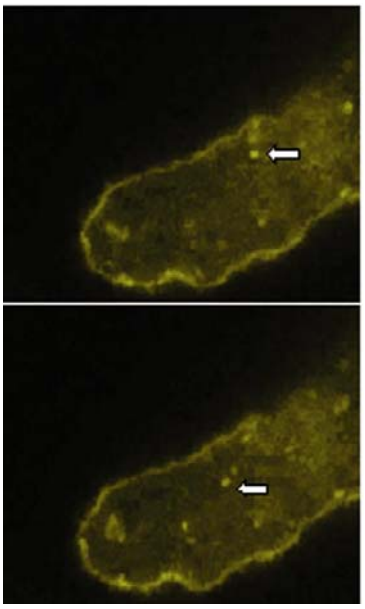

Figure 20. Movement of eYFP:EAAT1 in 293 cells. Time elapsed between frames is 1 second.

With the tagged transporter in hand, examination of its movement in cells could now be performed. Images taken every 0.5 seconds showed intriguing movement of small puncta in the cells (Fig. 20) and had the project continued, perturbation of the system with IGF, PI3K inhibitors, and mutants of WNK1, SGK1 and Nedd4-2 would have been employed.

Measuring aspartate uptake in cells.

The continuation of this project would hinge upon the ability to measure changes in glutamate transport. An assay using ^{3}H-D-aspartate was widely used in this field (Arriza et al., 1994). The D enantiomer of aspartate was preferable because it was metabolically inert. After demonstrating that the assay was functional (Fig. 21 and 22), CFP:EAAT1 and eYFP:EAAT1 were tested to ensure that the fluorescent tags did not inhibit transport (Fig. 23).

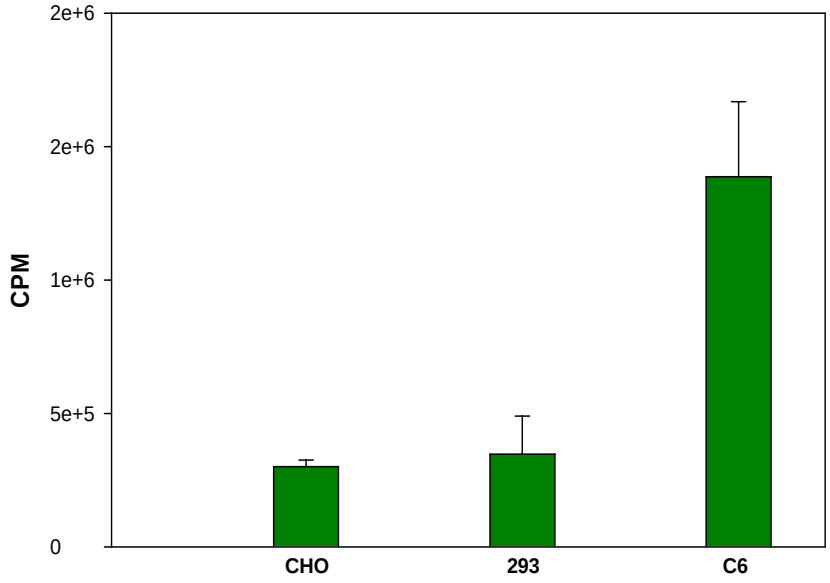

Figure 21. D-aspartate uptake in untransfected cell lines. Indicated cell lines were incubated with ^{3}H-D-aspartate for 15 minutes (see Methods). Uptake was the strongest in C6 glioma.

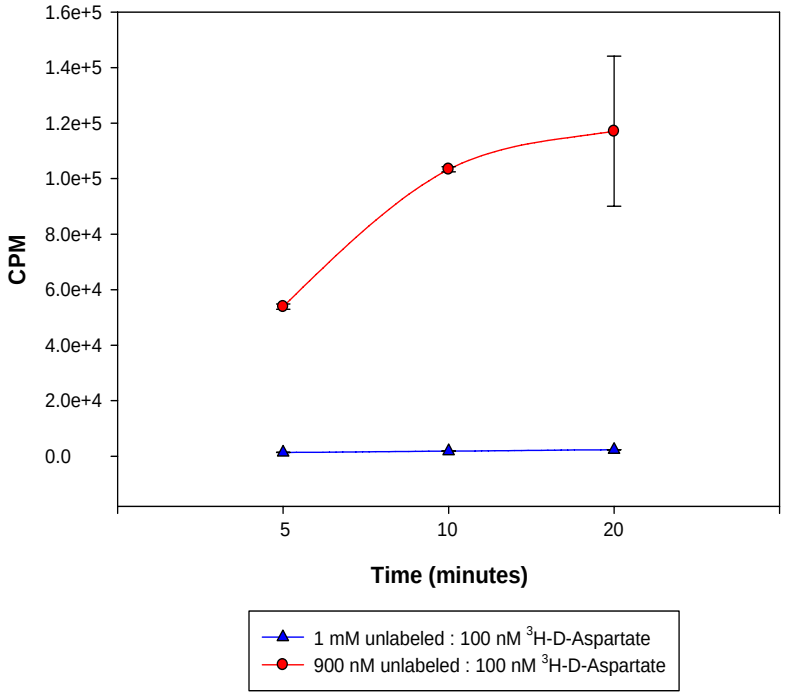

Figure 22. D-aspartate uptake in C6 glioma. Excess unlabeled aspartate is able to compete for uptake of ^{3}H D-aspartate. Error bars are SEM**.**

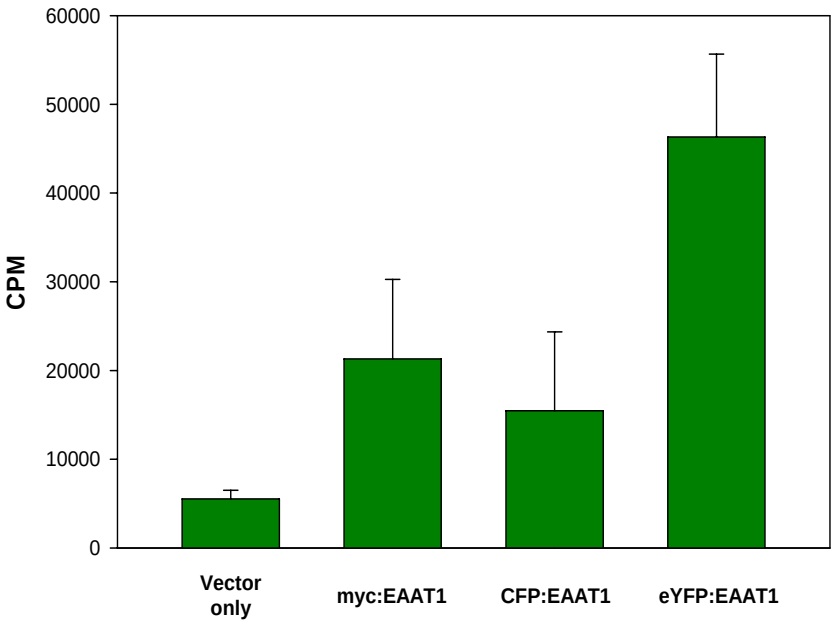

Figure 23. ^{3}H D-aspartate uptake in 293 cells. Overexpression of the tagged EAAT1 constructs demonstrated their functionality.

A problem that limited this project was the inability to extricate the function of EAAT1 from that of 2,3, and 4. Blots of C6, 293 and HeLa cells show that, in contrast to the literature, glioma cells express all four transporters (Fig. 24). One method that was hoped would address this issue was to apply EAAT type-specific inhibitors in a combination that would enable the isolation of EAAT1 function. This approach failed (Fig. 25 and Table 2). Although DHK has an IC_{50} of 80 μM for EAAT2, it has identical IC_{50}s for both EAAT1 and 3 (3 mM) and this means EAAT1 function cannot be separated from that of EAAT3 by this inhibitor.

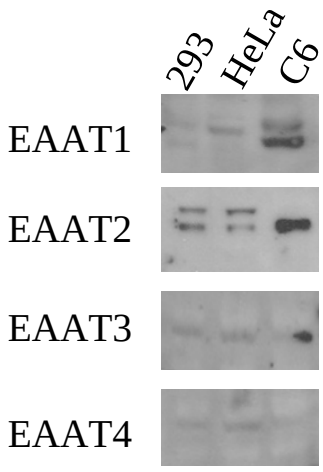

Figure 24. Expression of EAATs 1-4 in 293, HeLa, and C6 glioma cells. Four cell lines tested (including SH-SY5Ycells, not shown) express all four transporters.

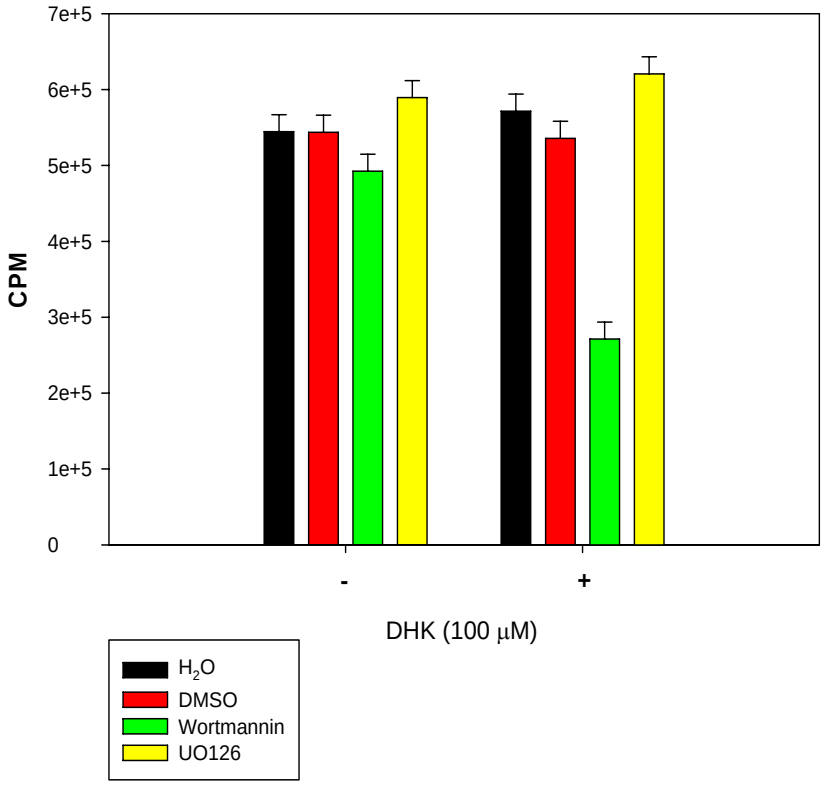

Figure 25. Application of EAAT2-specific inhibitor DHK. C6 glioma cells were preincubated with or without DHK and specified inhibitors for 30 minutes. D-aspartate uptake was assayed for 20 minutes.

As mentioned, the literature stated that glioma cells express only EAATs 1 and 2 so the strategy of using DHK to allow selective measurement of EAAT1 seemed logical until it was discovered that C6 glioma cells express all four EAATs. Wortmannin reveals a potential PI3K effect but extrication of EAAT1 from 3 and 4 remained an issue. Insulin-like growth factor (IGF1) has a well documented effect for activating PI3K, which in turn activates Akt. The application of IGF1 to 293 cells over-expressing EAAT1 did not show the predicted result, an increase in D-aspartate uptake.

There is evidence that only EAAT2 resides in cholesterol and sphingolipid-enriched membrane domains (Butchbach et al., 2004) which could be specifically disrupted with

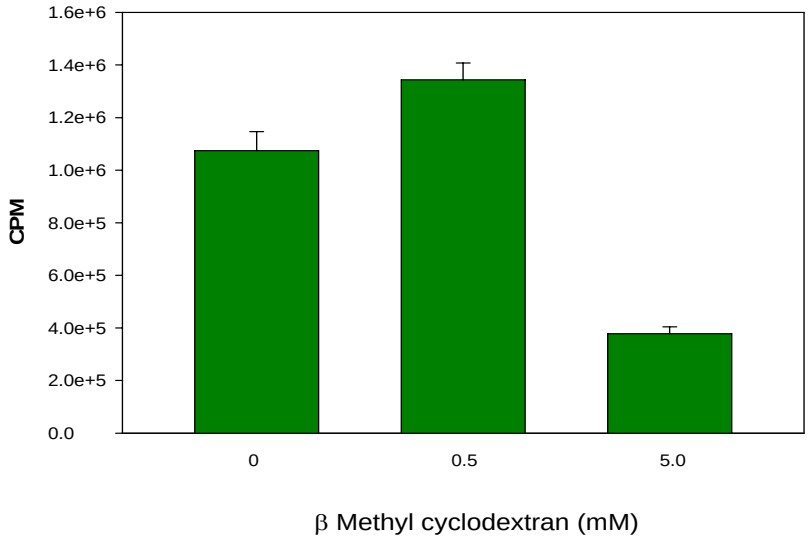

Figure 26. Application of methyl-β-cyclodextrin to C6 glioma cells. Cells were pre-treated with mβcd for one hour prior to assaying for D-aspartate uptake. Treatment decreased uptake approximately 60% but significant cell-death was seen at this concentration and above.

methyl-β–cyclodextrin. If the function of EAAT2 was dependent upon the integrity of lipid rafts, the application of β methyl cylcodextran could allow the function of EAAT1 to emerge. Unfortunately, as noted above, there was no way to confirm that the effect seen was due to a specific inhibition of EAAT2 because it was not realized until later that all 4 EAATs are present in most cells (Fig. 26).

WNK1, SGK1, and Nedd4-2 do not affect EAAT1 function.

Despite the setbacks detailed above, it was hoped that the predicted effect of SGK1 and Nedd4-2 could be tested. If WNK1-dependent SGK1 phosphorylation of Nedd4-2

causes an increase in EAAT1 function, then over-expression of kinase dead SGK1 (SGK1 K127M), which functions as a dominant negative, would be expected to increase EAAT1 uptake by relieving Nedd4-2 inhibition. This was not the case (Fig. 27). Furthermore, over-

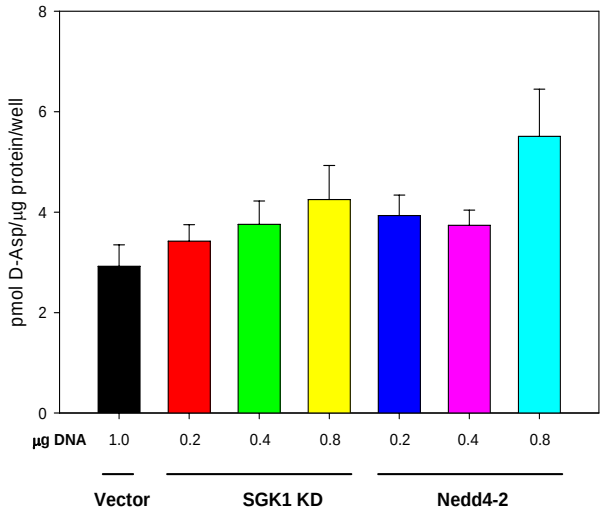

Figure 27. Over-expression of EAAT1, kinase-dead SGK1 and Nedd4-2 in 293 cells. Increasing amounts of DNA for SGK1 kinase-dead and Nedd4-2 were assessed for their ability to decrease D-aspartate uptake.

expression of Nedd4-2 should also cause a reduction in D-aspartate uptake by any EAAT that is controlled by this mechanism. In contrast, no effect was observed. Likewise, expression of WNK1 1-220 with the Akt phosphorylation site mutated to alanine and the use of the PI3K-inhibitor wortmannin would be expected to obviate the WNK1-dependent activation of SGK1, resulting in greater Nedd4-2 activity and a concomitant reduction in EAAT1 uptake (Fig. 27 and 28). Such effects were not observed. Finally, if Nedd4-2 ubiquitinates EAAT1,

immunoprecipitates of EAAT1 from cells over-expressing HA-tagged ubiquitin should be ubiquitinated. No such effect was observed (data not shown).

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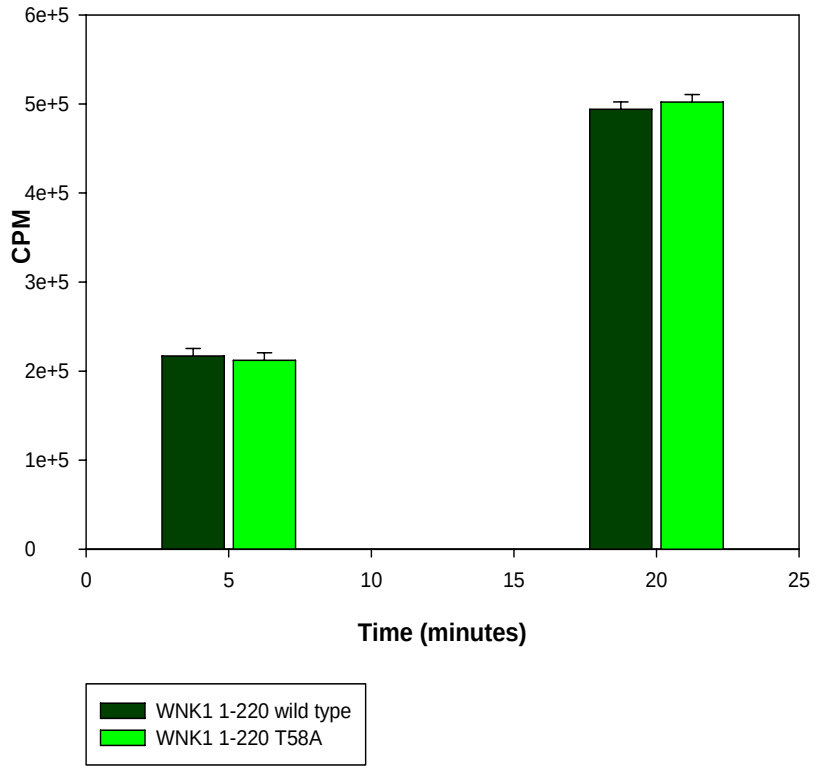


Figure 28. Over-expression of WNK1 in C6 glioma cells. C6 glioma cells were nucleofected (Amaxa) with WNK1 1-220 wild type or the Akt phosphorylation mutant T_{58}**A.** Nucleofection (electroporation) consistently produced transfection efficiencies between 70-90%.

Determining interacting proteins

The predicted cytosolic amino-terminal (residues 1-49) and carboxy-terminal (residues 462-549 and 503-549) tails of EAAT1 were fused to the maltose binding protein (MBP) creating three constructs: N, C_{long}, and C_{short} respectively. These constructs were expressed in bacteria. The proteins were purified by affinity chromatography and left bound to the amylose resin. Rat brain cytosol from the endocytosis assay (Chapter 2) was added to

each construct and MBP alone, the resins were washed and bound protein was analyzed on a 20 cm 4-20% polyacrylamide gel in SDS and silver staining in an attempt to find interacting proteins. No significant differences were evident despite three attempts (data not shown). Were Nedd4-2 a direct interactor, this approach would have been expected to show a distinct band at approximately 108 kDa. There was no such band significant enough to warrant mass spectrometric analysis.

Support for the WNK1/SGK1/Nedd4-2 regulation of EAATs

One positive result was obtained when WNK1 gene-trap MEFs were made by Dr. Anthony Anselmo in our lab from embryos obtained from the lab of Chou-Long Huang. The gene-trap strategy fused β-geo to the first exon of WNK1 creating heterozygous and homozygous WNK1 1- 253:β-Gal expression (Zambrowicz et al., 2003). Because these

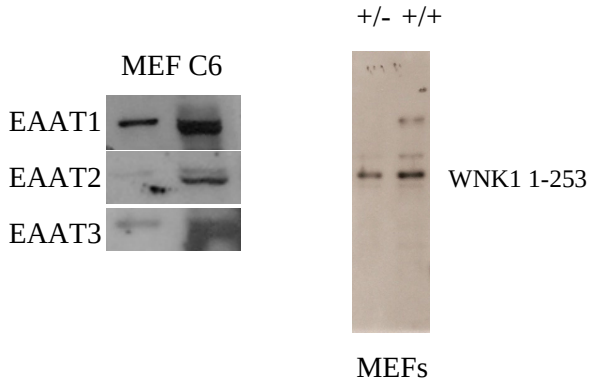


Figure 29. Expression of EAATs and WNK in MEFs. EAATs 1-3 are expressed in the WNK1 gene-trap MEFs. The WNK1:b-geo fusion is present in both heterozygous and homozygous gene-trap MEFs.

MEFs expressed EAATs (Fig. 29), uptake assays were performed. The model would predict that the amino-terminal fragment of WNK1 that our laboratory has shown to activate SGK1 *and* that is present in the gene trap MEFs would limit the activity of Nedd4-2, leading to increased EAAT activity (Fig. 30). This was indeed the result. Unfortunately, the MEFs did not survive more than five passages, despite the use of low oxygen culture conditions

suggested by Dr. Jerry Shay (www4.utsouthwestern.edu/cellbio/shay-wright/research/Low%20Oxygen%20Setup.0305.pdf). Even if the MEFs were viable, isolation of EAAT1 function would prove to be limiting and the chance that other glutamate/aspartate transporters were expressed in these MEFs could not be ruled out.

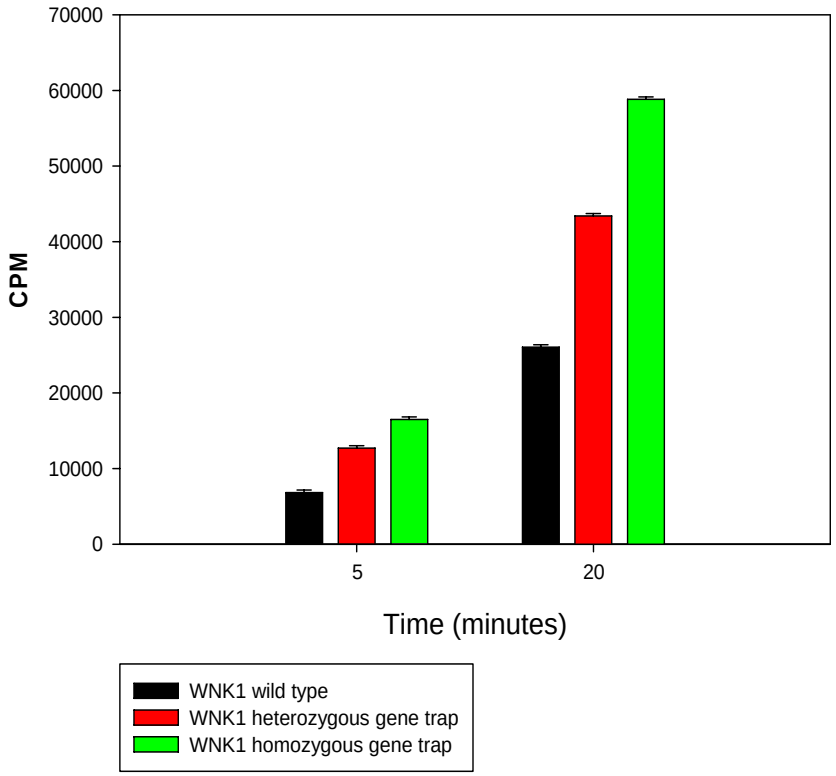


Figure 30 . D-aspartate uptake in gene-trap MEFs. MEFs expressing WNK1 1-253 in one allele or both alleles were compared to control MEFs from littermates homozygous for WNK1 wild type.

Discussion

Perhaps the single greatest shortcoming of this project was the reliance upon the data of one laboratory. Nearly every effort made to replicate these data produced no result. The lack of an effect from SGK1 kinase-dead, Nedd4-2 over-expression, IGF1 treatment, and

WNK1 1-220 $T_{58}A$ combined with the lack of HA-tagged ubiquitination lead us to conclude that, after more than a year, the project had run its course. The one positive outcome, the altered D-aspartate uptake in the gene-trap MEFs, was unsustainable as the MEFs would not survive passage. Another fact to consider in the analysis of this project lies in the absence of PY motifs in the predicted cytosolic tails of EAAT1. If this transporter is in fact regulated by Nedd4-2, the absence of such motifs, shown to be essential for Nedd4-2 WW domain binding, is curious.

Thus, this project was shelved. The departure of Dr. Xu, who had pioneered the research in WNK1 in our laboratory, was a great loss for our laboratory and this researcher. Nevertheless, the project he had managed for many years was now available. This serendipitous event and the lack of progress lead to work on the final project, discussed in the following chapter. The final chapter will include more discussion on this project.

CHAPTER IV

Introduction

As mentioned previously, SGK1 was identified as a WNK1 interacting protein in a yeast two-hybrid screen performed with WNK1 1-555 as the bait. This result preceded the discovery of a genetic linkage to PHAII by several years and, as such, we were left to discern *de novo* how WNK1 could interact with this protein.

IGF1

IGF1 activates SGK1 by a PI3K- and PDK-dependent mechanism. IGF1 activation upregulates outward potassium current in cells over-expressing the potassium channels Kv1.1-3 (Gamper et al., 2002). Work in our laboratory showed that siRNAi depletion of WNK1 abrogated the IGF1-dependent activation of SGK1 (Xu et al., 2005b).

PI3K

When 293 cells are treated with IGF1, SGK1 activity is increased in a PI3K-dependent manner and this activation is ablated by the pre-treatment of cells with wortmannin (Park et al., 1999). Additionally, 293 cells expressing a $S_{422}A$ mutant of SGK1 are no longer responsive to IGF1 or PI3K (Kobayashi and Cohen, 1999). Both PI3K and Akt contain a Plesktrin homology (PH) domain which is responsible for binding to phosphoinositides and is necessary for membrane localization. SGK1 lacks a PH domain, containing instead a carboxy-terminal hydrophobic (H) domain whose phosphorylation at S_{422} is both necessary for activation and PI3K-dependent (Kobayashi and Cohen, 1999; Park et al., 1999). However, the kinase responsible for phosphorylation at S_{422} remains undefined. One study showed that PI3K may affect ENaC open-probability and not affect

membrane localization (Tong et al., 2004). However, this study used excised patches from CHO cells, which cannot directly address insertion of new or retention of previously present ENaCs.

Akt

As mentioned previously, PDK is an activator of Akt. Work in our laboratory and others (Vitari et al., 2004) showed that Akt phosphorylates WNK1 at residue T_{58}. Mutation of threonine 58 to alanine results in the substantial reduction of PI3K- and IGF1-dependent activation of SGK1.

SGK1

Although generally SGK1 is broadly expressed in tissues and cell lines, immunofluorescence analysis showed that it was strongly expressed in the collecting ducts of embryonic mouse kidneys (Huber et al., 2001). Several lines of evidence suggest that SGK1 is involved in sodium balance. First, mineralocorticoids, which are among the more potent activators of SGK1 (Pearce, 2003; Fuller and Young, 2005), have an established role in the upregulation of sodium uptake in the aldosterone-sensitive distal nephron (Alvarez de la Rosa and Canessa, 2003). Second, *sgk1* -/- mice have impaired sodium retention on a sodium-deficient diet, despite having enhanced sodium uptake in the proximal nephron (Wulff et al., 2002). Moreover, *sgk1* -/- mice are hypotensive despite elevated levels of the mineralocorticoid aldosterone which strongly upregulates apical ENaC localization. Third, SGK1 is activated in response to cellular volume changes, both shrinking (Waldegger et al., 1997; Warntges et al., 2002) and swelling (Rozansky et al., 2002).

Nedd4-2

Of the two Nedd4 family members, Nedd4-2 and not Nedd4-1 is responsible for ubiquination of ENaC (Kamynina et al., 2001). Heterologous expression studies have shown that Nedd4-2 reduces ENaC current and surface expression of ENaC (Abriel et al., 1999; Goulet et al., 1998). Splice variants lacking combinations of WW domains do little to perturb the effect of Nedd4-2 activity towards ENaC as all four WW domains bind to all ENaC PY motifs. However, the presence of functional ENaC PY motifs is critical to its interaction with Nedd4-2 as neither the surface localization nor the activity of ENaC mutants lacking the PY motifs were affected when expressed in *Xenopus* oocytes (Abriel et al., 1999; Goulet et al., 1998). Inhibition of Nedd4-2 by antisense oligonucleotides and siRNAi leads to a 2-fold increase in endogenous ENaC current and a similar increase in the number of channels in A6 cells (Malik et al., 2005).

ENaC

As discussed in Chapter 1, ENaC is primarily responsible for the regulation of sodium in the aldosterone-sensitive distal nephron. The multimeric nature of ENaC is certain, and although the precise composition of subunits is not universally agreed upon, there is general consensus that a functional ENaC is comprised of 2 α, 1 β and 1γ subunits. Mutations in ENaC are linked to Liddle's syndrome, a genetic form of hypertension (Staub et al., 1997), and occur in the cytoplasmic tails of the β and γ subunits, affecting the functionality or even the very presence of the PY motifs. These mutations obviously implicate Nedd4-2 which binds to ENaC via these PY motifs.

Thus, a signaling pathway begins to emerge involving IGF-1, PI3K, Akt, SGK1, Nedd4-2, and ENaC. Because WNK1 interacts with SGK1 by yeast two-hybrid and co-immunoprecipitation, and additionally WNK1 is phosphorylated by Akt, our laboratory sought to explore the interaction further. We demonstrated that SGK1 is not a substrate of WNK1 (Xu et al., 2005a) and also that the amino-terminal 220 residues (a fragment that lacks the catalytic domain) bind to SGK1. Moreover, the fragment of WNK1 159-491 does not bind as well. Analysis of the mechanism of activation by WNK1 1-220 forms the basis of the final project.

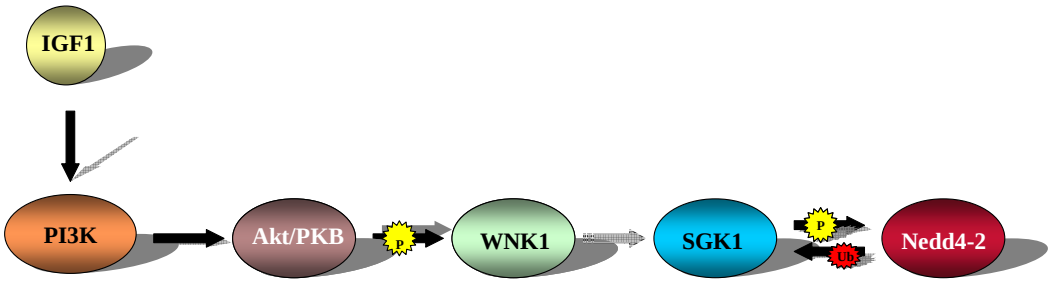


Figure 31. Proposed pathway for WNK1 activation of SGK1. IGF1 activates PI3K which activates Akt. Akt phosphorylates P WNK1 at threonine 58. WNK1 then activates SGK1 by an unresolved mechanism. SGK1 phosphorylates and is ubiquitinated Ub by Nedd4-2.

Rationale

IGF-1 binds to its receptor, which activates the insulin receptor substrate (IRS). IRS then binds the SH2-domain containing phosphatase, SHP-2, and the regulatory subunit of PI3K, p85. This then activates the catalytic subunit of PI3K, p110 leading to the activation of PDK and Akt (Cantley 2002). Seminal work by a colleague, Bing-E Xu, demonstrated that WNK1 is implicated in the regulation of ENaC by a IGF-1/PI3K/Akt/SGK/Nedd4-2-

dependent signaling pathway (Xu et al., 2005a & 2005b) (Figs. 31 and 32). What remains unresolved is how WNK1 activates SGK1.

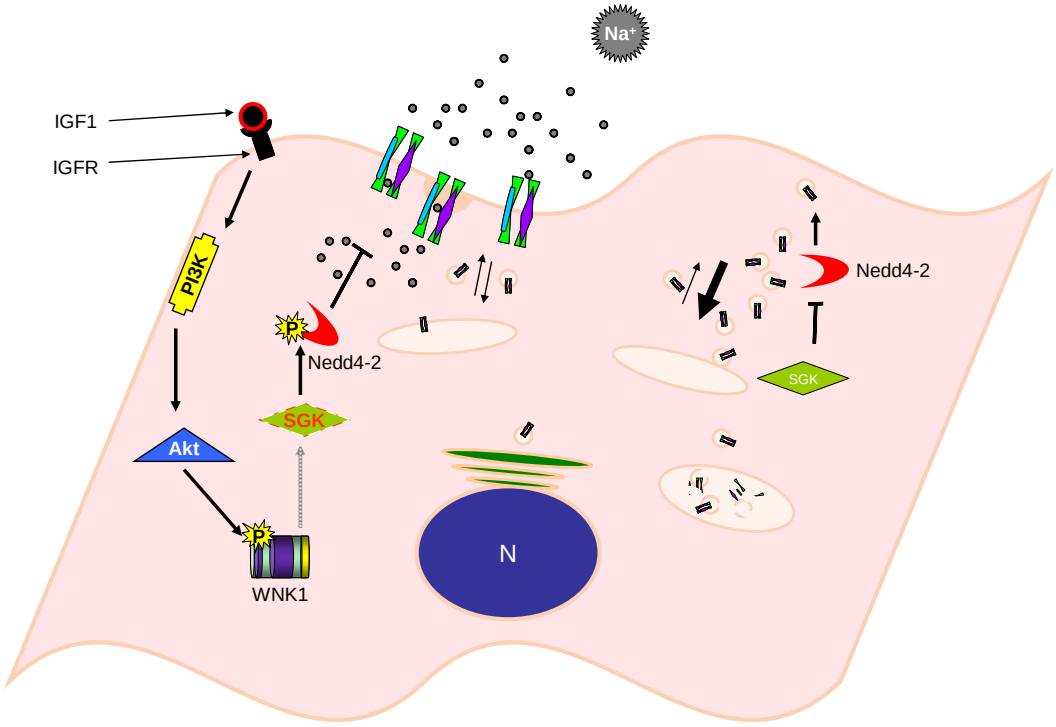


Figure 32. Schematic of proposed pathway of WNK1 activation of SGK1. IGF1 binding leads to PI3K activation of Akt which then phosphorylates WNK1. WNK1 activates SGK1 by an unknown mechanism. SGK1 phosphorylates Nedd4-2 which blocks its ability to interact with ENaC leading to increased sodium uptake. The right side of the cell represents ENaC regulation in the absence of WNK1. A larger portion of ENaC is internal and may be lysosomally degraded or stored in recycling endosomes.

Our laboratory along with that of Chou-Long Huang demonstrated that: WNK1 activation of SGK1 is IGF1-, PI3K-, and Akt-dependent; the amino-terminal 220 residues of WNK1 are sufficient for activation; and this amino-terminal fragment is sufficient to raise

ENaC activity (Xu et al., 2005a & 2005b). These analyses were performed in 293 and CHO cells as well as *Xenopus* oocytes.

That the amino-terminal fragment of WNK1 was sufficient to cause ENaC activation provided the rationale for the final project detailed below. This project would subsume three main objectives: first, based on the fact that WNKs can regulate each other, determine if WNKs 2, 3, and 4 are implicated in the regulation of this pathway; second, identify proteins that would interact with the amino-terminal piece(s) of WNK(s); and third, identify the minimally necessary fragment of WNK1 for SGK1 activation.

Methods

Reagents. The peptide substrate Crosstide was purchased from Upstate. Mouse monoclonal antibodies to the flag and HA epitopes and wortmannin were purchased from Sigma.

Tissue culture. 293 cells were grown in Dulbecco's modified Eagle's medium containing 5.5 mM glucose, 10% fetal bovine serum, pH 7.4, 2 mM L-glutamine, streptomycin and penicillin (100 units/ml) at 37 °C in 5% CO_2. DCT cells were grown in Dulbecco's modified Eagle's medium with 2 mM L-glutamine, 50 mM sodium pyruvate, and streptomycin and penicillin (100 units/ml), and 10% fetal bovine serum, at 37° C in 10% CO_2. Transfection was accomplished by $Ca_3(PO_4)_2$ using standard methods (Sambrook and Russell).

Protein purification. Munc18c in pGEX was a gift from Csiro Labs. Plasmids were transformed into chemically competent BL21 E. coli bacteria via standard protocol. A single colony was grown in 1000 ml Luria-Bertani Broth (LB) at 37° C shaking at 250 rpm until OD595 of 0.6-0.8 was reached. The culture was induced with 50μM isopropyl β-D-1-thiogalactopyranoside for 4 hours. Bacteria were pelleted in a Beckman J6B centrifuge with

a TS4.2 rotor at 4° C and 2700 x g for 15 minutes. The pellet was lysed in 30 ml of lysis buffer (50 mM Tris pH 8, 200 mM NaCl, 1% Triton X-100) and sonicated (40% amplitude, 1 second pulses, 3 cycles of 10 pulses each). The lysate was clarified by centrifugation (4° C /18,000 x g/ 30 minutes) and batch bound to glutathione agarose (4° C/rocking/1 hour). Resin was transferred to a column, washed with 50 bed volumes of buffer (50 mM Tris pH 7.4, 200 mM NaCl), eluted with 10 ml of elution buffer (50 mM Tris, 200 mM NaCl, 10 mM reduced glutathione, 1 mM DTT) and dialyzed overnight in wash buffer plus 10% glycerol. Protein concentration was assayed by the Bradford method, and purity was assessed by SDS-PAGE and Coomassie stain.

<u>In vitro kinase assay</u>: Over-expressed flag-tagged SGK1 was immunoprecipitated from cells (lysed in 50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 100 mM NaF, 0.2 mM Na_3VO_4 and 10% glycerol) and washed 3x with 20 mM Tris, pH 7.4, 1000 mM NaCl. GST-tagged Nedd4-2 (1 μg) or Crosstide (1 μg) was combined with the immunoprecipitated SGK1 in 30 μl reactions containing 10 mM HEPES, pH 8, 10 mM $MgCl_2$,1 mM benzamidine, 1 mM DTT, and 50 μM ATP containing 1 cpm/fmol γ-^{32}P ATP. Reactions were incubated at 30° C for 30 minutes. Nedd4-2 reactions were stopped by the addition of 7.5 μl of 5x SDS sample loading buffer and boiling for 2 minutes and proteins were resolved by SDS-PAGE, autoradiography, and liquid scintillation counting. Crosstide assays were stopped by placing on ice for 2 minutes. 25 μl of each reaction was transferred to Whatman P81 ion-exchange cellulose and washed 5x with 200 ml of 0.5% H_3PO_4. The cellulose was dried and spots were cut for analysis by liquid scintillation counting.

Results

Amino-termini of WNK1 and WNK4 activate SGK1

It has been demonstrated that the amino-terminus of WNK1, residues 1-159, is sufficient to activate SGK1 towards Nedd4-2 and, ultimately, ENaC (Xu et al., 2005a). This effect was replicated as the first step in this project and the optimal ratio of WNK1 1-220 DNA to SGK1 DNA was shown to be approximately 1:2 (Fig. 33). Determining the precise ratio was complicated by the fact that a relatively large amount of DNA was needed for WNK1 1-220 expression, thus limiting the amount of SGK1 that could be used without inducing toxicity. As the amino terminus of WNK2 was unavailable, only WNK3 and WNK4 were assessed for their ability to activate SGK1. Full-length WNK4 but not WNK3 was able to activate SGK1

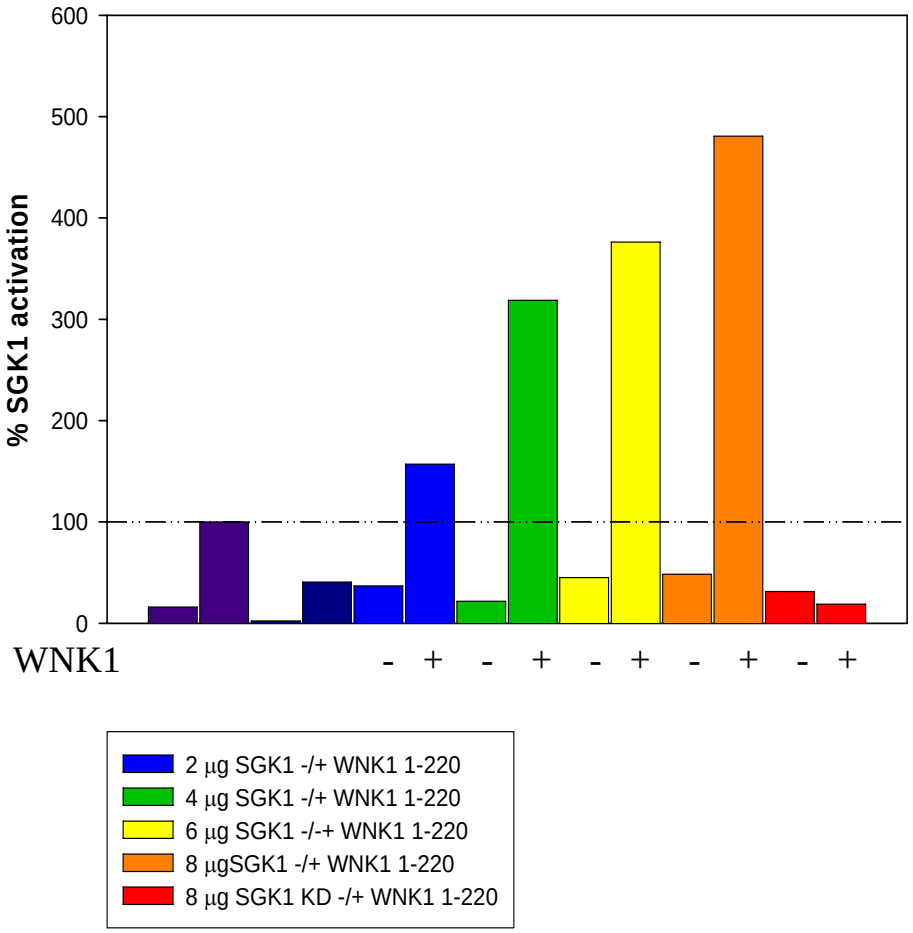


Figure 33. WNK1 activation of SGK1. WNK1 -1-220 and SGK1 full-length were overexpressed in 293 cells and used in an immunoprecipitate kinase assay directed against Crosstide. The first four columns are myc x flag, myc x SGK1, WNK1, myc x SGK1 kinase dead, and WNK1 1-220 x flag. SGK1 without WNK1 (lane 2) was used as the baseline.

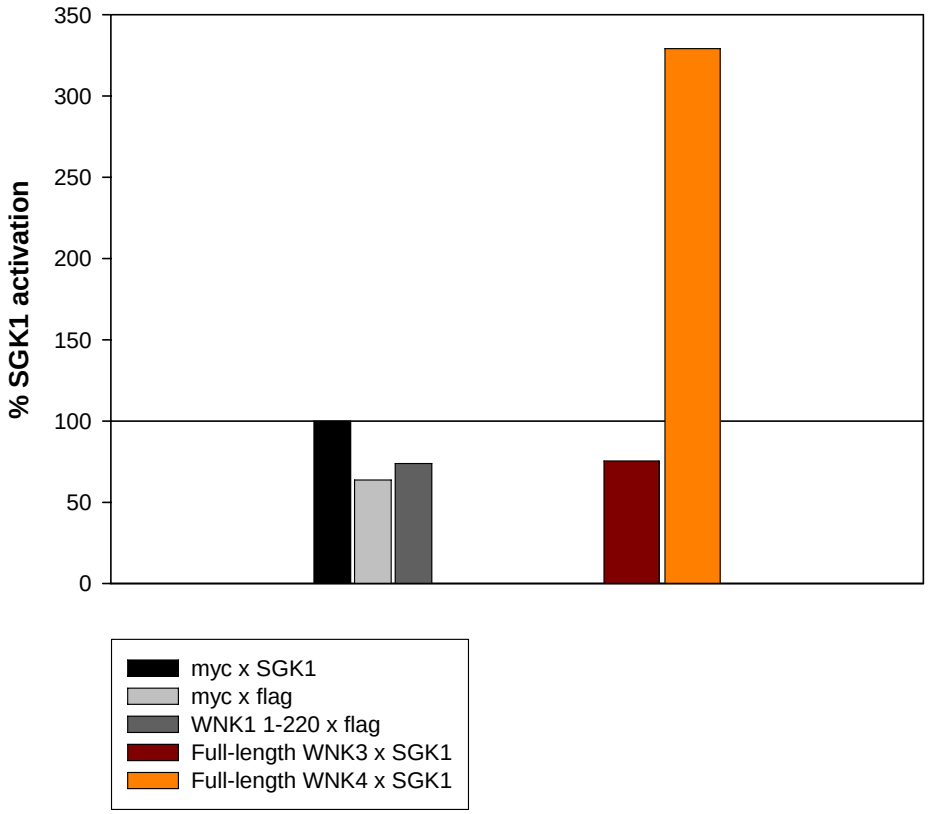


Figure 34. WNK4 activates SGK1 while WNK3 does not. Full-length WNK3 or WNK4 were over-expressed with SGK1 in 293 cells. SGK1 was immunoprecipitated and used in a kinase assay directed against Crosstide.

(Fig. 34). In fact, the amino terminal 144 residues of WNK4 were shown to activate SGK1 while WNK3 1-150 did not (Figs. 35 and 36). Interestingly, while WNK4 activates SGK1, it does not have the same effect on the downstream components of the pathway. Data from our laboratory and that of our collaborator, Dr. Huang, show that although WNK4 activates SGK, the outcome upon ENaC is not the same as WNK1. The inability of WNK3 to activate SGK1 is supported by research that showed that WNK3 was unable to increase ENaC activity (Leng et al., 2006).

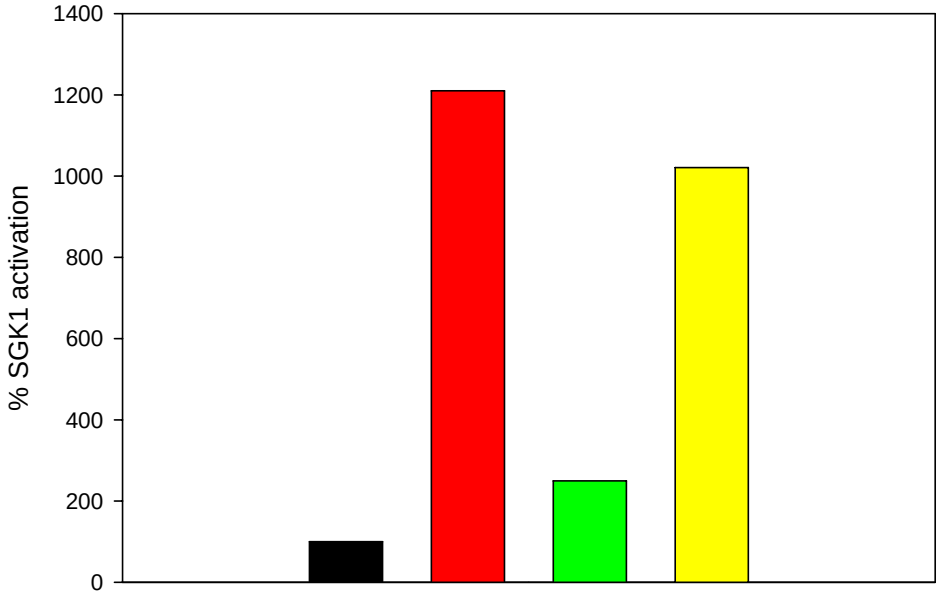


Figure 35. The N-terminal fragments of WNK1 and WNK4 activate SGK1. WNK1 and WNK4 fragments that precede the kinase domain are sufficient to activate SGK1 compared to vector alone or the Akt phosphorylation mutant $T_{58}A$.

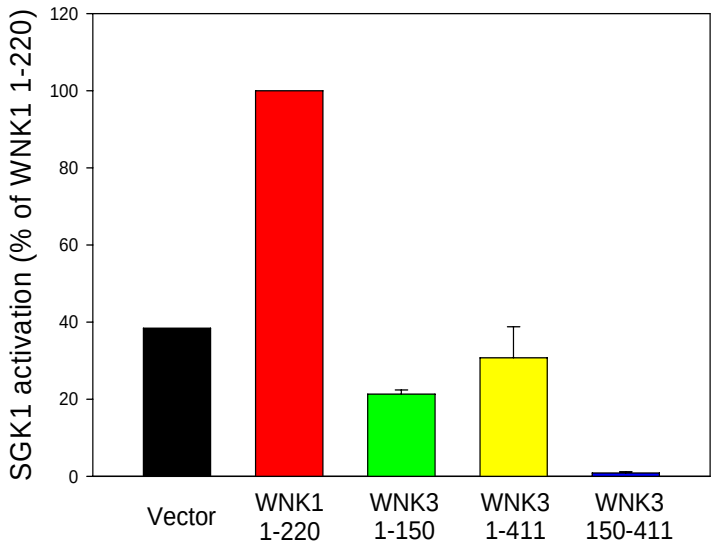


Figure 36. The N-terminal portion of WNK3 does not activate SGK1. WNK1 1-220 activation of SGK1 is greater than two-fold that of SGK alone (vector only). WNK3 amino-terminal pieces do not activate SGK1.

The question then becomes, what is the difference between the activators WNKs 1 and 4 and the non-activator WNK3? Cursory examination of the architecture of the WNK family shows that both WNK1 and WNK4 contain proline-rich motifs while WNK3 does not (Fig 37).

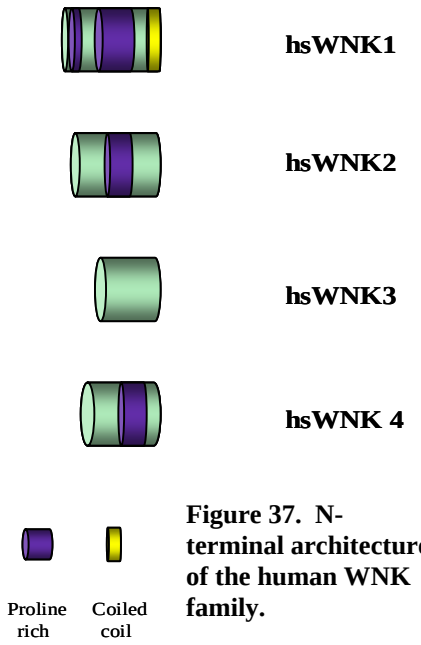


Figure 37. N-terminal architecture of the human WNK family.

Identification of proteins interacting with the WNK1 amino-terminus

The absence of proline-rich domains (PRD) in WNK3 and its corresponding inability to activate SGK1 made examination of the PRDs of WNK1 of interest. Full-length WNK1 has 24 PxxP SH3-interacting motifs while the amino-terminal 121 residues contain 4: $PxxP_{21}$, $PxxP_{97}$, $PxxP_{105}$, and $PxxP_{117}$. Scansite (www.scansite.mit.edu) prediction of potential interacting proteins is detailed in Figure 38.

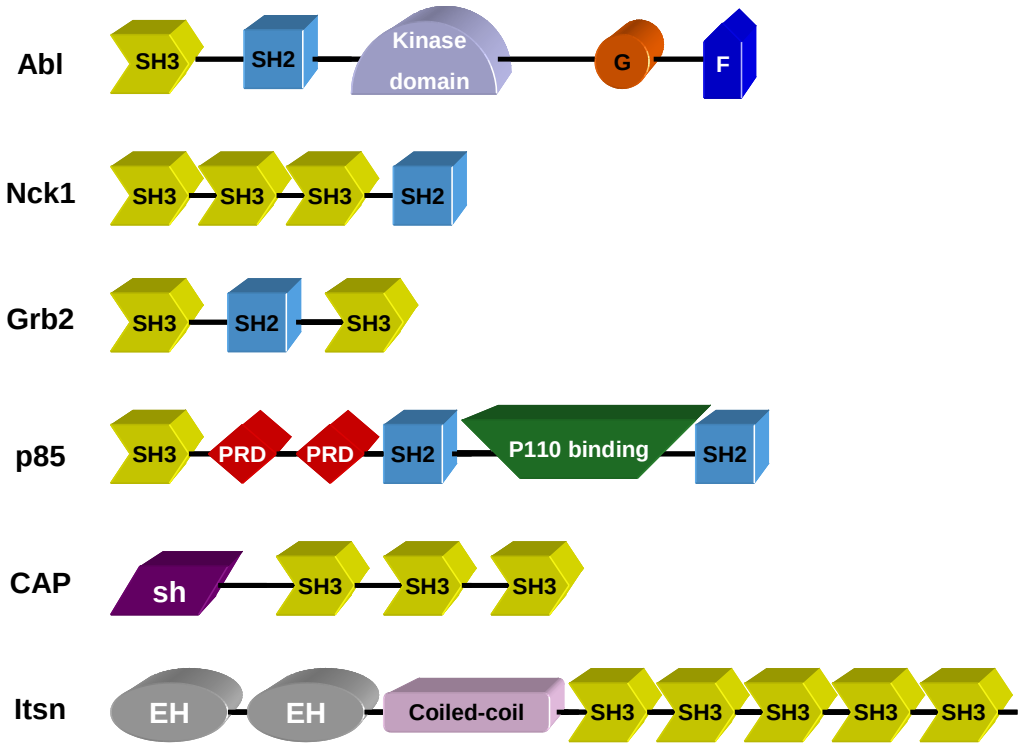


Figure 38. General architecture of the SH3 domain-containing proteins proposed to interact with WNK1. G – globular actin binding domain; F – filamentous actin binding domain; PRD – proline rich domain; sh – porcine hormone sorbin homology; EH – Eps15 (an EGFR tyrosine kinase substrate) homology domain.

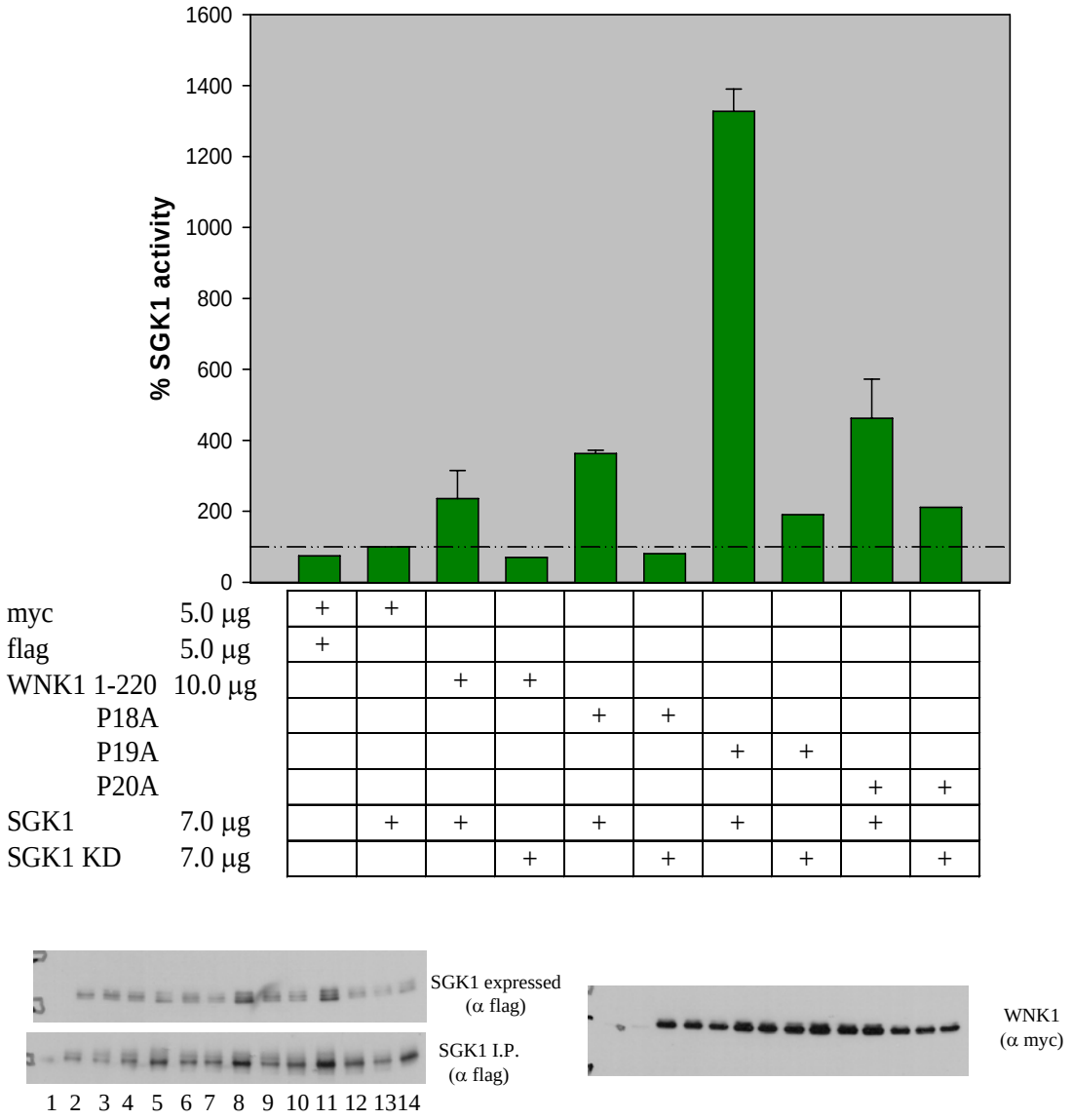


Figure 39. Activation by the PxxP single mutants. Single proline mutants of WNK1 1-220 PxxP motifs used to activate SGK1. Duplicates of each PxxP single mutants and SGK1 (lanes 3-4, 6-7, 9-10, 12-13) were compared to each single mutant and SGK1 kinase dead (lanes 5, 8, 11, and 14).

Initial tests using single mutations of the proline predicted by Scansite to be the important residue demonstrated that mutation of P_{19} to A resulted in increased activation of WNK1 1-220 towards SGK1 (Fig. 39). However, the use of single PxxP mutants was only

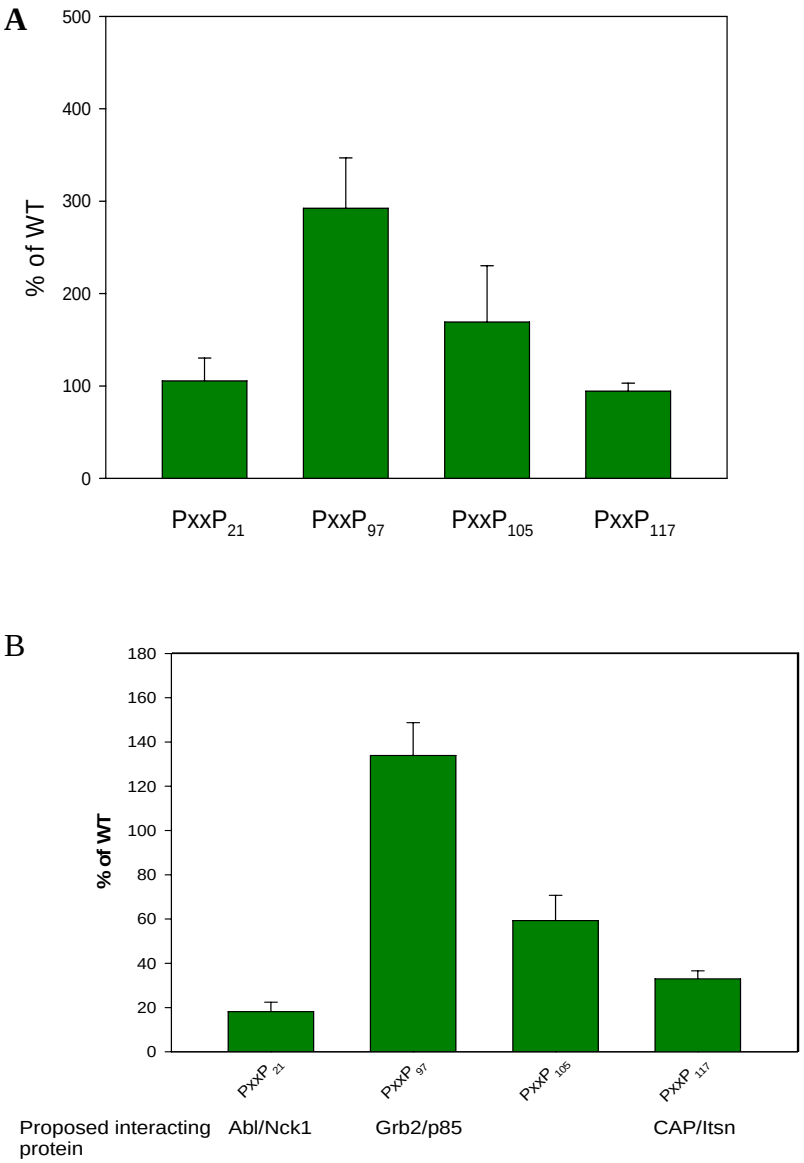


Figure 40. Activation of SGK1 by WNK1 1-220 PxxP complete mutants. *In vitro* kinase assays directed against Nedd4-2 (**A**) or Crosstide (**B**) show that while the trend is similar between the two different substrates (Nedd4-2 vs. Crosstide), the only consistant activation is by the $PxxP_{97}$ mutant.

an initial screen to determine if an effect would warrant further investigation. Therefore, four constructs were made with all prolines in each PxxP mutated to alanine creating $AAxA_{21}$, $AxxA_{97}$, $AxxA_{105}$, and $AAxA_{117}$. WNK1 1-220 wild type or PxxP mutant activation of SGK1 was directed against either recombinant Nedd4-2 or the peptide substrate Crosstide (Fig. 40). WNK1 $AxxA_{97}$ consistently elevated activation of SGK1 compared to wild type. Scansite predicted that P_{94} would interact with the molecular adaptor growth factor receptor-bound protein (Grb2) and P_{97} would interact with p85, the PI3K regulatory subunit. Initial co-immunoprecipitation of WNK1 1-220 with Grb2, Nck1, intersectin, and cbl-associated protein showed no interaction (Fig. 41).

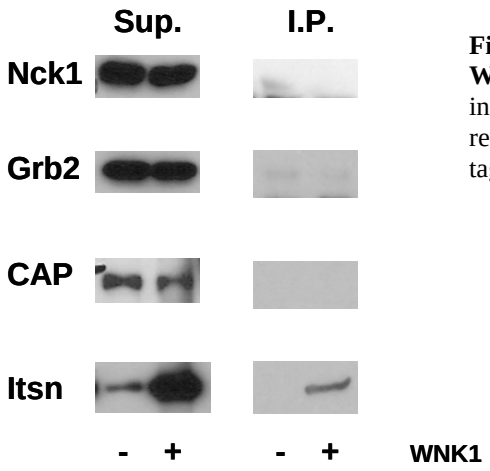


Figure 41. Co-immunoprecipitation of potential WNK1 interaction proteins. Proteins over-expressed in 293 cells were immunoprecipitated with their respective epitope tags and blotted for the respective tags on their proposed interacting proteins.

There is a potential interaction between WNK1 and Itsn. However, because two constructs (Grb2 and Itsn) were myc-tagged, a flag-tagged WNK1 1-640 was used to co-immunoprecipitate both Grb2 and Itsn. WNK1 1-640 and Itsn migrate similarly on an SDS gel and thus the potential interaction shown in Figure 37 must be evaluated further. Currently a flag-tagged WNK1 1-220 piece is being constructed which will address this problem.

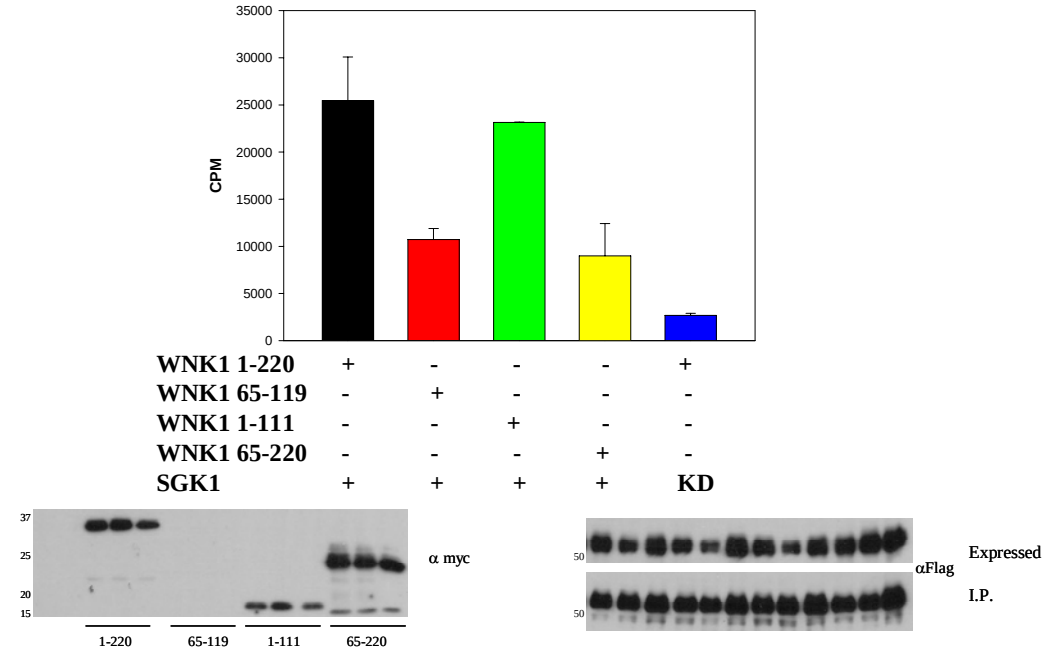
Lack of interaction between WNK1 1-220 and four of the six proposed interactors is perhaps not surprising. A yeast two-hybrid study conducted by Lisa Lenertz found no interacting proteins with the same piece of WNK1. Constructs for p85 and Abl are currently being obtained but no results are yet available. Failure to demonstrate interaction by co-immunoprecipitation may be a function of the location of the purported interaction. The predicted Abl/ and Nck1/PxxP21 interaction would occur within 19 residues of the epitope tag used to immunoprecipitate WNK1 1-220. Immunoprecipitating Nck1 with its epitope tag, hemagglutinin A, and blotting for WNK1 did not demonstrate an interaction, however.

WNK1 1-220 is important for SGK1 activation

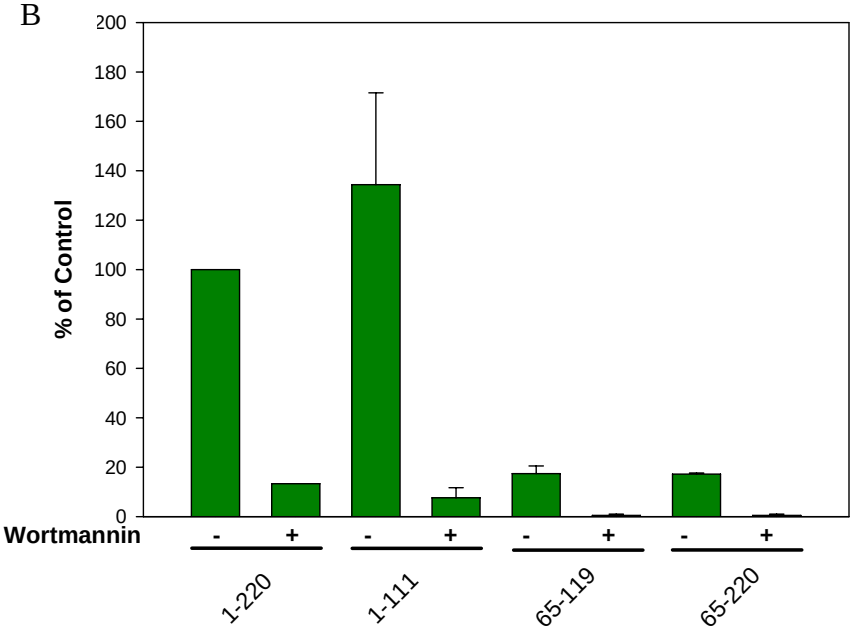
Work by Dr. Xu demonstrated that WNK1 1-220 activated SGK1 while WNK1 159-491 did not (2005a). This suggested that the fragment of WNK1 that was minimally needed for SGK1 activation was within residues 1-159. Accordingly, truncations were made based upon a secondary structure prediction program (Jpred, www.expasy.ch/tools/) that would either include the Akt phosphorylation site T58 (WNK1 1-111) or exclude it (WNK1 65-220 and WNK1 65-119). WNK1 65-119 was not an intentional design, but rather the result of a premature stop codon in the template. The presence of this stop codon is the most likely explanation as two separate PCR amplifications produced the same insert and the likelihood of two identical mutations in two successive runs is infinitesimally small.

When the fragments 65-119 and 65-220 of WNK1 are used to activate SGK1, a small but consistent effect is seen (Fig. 42a). However, this effect is wortmannin-sensitive (Fig. 42b).

A


35000
30000
25000
20000
15000
10000
5000
0
CPM
WNK1 1-220 + - - - +
WNK1 65-119 - + - - -
WNK1 1-111 - - + - -
WNK1 65-220 - - - + -
SGK1 + + + + KD
37
25
20
15
α myc
1-220
65-119
1-111
65-220
50
αFlag
Expressed
50
I.P.

B


200
180
160
140
120
100
80
60
40
20
0
% of Control
Wortmannin
- + - + - + - +
1-220
1-111
65-119
65-220

C

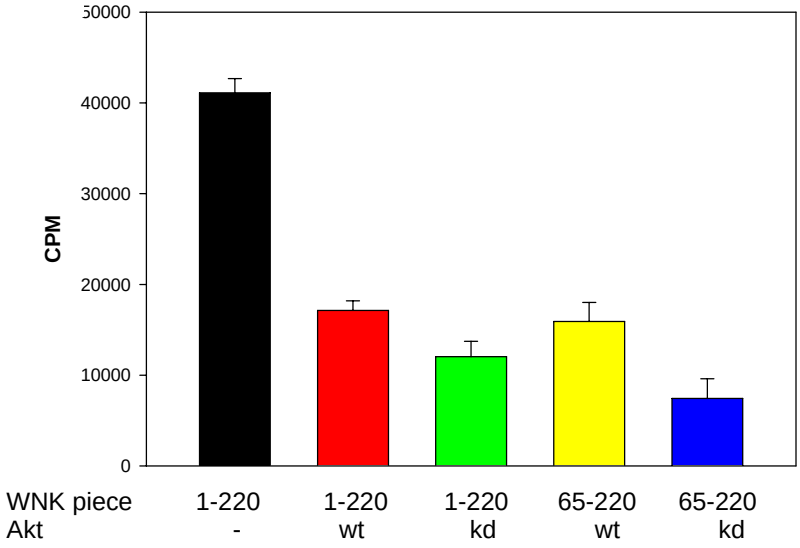


Figure 42. WNK1 fragments lacking T_{58} are still affected by PI3K-dependent regulators. A. WNK1 65-119 and 65-220 show modest activation of SGK1. Expression of 65-119 could not be verified by immunoblot despite attempting many alternate methods **B.** WNK1 65-220- and 65-119-mediated SGK1 activation is susceptible to Wortmannin. **C.** The activation of SGK1 by WNK1 65-119 and 65-220 is reduced by kinase-dead Akt.

Using the kinase dead construct of Akt reduces the WNK1 activation of SGK1. Assuming that the action of PI3K on this pathway is mediated solely through the phosphorylation of WNK1 T58 by Akt, it should not have the same effect on a piece of WNK1 lacking the Akt phosphorylation site T_{58} but it did (Fig. 42c). This apparent contradiction might be explained by the fact that WNK1 exists as a tetramer (Xu et al., 2002). If over-expression of this piece of WNK1 enhances oligomerization with endogenous, full-length protein for example, that would explain the sensitivity to wortmannin of a piece that lacks the PI3K-dependent Akt phosphorylation site. Knocking down the expression of endogenous WNK1 with siRNAi might address this conflict.

Discussion

Data are accumulating supporting the supposition that the fragment of WNK1 responsible for the WNK1-dependent activation of SGK1 lies in the amino terminus. Again, as this fragment lacks the kinase domain, this activation is indirect which suggests that there must be intermediaries; proteins that propagate the signal from PI3K to SGK1 in a WNK1-dependent manner. The fact that co-immunoprecipitation and yeast two-hybrid analyses failed to identify any candidates should not be taken as contrary to this argument. Interactions may be transient, dependent on other proteins yet to be identified, or may rely on sub-cellular localization that could not be replicated *in vitro*. The next chapter will be used to further explore possible models and mechanisms of this signaling pathway and to discuss experiments to elucidate this physiologically important signaling cascade further.

CHAPTER V

Synopsis

Introduction

It is, hopefully, apparent by this point that the WNK family of protein kinases plays a vital role in the maintenance of ion regulation. There is simply too much evidence associating WNKs with proteins involved in vesicular traffic and ion transport to be dismissed as circumstantial. Nevertheless, the mechanism by which the WNKs exert this effect remains elusive. It is unlikely that WNKs are master regulators involved in systemic regulation or their identification would not have been so recent. Indeed, PHAII mutations in WNK1 and WNK4 have a relatively mild phenotype. Also, one would expect that in proteins as large as those of the WNK family, the potential for mutation is correspondingly large. Despite this possibility, WNK1 is an essential gene.

Chapter II: WNKs and vesicular trafficking.

Discussion

The connection between WNK1, Syt2, and Munc18c is intriguing but unresolved. One major difficulty lies in establishing a means of investigation. As noted above, the endocytosis assay was never successfully sensitive and although it would be a powerful assay, it is curious why only three laboratories publish data using this assay and those authors were all members of the laboratory that developed that assay. Otherwise, it would be expected that this assay would be widely employed by the myriad of researchers studying

this process. Nonetheless, data exist that link these proteins and some semblance of a model is needed.

Models

WNK1 binds to and phosphorylates Syt2. Because Syt2 has no catalytic function, this suggests that the interaction with WNK1 somehow alters the ability of Syt2 to bind to other proteins of phospholipids. This assumption suggests two possibilities. First, phosphorylation of Syt2 in its second C2 domain may disrupt its ability to bind to the μ2 subunit of AP2. In this manner, WNK1 would reduce endocytosis, preventing a cell from recovering channels and transporters from the cell surface. This possibility would support a model where the WNK1 over-expression that results in PHAII (caused by increased uptake of ions), could be a result of the increased plasma membrane residence of ENaC. Second, WNK1 phosphorylation of Syt2 has been shown to right-shift the calcium-dependent affinity of Syt2 for phospholipid vesicles. This shift in affinity can be interpreted as a reduction and, as such, the model could then be that this phosphorylation could disrupt the forward trafficking of membrane proteins by reducing the calcium-dependent fusion of vesicles laden with these transporters or channels. This possibility, however, conflicts with the data on WNK1 over-expression in PHAII patients. If PHAII patients have an excess expression of WNK1 and that leads to a reduction in the forward trafficking of channel-laden vesicles, ionic uptake would be reduced.

The interaction between Munc18c and WNK1 is also difficult to characterize. This interaction was observed using the first 555 residues of WNK1. However, data contained here demonstrate that Munc18c is not a WNK1 substrate. Recent data from our collaborator,

Dr. Thurmond, demonstrate that the residues including the kinase domain and a small amino-terminal piece of WNK1 interact with Munc18c. Should this be the case, potential interactors with WNK1 1-220 from Chapter 4 may provide a crucial link. In other words, several of the putative WNK1 interacting proteins, such as Nck1 and p85, contain SH2 domains as well as SH3 domains. The latter are predicted to interact with the PxxP motifs of WNK1 while the former are known to interact with tyrosine-phosphorylated proteins. Considering that recent data demonstrate that Munc18c is tyrosine phosphorylated, this presents an interesting avenue of research.

Future directions

Furtherance of this project awaits the creation or adaptation of a facile assay. The Smythe Schmid endocytosis assay is *not* that assay. Given the sheer number of investigators researching this topic, it is unlikely that this author can make any salient proposals. The study of WNK1 phosphorylation of Syt2 is complicated by the fact that this phosphorylation is not robust. However, one immediately obvious approach would be to express phosphomimetic constructs of Syt2, T202E and/or T386E, in *in vivo* or *in vitro* and assess their effect on the internalization of transferrin, for example.

Speculation on suggestions for the examination of Munc18c is even harder. But one tactic would be to identify the specific residues of Munc18c that are required for binding to WNK1. If WNK1 binds to the area of Munc18c that contains the newly identified tyrosines whose mutation ablates glucose-stimulated insulin exocytosis, a model could be envisioned where WNK1 binding to Munc18c alters this tyrosine phosphorylation, thereby affecting exocytosis. Moreover, Dr. Thurmond has data that shows that insulin secretion is dependent

upon a WNK1/Munc18c interaction. It could be that WNK1 binding Munc18c is necessary for recruiting and/or coordinating the unspecified tyrosine kinase. This possibility makes the elucidation of WNK1 binding partners particularly interesting, especially considering that the 4 of the 6 interactors discussed in Chapter 4 contain tyrosine-phosphorylated protein interacting SH2 domains.

Chapter III: WNK1 and EAAT1

Discussion

The fact that one laboratory is responsible for the majority of the research upon which this project was based makes the argument for further research tenuous at best. Despite this, the overlap of regulatory proteins between this project and the next support some speculation.

Models

Kinase-dead SGK1, over-expression of Nedd4-2, and treatment with IGF1 had no discernable effect on EAAT1 activity. Based on these results, it would seem that the model proposed, suggesting that WNK1 activity would increase the activity of EAAT1, is not valid. Nevertheless, the actual mechanism may be valid while the means used to analyze the system were inappropriate.

There are no data to suggest that endocytosis of EAAT is constitutive or stimulus driven. As such the WNK1-dependent reduction in EAAT1 endocytosis may not, in and of itself, be sufficient to cause a measurable level of reduction in EAAT1 activity. It is possible that EAAT1 plasma membrane time-in-residence is sufficiently long that recovery by endocytosis is a relatively rare event. Conversely, the addition of aspartate to these cells might cause a profound increase in the transport of EAAT1 to the plasma membrane,

especially in cells specifically tasked with recovery of glutamate and aspartate *in vivo*. As such, the addition of substrate designed to measure the activity of the transport may irreparably perturb the system.

Future directions

Given this possibility, the first step in pursuing this project would be to determine the lifetime of EAAT1 in general as well as the time spent in plasma membrane residence. The overall lifetime of the protein would be a significant first step in examining the question of whether or not EAAT1 is recycled or merely degraded upon endocytosis and pulse-chase is a proven method to analyze this question. To address the time that EAAT1 spends on the plasma membrane, biotin labeling and cellular fractionation could be employed under basal (low glutamate) and stimulatory environments.

Chapter IV: WNK1, SGK1, Nedd4-2, and ENaC.

Discussion

In the research described above, WNK1 connects two established signaling pathways. The mechanism by which WNK1 is activated by IGF, PI3K, and Akt is beginning to be resolved and the mechanism of the downstream components (SGK1/Nedd4-2/ENaC) has been well researched. What remains enigmatic is the precise nature of the role of WNK1. While the data in this document have hopefully added to the overall understanding of the role of WNK1 in ENaC regulation, another contribution will be the intercalation of data into several testable models.

Models

Our current understanding is represented in figure 32. However, the indirect role of WNK1 in this pathway is certainly less than satisfying. Herein, each of the six suggested SH3-containing proteins will be examined. Assuming that they are actual WNK1-interacting proteins, possible mechanisms will be discussed.

1. Abl – Abl is most notably known as a transforming factor when a genetic translocation fuses Abl to BRC, leading to chronic myelogenous leukemia (reviewed in Ren, 2005). The tyrosine kinase activity of Abl is required for transformation and is sensitive to the imatinib kinase inhibitor Gleevec. The non-transforming function of Abl receives markedly less attention but it is known to be a non-receptor tyrosine kinase that shuttles between the plasma membrane and the nucleus (di Bari et al., 2006). Abl is characterized by one SH3 and one SH2 domain that lie amino-terminal to the kinase domain as well as carboxy-terminal globular and filamentous actin interacting domains. A connection between WNK1 and a noted actin polymerization mediator such as Abl could provide clues as to the localization of the WNK1/SGK1/Nedd4-2/ENaC signaling complex as well as the mechanisms of its function.
2. Nck1 – Characterized by three SH3 domains and one SH2 domain, Nck1 interaction with WNK1 is potentially very exciting. Nck1 has been implicated in the regulation of cytoskeletal rearrangement in such processes as cytokinesis, neuronal outgrowth, and actin polymerization (Bladt et al,. 2003; Rivera et al., 2004; Holland et al., 1997). The SH2 domain of Nck1 has been shown to bind to

several tyrosine phosphorylated proteins including platelet-derived growth factor receptor, epidermal growth factor receptor, and vascular endothelial growth factor receptor. A complex formed between WNK1, Nck1, and the IGF1 receptor would not require a large intellectual leap and would further provide a tantalizing link between the activation of this pathway by IGF1 and its downstream target, WNK1. Also, the SH3 domain of Nck1 has been shown to bind to the p21-activated kinase (Pak1) which can then lead to actin polymerization or membrane ruffling (Bladt et al., 2003).

3. Grb2 – Grb2 associates with Sos via its SH3 domains (Sastry et al., 1995). Upon activation by growth factors, the SH2 domain of Grb2 binds to receptor tyrosine kinases bringing Sos with it which activates the MAPK pathway via Ras (Rozakis-Adcock et al., 1993). Connection of WNK1-dependent activation of SGK1 to Grb2 provides another potential link to IGF.
4. p85 – The most direct connection of any of these molecular adaptors to WNK1 is obviously p85. Because WNK1-dependent activation of SGK1 is reliant on PI3K, WNK1 binding the regulatory subunit of PI3K would be the most intuitive. Moreover, data in our laboratory show that the requirement of PDK for WNK1 activation of SGK1 is PI3K-independent. PDK is an activator of the PI3K-target Akt and of SGK1 itself, thus, the conundrum of PI3K/PDK independence. If p85 binds directly to WNK1 $PxxP_{97}$, p110 may be in direct apposition to Akt binding at WNK1 T_{58}, thus obviating the need for PDK activation.

5. CAP – Cbl-associated protein, or CAP, is an accessory factor for the ubiquitin ligase Cbl. Cbl is a member of the RING (really interesting new gene) family of ubiquitin ligases which have no intrinsic ligase ability, depending instead upon the high-energy thioester bond formed between the E2 family of ubiquitin activating proteins and ubiquitin itself. Cbl has been shown to be essential for the internalization and degradation of insulin-like growth-factor receptor and is also a substrate of Nedd4-2 (Rotin, 2000). One could then envision a scenario where WNK1 binding to CAP releases Cbl to internalize IGFR, thus terminating the signal. Additionally, over-expression of CAP has been shown to induce stress fiber and focal adhesions in NIH-3T3 cells (Ribon et al., 1998).
6. Itsn – Intersectin was discovered in a yeast two-hybrid screen for proteins that interact with SNAP-25 (Okamoto et al., 1999). Part of the exoocytic machinery, neuronal SNAP-25 and its ubiquitously expressed homolog SNAP-23 form the heterotrimeric SNARE complex with their cognates synaptobrevin and syntaxin. Itsn is rife with SH3 domains and WNK1 $PxxP_{117}$ is predicted to bind to the most amino-terminal one, SH3A. Other proteins that bind to Itsn SH3 domains are dynamin and synaptojanin (Okamoto et al., 1999) providing yet another possible connection of the WNK1-dependent SGK1 activation for the down-regulation of ENaC to the endocytic machinery. Indeed, over-expression of the SH3 domains of Itsn blocks epidermal growth-factor receptor endocytosis (Tong et al., 2000).

The aspect of this project that has perhaps generated more questions than answers comes from the data that show a small Akt-independent SGK1 activation effect. The amino-terminal fragments of WNK1 that lack the T_{58} Akt phosphorylation site still activate SGK1. This effect can be dismissed as a vagary of the assay were it not for the consistent result obtained by Dr. Xu that shows WNK1 T_{58}A activation is approximately 10-15% of wild type WNK1. It is for this reason that another facet of this signaling cascade must be considered. This seemingly anomalous possibility combined with the PI3K-independent PDK activation of SGK1 makes explicit that we are tyros at this time.

Finally, the crosstalk between the WNK family members is only now beginning to be appreciated. For example, the fact that WNK1 and WNK4 activate SGK1 to differing ends demonstrates the complexity of this task. The other notable instance is the data that show WNK1 and WNK4 work at cross purposes in the regulation of NCCT. Whether the WNK family is the nexus of the regulation of ENaC specifically and ion transport in general, remains to be determined.

Future directions

Examination of the purported interacting proteins may be facilitated by moving the epitope tag to the carboxy-termini. WNK1 interactions with Nck1 and Abl in particular, may not be realized because of the closeness of the epitope tag and the PxxP motif. Far Western analysis could also be employed to examine these putative interacting proteins.

Another distinct and sobering possibility is that WNK1 does not interact with any of the six predicted partners. WNK1 binding could instead cause allosteric changes that lead to the elevated activity of SGK1. Structural studies of a WNK1 1-220/SGK1 complex, already

underway in our laboratory, might shed light on this possibility. An additional approach would be direct kinetic analysis of the effects of purified WNK1 amino-terminal pieces on an *in vitro* kinase assay pairing SGK1 with Nedd4-2. If a WNK1 amino-terminal fragment was sufficient to cause an elevation in SGK1 activity, the regions of SGK1 necessary for binding the WNK1 fragment could be determined. Mutation of these residues and a corresponding drop in the WNK1-mediated activation would be powerful support of this hypothesis.

Conclusions

Work on WNK can be daunting, owing to its immense size and lack of defined domains. In such a case, biochemical analysis is still *de rigueur*. Parsing proteins to determine function is what remains, given the fact that WNK1 knock-out is embryonic lethal. The kinase domain comprises less than one-sixth of WNK1, which could suggest that catalytic activity is so specialized, due to its unique structure, that it becomes subordinate to its scaffolding function. If this is the case, then WNK1 discovery has only just begun and the cell-biological techniques employed by other laboratories are unlikely to divine any precise mechanistic details of WNK1 function. Discovery of a *bona fide* substrate of WNK1 would help to further the project. As yet, the best candidate is the oxidative stress-response kinase (OSR1) which provides yet another tantalizing link to the process of cellular ionic flux. Further research on the actions of WNK family kinases will help to unravel the complexities of kidney function and potentially provide relief for the millions of people suffering from hypertension.

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VITAE

Charles John Heise was born in Beatrice Nebraska, the fourth child of Mary Lee and Herman Heise. After studies at Colorado State University, he completed his Bachelor of Arts in Aviation Business Management in 1988 at Metropolitan State College in Denver Colorado. Following five years of work in varied pursuits, he matriculated at California State University, Chico where he earned a Masters of Arts in Psychology while simultaneously completing the requisite courses for admission to the University of Texas Southwestern Medical Center's Molecular and Cellular Biology graduate program. He worked full-time at night to fund these endeavors, attending school full-time during the day. Needless to say, graduate school has been a welcome respite from such strenuous work.

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Education:

Ph.D., University of Texas Southwestern Medical Center, Biological Chemistry, 2007
M.A., California State University, Chico, California, Psychology, 1999
B.S., Metropolitan State College, Denver, Colorado, Aviation Business Management, 1986

Professional Experience:

2001-2007 - Graduate Student, University of Texas Southwestern, Dallas, Pharmacology
1998-2000 - Research Assistant, California State University, Chico, Biology
1996-1999 - Graduate Teaching Assistant, California State University, Chico, Psychology

Expertise and Interests:

My expertise includes basic molecular biological techniques, a variety of microscopic techniques such as fluorescence, scanning electron, transmission electron as well as enzyme kinetics, protein purification, FPLC, sub-cloning, site-directed mutagenesis, sub-cellular fractionation, pulse-chase, and analytical ultracentrifugation. Additionally, I mentored several rotation students and undergraduate summer students.

Research:

My research has centered on the novel serine/threonine protein kinase, WNK1 (with no K/lysine) first discovered and characterized in our lab. This protein is implicated in blood pressure homeostasis, ostensibly at the level of ion transporter regulation.

In our original screen, WNK1 was shown to interact with the serum and glucocorticoid-inducible kinase 1 (SGK1) and Munc18c. Further screens by another graduate student in our lab identified synaptotagmin 2 (Syt2) as a WNK1 interactor. Using the connection of WNK1 with two proteins involved in endocytosis and exocytosis, I characterized the endocytic relationship between WNK1 and Syt2 and Munc18c using the scrape load assay of Schmid and Smythe as well as subcellular fractionation.

Another lab demonstrated that SGK1 and another protein of interest to our lab, the E3 ubiquitin ligase neuronal cell precursor-expressed developmentally-down regulated protein 4-2 (Nedd4-2), were involved in the regulation of a family of glutamate/aspartate transporters, the excitatory amino acid transporters (EAATs). I endeavored to link WNK1 upstream of SGK1 and Nedd4-2 using ^{3}H-Asp uptake assays, live-cell fluorescence microscopy and co-immunoprecipitation.

Currently I am focused on the signaling pathway of the WNK family members on the ion channel ENaC (epithelial Na^+ channel). Our lab has shown that WNKs 1 and 4, both proteins linked to a type of hereditary hypertension, are involved in the activity of ENaC via SGK1 and Nedd4-2. I am using *in vitro* kinase assays to examine the impact of the N termini of WNK family members on this pathway. It would appear that the SH3 binding motifs in this region of WNK1 are essential for this influence and to that end, I am performing a pseudo scanning alanine mutagenesis of these motifs to elucidate the necessary residues.

Funding Received:

National Institutes of Health (NIH): Recipient of training in the NIGMS, Cellular and Molecular Biology Training Grant, $50,000, from 2004 to 2006.

Other Expertise:

From 1988 to 1999, I was employed in business management and social work.

Publications:

- Oh, E, **Heise, CJ**, English, JM, Cobb, MH, and Thurmond, DC. WNK1 is a novel regulator of Munc18c-Syntaxin 4 complex formation in SNARE-mediated vesicle exocytosis. In Preparation.

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Conference Proceedings/Abstracts:

- Oh, E, **Heise, CJ**, English, JM, Cobb, MH, and Thurmond, DC. (2007). WNK1 is a novel regulator of Munc18c-Syntaxin 4 complex formation in SNARE-mediated vesicle exocytosis, Abstract, American Diabetes Association, Chicago, Il.

- **Heise CJ**, Xu BE, Stippec S and Cobb MH (2006). WNK1 increases ENaC activity via SGK1 and Nedd4-2, Abstract and poster, Cytoskeletal Dynamics Conference, Montreal, QC.

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