WITH NO LYSINE (WNK) FAMILY PROTEINS AND THEIR INTERACTIONS

WITH DOWNSTREAM KINASES

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DEDICATION

I would like to thank my family for all of the support to get me to this point in my life; my girlfriend, Pooja Paranjpe, for putting up with the vagaries of my path through endless education; the Medical Scientist Training Program and all of my friends for taking this route with me; members of the Cobb lab for a great experience these past four years; and most especially, Melanie Cobb, for being an amazing mentor who has had incredible faith in me, and without whom this dissertation would not be possible.

WITH NO LYSINE (WNK) FAMILY PROTEINS AND THEIR INTERACTIONS WITH DOWNSTREAM KINASES

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With no lysine (WNK) kinases are a family of protein kinases characterized by unusual kinase domain architecture. These large proteins, divergent outside of a kinase core and protein-protein interaction motifs, have been associated with pseudohypoaldosteronism 2, a form of Mendelian-inherited hypertension, and numerous downstream effectors that regulate vesicle trafficking, membrane protein localization, and ion handling. This study shows that WNK2 is also a functioning protein kinase with the same unusual kinase domain architecture and regulation by an autoinhibitory region. Like WNK1, WNK2 is able to signal to the extracellular-signal regulated kinase 5

(ERK5) pathway. One effector for WNK1 is oxidative stress responsive 1 (OSR1), a sterile20-like kinase. All four WNKs are able to phosphorylate OSR1 and stimulate its activity toward an ion transporter substrate, to roughly a similar degree. The WNKs have similar kinetic properties, with K_m toward OSR1 in the micromolar range and k_{cat} near 1 min^{-1}. No significant differences in activity toward OSR1 were seen for a mutant kinase domain at a site divergent among the WNKs that shows differential binding to substrate. Analysis of the phosphorylation sites of OSR1 reveals multiple sites along the activation loop that can promote increased activation if carrying a negative charge. It is unknown if these sites are phosphorylated in vivo. However, a second site of WNK1 phosphorylation just outside of the OSR1 kinase domain does not seem to affect WNK-OSR interactions. Further studies of interactions of the WNKs with their downstream effectors will reveal unusual functions for this unique family of proteins.

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PRIOR PUBLICATIONS

Lenertz, L.Y., Lee, B.H., Min, X., Xu, B.E., Wedin, K., Earnest, S., Goldsmith, E.J., Cobb, M.H. (2005). Properties of WNK1 and implications for other family members. *280*, 26653-26658.

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AGC kinases – containing protein kinase A, protein kinase G, protein kinase C

APRR1 – *Arabidopsis* pseudo response regulator 1

ATP – Adenosine triphosphate

ATPase – Adenosine triphosphatase

BMP – Bone morphogenetic protein

C. elegans – *Caenorhabditis elegans*

C terminus – Carboxy terminus

CD region – Common docking region

CFEX – Chloride-formate exchanger

CFTR – Cystic fibrosis transmembrane conductance regulator

CHO – Chinese hamster ovary

COUP-TFII – Chicken ovalbumin upstream promoter transcription factor II

DCT – Distal convoluted tubule

DMEM – Dulbecco's modified Eagle's medium

DTT – Dithiothreitol

E. coli – *Escherichia coli*

EAAT1 – Excitatory amino acid transporter 1

EDTA – Ethylenediaminetetraacetic acid

EGF – Epidermal growth factor

ENaC – Epithelial sodium channel

ERK – Extracellular signal-regulated kinase

ESCRT – Endosomal sorting complex required for transport

FBS – Fetal bovine serum

GABA – γ-amino butyric acid

GFP – Green fluorescent protein

GLUT4 – Glucose transporter 4

GST – Glutathione-S-transferase

GTPase – Guanine triphosphatase

HA – Hemagglutinin

HCK – Hemopoeitic cell kinase

HEK – Human embryonic kidney

HEPES – 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid

HIV-1 – Human immunodeficiency virus-1

Hsp70 – Heat shock protein 70

IGF1 – Insulin-like growth factor 1

IMCD – Inner medullary collecting duct

IPTG – Isopropyl β-D1-thiogalactopyranoside

JNK – c-Jun N-terminal kinase

KCC3 – Potassium chloride cotransporter 3

KS-WNK1 – Kidney-specific WNK1

LINGO1 – Leucine-rich repeat and immunoglobulin domain-containing Nogo receptor-interacting protein 1

MAP kinase – Mitogen-activated protein kinase

MAP4K – Mitogen-activated protein kinase kinase kinase kinase

MBP – Myelin basic protein

MDCK – Madin-Darby canine kidney II

MEK – MAP kinase ERK kinase

MEKK – MEK kinase

NCC – Sodium chloride cotransporter

NKCC – Sodium potassium two chloride cotransporter

N terminus – Amino terminus

N-WASP – Neuronal Wiskott-Aldrich syndrome protein

Ni-NTA – Nickel-nitrilotriacetic acid

OSR1 – Oxidative stress-responsive 1

PAI-1 – Plasminogen activator inhibitor 1

PAK1 – p21-activated kinase 1

PBS – Phosphate-buffered saline

PCR – Polymerase chain reaction

PDK1 – 3-Phosphoinositide-dependent kinase 1

PF1/2 – Proline-alanine-rich Ste20-related kinase fray domain 1/2

PHA2 – Pseudohypoaldosteronism type 2

PI3K – Phosphatidylinositol 3-kinase

PKB – Protein kinase B / Akt

PMSF – Phenylmethylsulfonylfluoride

Rac1 – Ras-related C3 botulinum toxin substrate 1

RhoA – Ras homolog gene family, member A

RNA – Ribonucleic acid

RNAi – Ribonucleic acid interference

ROMK – Renal outer medullary potassium channel

RSK – Ribosomal protein S6 Kinase, 90-kiloDalton

S6K – p70 or ribosomal protein S6 Kinase, 70-kiloDalton

SDS – Sodium dodecyl sulfate

SDS-PAGE – SDS-polyacrylamide gel electrophoresis

SGK1 – Serum/glucocorticoid-regulated kinase 1

SH3-domain – Src homology 3 domain

siRNA – Small interfering RNA

SLC12 – Solute carrier family 12

SLC26A9 – Solute carrier family 26 member 9

SNARE – Soluble N-ethylmaleimide-sensitive factors attachment protein receptors

SNP – Single nucleotide polymorphism

SPAK – Sterile20-related proline- and alanine-rich kinase

Syt2 – Synaptotagmin 2

TBST – Tris-buffered saline with 0.05% tween-20

TGFβ – Transforming growth factor β

TGFβR – TGFβ receptor

TIE2 – Tyrosine kinase with immunoglobulin and EGF homology domains 2

TNF – Tumor necrosis factor

TRPV4 – Transient receptor potential cation channel V4

VAMP2 – Vesicle-associated membrane protein 2

VEGF – Vascular endothelial growth factor

VPS4a – Vacuolar protein sorting 4A

WNK – With no (K) lysine kinase

ZO1 – Zona occludens protein 1

CHAPTER ONE
Introduction

WNKS AND THEIR INTERACTIONS WITH OSR1

WNKs and OSR1

The with no lysine (WNK) kinases are a unique family of protein kinases characterized by their unusual kinase domain architecture. Instead of the catalytic lysine in β strand 3 conserved in all other functional kinase domains, the WNKs contain a cysteine. An alternate lysine in β strand 2 functions in its place. The four mammalian genes of the WNK family code for long proteins, 1200 to 2400 amino acids in length. However, outside of the kinase domain, there are no other known folded domains in the protein. There are some conserved areas, namely an autoinhibitory region and two coiled-coil containing regions, but otherwise the proteins are highly divergent. They do contain multiple protein-protein interactions motifs, such as PxxP and coiled-coils, suggesting that the proteins function as protein scaffolds.

WNKs have been shown to interact with a number of different functional signaling pathways, including downstream on various mitogen-activated protein (MAP) kinase cascades, apoptosis regulation, and growth factor signaling. But the majority of WNK target pathways have been involved in the regulation of membrane trafficking or membrane ion transporter function. These pathways include regulation of synapse proteins, stimulated release of vesicles, recycling of membrane protein, and adjustment of solute handling. Key WNK interactors in the regulation of ion transport are the oxidative stress responsive 1 (OSR1) and sterile20-related proline- and alanine-rich kinase (SPAK)

proteins. These proteins bind to conserved motifs on WNK1 and their membrane targets to regulate ion transport activity and membrane localization.

The physiologic relevance of WNKs was revealed by linkage of WNK1 and WNK4 mutations to an inherited form of hypertension, pseudohypoaldosteronism 2 (PHA2). Patients have disrupted ion transport regulation as reflected in hypertension, hyperkalemia, and hyperchloremic metabolic acidosis. WNK1 is also an essential protein during development, as knockout mice die *in utero* of failed vascular system remodeling.

The study of the WNK family has focused on these two disease-linked family members, WNK1 and WNK4. However, WNK2 and WNK3 also contain the OSR1/SPAK-interaction motifs, as discussed in greater detail below. As these proteins, aside from WNK4, are all widely expressed, WNK-OSR/SPAK interactions are likely to play key roles in physiologic functions throughout the body. This study shows that the WNK proteins recognize and activate OSR1, and characterizes their biochemical interactions.

WNK2 characterization

At the same time WNK1 was originally cloned, a fragment of the second WNK family member, WNK2, containing the putative kinase domain and some flanking sequence was discovered. Initial characterization of this protein revealed that it was also a functioning protein kinase, dependent on the same β strand 2 lysine as WNK1. This fragment contained a region conserved with WNK1 that had autoinhibitory function. Expression of this fragment inhibited both WNK2 and WNK1 activity. The equivalent fragment in WNK1 could also inhibit WNK2 activity. This provides a potential

mechanism for WNK isoforms to regulate each other in vivo. WNK2 is able to activate the extracellular-signal regulated kinase 5 (ERK5) signaling cascade when expressed in cells.

WNKs activate OSR1

All four WNKs are able to phosphorylate OSR1, to roughly equal degrees. They are also able to stimulate OSR1 activity toward the model substrate sodium potassium two chloride cotransporter 2 (NKCC2). With each isoform of WNK, OSR1 activity is increased 60- to 100-fold. Kinetic characterization of the WNK proteins reveals that they have K_m toward OSR1 on the order of 1.5 to 4.3 μM. The WNKs are slow enzymes, with k_{cat} on the order of 1 min^{-1}, with the exception of WNK2 at 0.05 min^{-1}. These data suggest that all four WNKs are biochemically competent to act through OSR1 and SPAK and may cause physiologic effects outside of the kidney.

One difference noted in the otherwise highly conserved kinase domains is an OSR1-interaction motif, RFXV, which is conserved in WNK1-3 but lost in WNK4. The valine conserved in WNKs 1-3 has previously been shown to be required for WNK1 interaction with the vesicle fusion regulator synaptotagmin2 (syt2). However, mutation of the valine did not affect the ability of the kinase domain of WNK1 to phosphorylate OSR1 or to stimulate OSR1 activity. Further study of the V318E mutation in the context of the full length protein will be required to determine a role for the kinase domain RFXV motif in WNK function.

Analysis of OSR1 possible phosphorylation sites

Many protein kinases are phosphorylated at multiple sites on their activation loop to allow for controlled regulation of their activity. OSR1/SPAK contain a long activation loop with multiple possible phosphorylation sites. These sites were mutated to phosphomimetic aspartate residues to examine possible roles in regulation. Mutation of the known WNK1 primary phosphorylation site on OSR1, T185, resulted in a protein with increased activity over wild type. However, that protein was only ~10% as active as protein activated by WNK1. While mutation of the neighboring sites T173D and T178D had little effect on activity, double mutants with either site and T185 were more active than either wild type or T185D alone. Yet these mutants still had an activity that fell short of WNK1-stimulated OSR1 activity.

A second known WNK1 phosphorylation site, S325, lies outside of the kinase domain in a region that could provide allosteric regulation. However, the S325A mutant had activity no different from wild type. The S325E mutant was more active than wild type after stimulation with WNK1; its presence also seemed to increase WNK1 autophosphorylation. However, no significant effect on OSR1 activity was noted.

It is still unclear if the activation loop threonines are phosphorylated *in vivo*. However, it is interesting that OSR1/SPAK kinase domains exist as dimers, with the long activation loop swapped between domains. It is possible that phosphorylation of these sites regulates protein dimerization and therefore affects either their activity directly or the ability of activators and inhibitors to reach the active site.

These results suggest that further study of the WNK family and its interaction with the downstream effectors OSR1/SPAK will reveal novel methods of physiologic regulation.

CHAPTER TWO

A REVIEW OF THE LITERATURE

Cloning and characterization of the WNK kinases

The WNK proteins are a family of four protein kinases first noted for their unusual active site conformation. WNK1 was originally discovered in a screen for new members of the MAP kinase-ERK kinase (MEK) family of proteins, and is a large protein of 2382 residues in humans (Xu et al., 2000). In the amino- (N)-terminal portion there is a kinase domain. All active kinase domains known when these studies began had a conserved lysine residue in β strand 3 that functions to bind and stabilize the β-phosphate of adenosine triphosphate (ATP) in the active site. All four WNK proteins are catalytically active but have a cysteine in place of this conserved lysine, leading to their name *w*ith *n*o *K*=lysine (WNK). Instead, a distinct lysine residue is conserved in kinase β strand 2, the phosphate anchor ribbon, of WNK proteins that in other kinases is a conserved glycine. When this lysine is mutated, a kinase-inactive mutant is created. Interestingly, "reversing" this atypical structure by mutating the cysteine to lysine and the lysine to glycine recapitulates the traditional kinase domain architecture but does not restore catalytic function (Xu et al., 2002a). This indicates that the remaining structure of the WNK kinase domain favors the placement of the ATP-stabilizing lysine in its unusual position. This may affect WNK interactions with substrates or regulators, as β strand 2 is more exposed on the exterior of the domain and generally is more flexible than β strand 3 (Fig. 2-1).

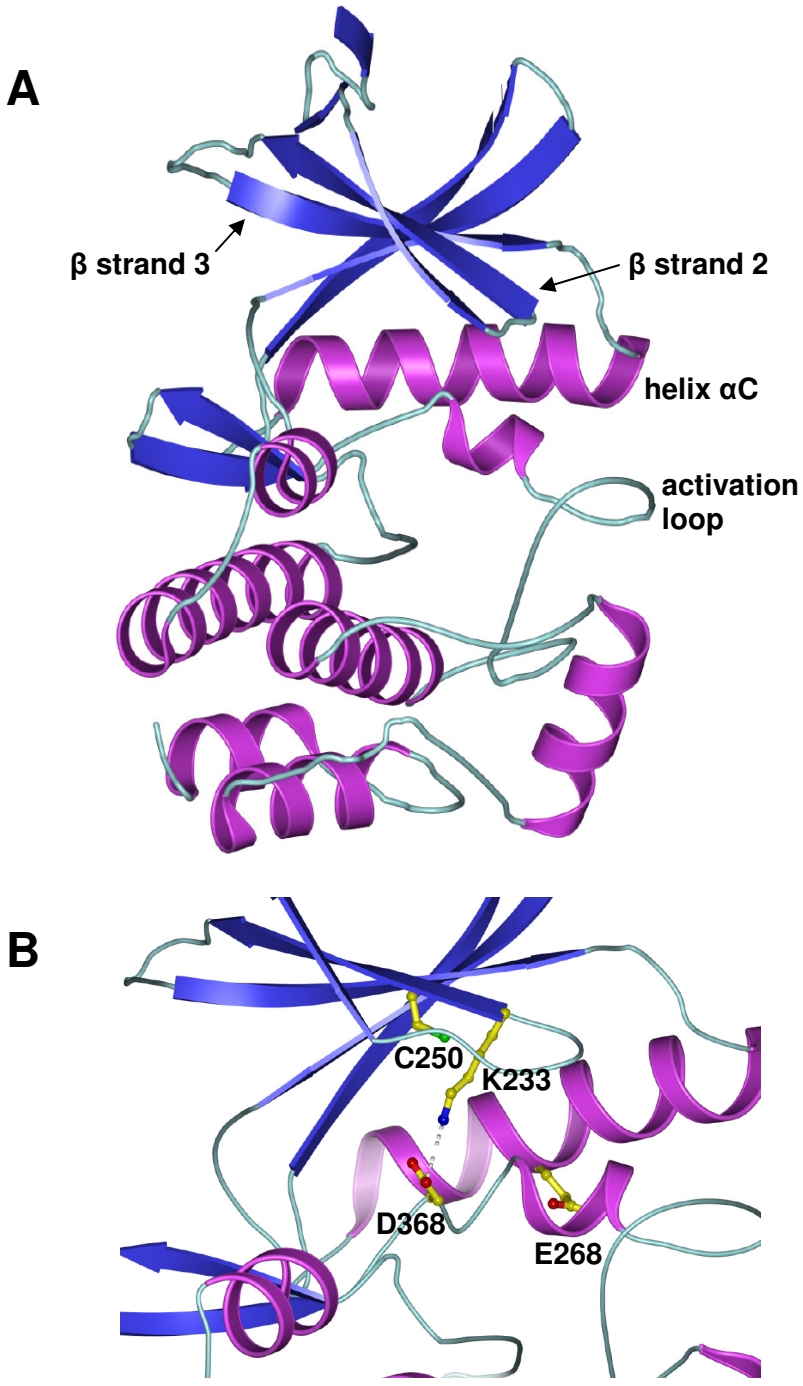


Fig. 2-1 Crystal structure of WNK1 A) Kinase domain of WNK1 in ribbon format. B) Enlargement of active site. Note K233, the catalytic lysine in β strand 2, and C250, the normal site of lysine in β strand 3. Adapted from Min et al., *Structure* 2004.

In mammals there are four WNK proteins, characterized by their atypical kinase domain (Verissimo and Jordan, 2001). In the kinome tree, WNKs are not closely related to any other kinases and sit on their own branch. They are most closely related to sterile20 proteins. The four mammalian WNKs share 90% similarity over their kinase domains, located in the N terminus. Just carboxy (C)-terminal to the kinase domain is a conserved 60 residue auto-inhibitory region, with a similar conserved second sequence nearer to the C terminus. A fragment containing this region is able to inhibit WNK1 activity on its own or as part of a single protein chain including the kinase domain. The autoinhibitory region of WNK1 is able to inhibit the kinase domains of other WNK kinases, and vice versa (Xu et al., 2002a). This autoinhibitory effect is dependent on two phenylalanine residues, at positions 524 and 526 in the rat sequence, that are conserved among species and WNK paralogs; when these residues are mutated, auto-inhibition is lost. At the end of the autoinhibitory sequence is a short stretch of ten conserved acidic residues (Verissimo and Jordan, 2001). This family consists of large proteins, between 1200 and 2400 residues in length and 130 kDa to 250 kDa in size (Fig. 2-2). However, there are no other known or predicted folded domains. The only other conserved sequence is a small region in the C terminus of all four WNKs that contains a coiled-coil domain. There are also a number of PxxP motifs, 29 in rat WNK1, that are typical binding sites for Src homology 3 (SH3) domain-containing proteins, and other coiled-coil structures indicating the importance of protein-protein interactions for these kinases.

WNK homologs have also been identified in multiple eukaryotes. There is a single WNK in *Caenorhabditis elegans* and *Drosophila melanogaster,* both of which

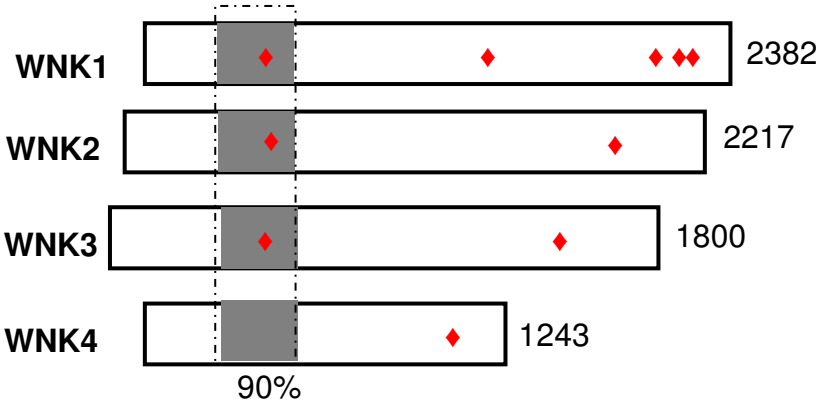


Fig. 2-2 Domain structure of WNK kinases. There are four WNK kinases in humans, with only one known domain, a Ser/Thr protein kinase domain (grey box). The WNKs share 90% identity over the kinase domain, but are highly divergent over the remainder of their varying lengths. Numbers to right indicate number of amino acid residues. Red diamonds signify RFXV motifs.

most closely resemble WNK3, and at least nine homologs in *Arabidopsis thaliana* (Nakamichi et al., 2002; Verissimo and Jordan, 2001). In plants, the WNK family is known to bind to and *in vitro* phosphorylate the *Arabidopsis* pseudo response regulator 1 (APRR1) family of proteins involved in establishing circadian rhythms in higher plants, and are expressed in a diurnal rhythm themselves (Murakami-Kojima et al., 2002; Nakamichi et al., 2002). WNK homologs have also been identified in the filamentous fungus *Phycomyces* and the unicellular flagellate *Chlamydomonas*, showing that this family is conserved to early eukaryotic lineages.

Mammalian WNK1 is expressed ubiquitously in tissues, with multiple splice variants detected. In the kidneys in particular, an alternative promoter is utilized to create a protein with an alternative exon 4 with the balance of the remaining protein to the C terminus. This eliminates almost the entire kinase domain (Xu et al., 2002b). Exon 4a is a short, cysteine-rich region at the N terminus which contains a predicted palmitoylation site, which has not been shown to be functional. This form, called kidney-specific WNK1 (KS-WNK1), is expressed specifically in the renal cortex, primarily in the distal convoluted tubule (Delaloy et al., 2003). Full length WNK1 is also expressed in the kidney, but more diffusely and at lower levels than KS-WNK1, as determined by *in situ* hybridization. Full length WNK1 is also found in most other tissues studied, and specifically in the polarized epithelium of numerous organs (Kahle et al., 2004; Verissimo and Jordan, 2001). This includes cholangiocytes in the hepatic bile ducts, cuboidal epithelial ductal cells of the pancreas, and columnar epithelium in the epididymis. But full-length WNK1 is also expressed in various portions of the brain in adults and embryonic tissue, and in heart and skeletal muscle (O'Reilly et al., 2003).

Developmentally, WNK1 is ubiquitously expressed as soon as day E8-9, with enrichment in the cardiovascular system (Delaloy et al., 2006; Xie et al., 2009). A number of additional splice variants have been identified, mainly those lacking exons 11 and 12 or lacking exons 9,11, and 12 (O'Reilly et al., 2003). These exons have no known function, are located between two of the coiled-coil domains after the kinase domain, but are 82% conserved between mammalian species (Delaloy et al., 2003). Interestingly, the rat clone of WNK1 in the database corresponds to the human splice variant lacking exons 11 and 12. It is not known whether a full-length WNK1 clone including those exons is expressed in rats.

WNK2 is most highly expressed in heart and skeletal muscle, and also found in the brain, small intestine and colon (Moniz et al., 2008; Verissimo et al., 2006). In HeLa cells, WNK2 is shown to be localized to the plasma membrane in a manner dependent on the C-terminal 200 residues, which include the coiled-coil domain (Moniz et al., 2008). WNK3 is primarily localized in the brain, along with expression in the lung, kidney, liver, and pancreas, with a brain-specific splice form containing exon 18b. Specifically, it is expressed in regions of the frontal cortex, hippocampus, and basal ganglia (Holden et al., 2004). WNK3 is found throughout the cell, but localizes to the nucleus upon induction of apoptosis (Verissimo et al., 2006). WNK4 is primarily expressed in the kidney, in the distal convoluted tubule and cortical collecting duct. Like WNK1, WNK4 protein is made at lower levels in polarized epithelium in brain, colon, heart, liver, prostate, lung, and pancreas (Kahle et al., 2004; Verissimo and Jordan, 2001; Wilson et al., 2001). WNK4 is found in the cytoplasm and localized at intercellular junctions, co-localizing with zona occludens proteins.

Disruptions of WNK function lead to human disease

Interest in the WNK family was increased when it was discovered that mutations in certain family members were responsible for a familial form of hypertension, PHA2 (Wilson et al., 2001). PHA2, also known as Gordon's syndrome, is a Mendelian inherited disease that causes hypertension, hyperkalemia, and hyperchloremic metabolic acidosis, often manifesting before age 30 (Achard et al., 2003). (The nomenclature is confusing—the disease is related to hypoaldosteronism because of the hyperkalemia. PHA1 patients are hypotensive, in contrast with PHA2). These patients have a normal glomerular filtration rate, normal aldosterone level, lowered renin levels, and respond well to thiazide diuretics. Two separate groups of mutations were found to cause Gordon's syndrome. The first is a large deletion in the first intron of WNK1 which causes overexpression of the full-length and kidney-specific WNK1 isoforms (Delaloy et al., 2008; Wilson et al., 2001). The other cluster of mutations, PHA2B, is located in the coding sequence of WNK4. These are missense mutations conserved in a segment with ten negatively-charged residues C-terminal to the kinase domain and a coiled-coil motif. All of the mutations detected so far are charge-changing. No function is defined yet for this region, although a mouse knock-in model heterozygous for one of the WNK4 mutations (D561A) shows increased phosphorylation of the kinases OSR1 and SPAK and increased apical localization of the renal transporter sodium chloride cotransporter (NCC) (Wilson et al., 2001; Yang et al., 2007c). This suggests these mutants could show increased WNK4 activity, perhaps through increased binding affinity for OSR1/SPAK.

There are two main hypotheses to explain the electrolyte abnormalities found in PHA2. The first is that the changes are driven by enhanced proximal reabsorption of

sodium. Sodium absorption in the proximal tubule, loop of Henle, and distal convoluted tubule typically pass through electroneutral mechanisms such as NKCC2 and NCC. These electroneutral processes absorb approximately 95% of the sodium filtered in the glomerulus. In contrast, sodium absorption in the collecting duct is typically electrogenic, passing through the epithelial sodium channel (ENaC). This causes polarization of the epithelial membrane and provides the driving force for potassium and hydrogen secretion. Increased proximal absorption of sodium, such as through higher activity of NKCC2 and NCC, leads to decreased delivery of sodium to the distal tubule and therefore decreased polarization of the epithelial membrane. Increased sodium retention causes increased water retention and hypertension, while decreased potassium and hydrogen secretion causes hyperkalemia and metabolic acidosis. The first hypothesis is supported by the sensitivity of PHA2 patients to thiazide diuretics. The second hypothesis invokes the "chloride shunt" as the initial cause of symptoms. Chloride reabsorption, which can occur transcellularly or paracellularly, drives sodium reabsorption and reduces the polarization of the distal epithelium, thereby reducing the driving force for potassium and hydrogen secretion. Increased sodium and chloride reabsorption and potassium and hydrogen ion retention are the hallmarks of PHA2 symptoms. This hypothesis is supported by the fact that the electrolyte abnormalities of PHA2 are chloride-dependent and can be alleviated by infusion of sulfate in place of chloride.

While PHA2 is a rare disease, it is thought that understanding the pathophysiology behind it could help explain some component of the much more common problem, essential hypertension. Interestingly, the WNK4 gene is found in a region of a quantitative trait locus for hypertension demonstrated in humans and rats

(Monti et al., 2003). One paper examining coding sequence changes in WNK4 identified a single nucleotide polymorphism that segregated with hypertension in a population of white Americans (Erlich et al., 2003). However, no linkage could be identified when this single nucleotide polymorphism (SNP) was analyzed in an Anglo-Australian population (Speirs and Morris, 2004). A separate SNP in WNK4 was identified in a Japanese population as being associated with essential hypertension (Kokubo et al., 2004). The data are limited, but continue to provide hints that WNK proteins may play a role in the epidemic of hypertension and studying them could lead to greater understanding and novel interventions to treat it.

Disruption of the WNK1 gene in mice caused embryonic lethality between days 11.5 and 13.5 (Zambrowicz et al., 2003). WNK1 heterozygous mice developed hypotension but had no other obvious phenotype. This is consistent with the idea of a gene dosage-response relationship between WNK1 and blood pressure. In PHA2 patients, increased expression of WNK1 leads to hypertensive effects, while in mice, decreased expression of WNK1 caused hypotension. Extra-renal effects of the widely-expressed WNK1 were revealed in the WNK1 homozygous null mice. These mice died from a failure of angiogenic remodeling of their vasculature around day E11.5, with dysregulated expression of arterial and venous markers (Xie et al., 2009). Recently, suppression of vascular endothelial growth factor (VEGF)-notch signaling pathway by expression of chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) in venous-fated endothelial cells was identified as a determining factor in vessel identity (You et al., 2005). Disruption of this specification of arterial and venous fate leads to angiogenic defects. However, $WNK1^{-/-}$ mice showed no changes in expression of COUP-

TFII or Notch target genes, indicating that WNK1 acts on either a novel pathway regulating embryonic vessel fate or downstream of currently known Notch target genes. An endothelial-specific conditional knockout of WNK1 recapitulated the angiogenic defect, and expression of WNK1 driven by an endothelial promoter (tyrosine kinase with immunoglobulin and EGF factor homology domains 2 [TIE2]) was able to rescue the embryonic lethality of $WNK1^{-/-}$ mice. This rescue supports the hypothesis that other developmental defects seen in the knockout mouse, such as cardiac hypotrophy, are caused by a primary endothelial defect. However, endothelial expression only partially explains WNK1 function, as the endothelial-rescue mice were smaller than their heterozygous littermates and died around post-natal day 1.

Other physiological roles for WNK family proteins are still being discovered. For example, in studies of the kinase superfamily in a number of different cancers, multiple mutations in WNK1, WNK2, WNK3, and WNK4 have been reported (Davies et al., 2005; Greenman et al., 2007; Sjoblom et al., 2006; Stephens et al., 2005). A search for candidate tumor suppressor genes in adult gliomas found that the WNK2 gene is either deleted or abnormally methylated, causing up to a 100-fold decrease in expression (Hong et al., 2007). Methylation-induced silencing of the WNK2 gene was seen in meningiomas and adult gliomas, but was not found in other tumor types (Jun et al., 2009). WNK2 expression inhibited colony formation of a glioma cell line in a kinase activity-independent fashion, without affecting growth of a related glioblastoma cell line. This effect may be mediated by WNK2 signaling to the ERK1/2 pathway (see below). Besides roles in cancer, one group has reported a family with a microdeletion on chromosome X that affects three genes including WNK3 (Qiao et al., 2008). This family is characterized

by autism-spectrum disorder, midline facial defects, and intellectual disability, but it is unknown exactly what role, if any, WNK3 plays in these phenotypes. Further roles of WNK proteins in pathophysiology are still being explored.

Pleiotropic actions of WNKs

Activation and modification of WNK

In an attempt to explain the role of WNKs in these disease states and to identify new roles for these widely expressed proteins, much study has gone into finding activating conditions and downstream effectors. While many conditions have been studied, only osmotic stress has been consistently identified to cause increased WNK1 kinase activity. Both 0.5 M sorbitol and 0.5 M sodium chloride stimulate WNK1 activity in cells. Interestingly, hypotonic conditions also increase WNK1 activity. No effect on kinase activity has been detected after stimulation with growth factors, serum, or a number of other known kinase pathway stimulants (Lenertz et al., 2005; Xu et al., 2000).

WNK1 is phosphorylated by Akt/protein kinase B (PKB). There is a consensus phosphorylation site for AGC kinases in the N terminus of WNK1, proximal to the kinase domain. Alessi's group showed that Akt phosphorylates threonine 60 (in the human sequence) of WNK1 both *in vitro* and in cells (Vitari et al., 2004). Phosphorylation at that site was increased after stimulation of cells with insulin-like growth factor 1 (IGF1) and inhibited by a phosphatidylinositol 3-kinase (PI3K) inhibitor, and endogenous protein was found to be phosphorylated within two minutes of stimulation. 3-phosphoinositide-dependent kinase 1 (PDK1) was then mutated such that it no longer bound the effectors ribosomal protein S6 kinase-70-kiloDalton (S6K), ribosomal protein S6 kinase-90-

kiloDalton (RSK), or serum/glucocorticoid-regulated kinase 1 (SGK1). WNK1 phosphorylation was preserved in cells expressing this mutant PDK1, suggesting that Akt was responsible for the IGF-1 mediated effect. However, phosphorylation on Thr60 does not affect the catalytic activity of WNK1, either *in vitro* or when immunoprecipitated from cells, as detected by autophosphorylation and activity toward the model substrate myelin basic protein (MBP) (Vitari et al., 2004). In addition, inhibition of PI3K by wortmannin does not block the stimulation of WNK1 kinase activity by hyperosmotic stress (Xu et al., 2005b). In the same study, phosphorylation at Thr60 did not affect the subcellular localization of a green fluorescent protein (GFP)-WNK1 fusion protein in human embryonic kidney (HEK) 293 cells. However, work in our lab has shown that GFP-WNK1 does not localize in the same manner as endogenous WNK1 protein. It still remains to be seen if IGF stimulation affects the localization of endogenous protein.

WNKs and cell cycle regulation

WNK1 has been shown to act as a MAP4K upstream of ERK5. ERK5 is a MAP kinase most closely related to ERK2 that is stimulated by a number of proliferative and stress stimuli, including sorbitol/osmotic stress. It lies in a pathway downstream of MEK kinase 2/3 (MEKK2/3) and MEK5. WNK1 overexpression in cells is able to increase ERK5 activity, in a MEKK2/3- and MEK5α-dependent manner, largely without affecting other MAP kinase family members (Xu et al., 2004). WNK1 is able to co-immunoprecipitate with MEKK2/3 and phosphorylate them *in vitro*. Finally, WNK1 knockdown inhibits ERK5 activation in response to epidermal growth factor (EGF) stimulation. The role of WNK1 kinase activity is questionable in these experiments. A

catalytically inactive mutant of WNK1 is able to increase ERK5 activity, albeit at levels that are significantly less than wild-type WNK1. Phosphorylation of MEKK2/3 by WNK1 *in vitro* does not affect their kinase activities *in vitro.* However, either wild-type or inactive WNK1 in cells is able to stimulate MEKK3 activity toward a MEK6 substrate. MAP3Ks are able to be activated by oligomerization, suggesting that perhaps WNK1 acts as a scaffold to activate the MEKK2/3 pathway. Also of interest, while these upstream effects of overexpressed WNK1 were identified using a construct from the N terminus to the kinase domain, overexpression of a construct that also included the autoinhibitory region just C-terminal to the kinase domain did not increase ERK5 pathway activity (Xu et al., 2000). And while the catalytic activity does not seem to be required, WNK1 ablation by ribonucleic acid interference (RNAi) shows that WNK1 plays a required role in EGF-stimulated ERK5 activation. In addition, WNK1 catalytic activity itself is not activated by EGF stimulation, and ERK5 activity is only mildly increased by sodium chloride or sorbitol, the known stimulators of WNK1 kinase activity (Xu et al., 2000). This again suggests a possible role for WNK1 as a scaffolding protein, assembling the ERK5 pathway components together and stimulating their activity in a non-catalytic mechanism. The role of the kinase domain may be to aid in recognition of substrates for assembling on the scaffold without actually requiring their catalytic activity. Therefore, the kinase-defective mutant is perhaps less able to stimulate activation due to an altered binding recognition, and the auto-inhibitory domain can interfere with protein interaction. This auto-inhibitory domain, common in many protein kinases, also provides a possible mechanism for regulation of WNK1 activity, whether through inhibiting catalysis or scaffolding assembly. This also illustrates the difficulty in finding and confirming

downstream targets of WNK1, as the role of kinase activity, even in classic MAP kinase cascades, is questionable.

In contrast to WNK1, WNK2 has been shown to inhibit the ERK1/2 signaling pathway without affecting ERK5 (Moniz et al., 2007). EGF activation of ERK1/2 phosphorylation was greater in HeLa cells in which WNK2 was knocked down. In contrast, c-Jun N-terminal kinase (JNK), p38 MAPK, or ERK5 activation by EGF was unaffected by WNK2 knockdown. Over-expression of WNK2 inhibited ERK1/2 activation by EGF, but did not alter unstimulated ERK1/2 activity. Knockdown of WNK2 led to increased G_1/S transition and increased cell proliferation. Further studies revealed that this signaling was Raf-independent. Instead, WNK2 co-immunoprecipitated with RhoA from cells. WNK2 knockdown showed decreased Ras homolog family A (RhoA) and increased Ras-related C3 botulinum toxin substrate 1 (Rac1) activation, leading to increased activation of MEK1 via p21-activated kinase 1 (PAK1). (Moniz et al., 2008).

Regulation of cell survival by the WNK family has also been implicated by the interaction of WNK3 with the caspase-3 pathway. Caspase-3 is the final common member of the intrinsic and extrinsic cascades that lead to apoptosis, accomplished by proteolytic activation of procaspase-3. Upon induction of apoptosis, WNK3 moves from a cytoplasmic distribution to a nuclear pattern of localization. Overexpression of WNK3 promotes HeLa cell survival, and knockdown increases apoptosis in response to actinomycin D or etoposide (Verissimo et al., 2006). WNK3 in cells co-immunoprecipitates with procaspase-3 and heat shock protein 70 (Hsp70), but does not bind to other caspase family members. Knockdown of WNK3 in cells causes an increase in the number of cells which had activated caspase-3. As is the case with many other

effectors, the catalytic activity of WNK3 is not required to bind procaspase-3 or to inhibit apoptosis, nor are procaspase-3 or Hsp70 *in vitro* substrates of WNK3. Along with G_1/S transition regulation, moderation of the caspase cascade implicates WNK family members in modulation of cell proliferation.

WNKs – regulators of membrane trafficking

Much of the work on WNK effectors has focused on mechanisms of membrane trafficking. Synaptotagmins are a class of calcium-sensing regulators that interact with soluble N-ethylmaleimide-sensitive factors attachment protein receptor (SNARE) complex proteins to affect membrane trafficking and vesicle fusion. The presence of calcium stimulates synaptic vesicle fusion with the plasma membrane, mediated by synaptotagmins. Yeast two-hybrid screens with the kinase domain of WNK1 identified interactions with synaptotagmin 1 (Syt1) and Syt2 through their calcium-binding C2 domains (Lee et al., 2004). The C terminus of WNK1 is able to co-immunoprecipitate with Syt2, and immunofluorescence shows both Syt2 and WNK1 localized on insulin-containing vesicular structures in the rat insulinoma-derived cell line INS-1. *In vitro*, WNK1 interacts with Syt2 in a calcium-dependent manner and phosphorylates the Syt2 C2 domains. This phosphorylation decreases the ability of Syt2 to bind to phospholipid vesicles in the presence of a given calcium concentration. This interaction of WNK1 with the membrane trafficking proteins Syt1 and Syt2 provides a mechanism for WNK regulation of exocytosis and membrane protein recycling.

Another component of the SNARE complex along with synaptotagmins is Munc18c. Munc18 proteins interact with the syntaxin family to regulate vesicle fusion in

pancreatic beta cells, insulin granule release, and membrane localization of glucose transporter 4 (GLUT4) (Oh et al., 2007). The kinase domain of WNK1 binds to the N-terminal 172 residues of Munc18c in Chinese hamster ovary (CHO) cells and pancreatic beta cells, but catalytic activity is not required for the interaction. WNK1 and Munc18c are able to interact in the plasma membrane and cytosolic compartments, but WNK1 does not co-immunoprecipitate in complexes with the Munc18c plasma membrane partner syntaxin4. Expression of the N terminus of Munc18c disrupts full length Munc18c interaction with WNK1 but does not interfere with binding to syntaxin4. Expression of the fragment does not affect basal secretion of insulin from beta cells. However, it inhibits glucose-stimulated insulin secretion and prevents assembly of syntaxin4 into SNARE complexes with vesicle-associated membrane protein 2 (VAMP2). Presumably, WNK1 binds to Munc18c in cells to regulate SNARE complex assembly and regulate granule docking at the plasma membrane in preparation for insulin-stimulated vesicle fusion. This role does not require WNK1 catalytic activity. Overexpression of Munc18c causes inhibition of exocytosis; expression of the WNK1 active or inactive kinase domain reverses the inhibition. The role of Munc18c in modulating vesicle trafficking gives a further possible mechanism for WNK1 regulation of membranes and ion transporters.

Consistent with WNK1 interaction with the vesicle fusion regulator Munc18c, WNK1 also interacts with vacuolar protein sorting 4a (VPS4a) (Lenertz LY unpublished data), a member of the endosomal sorting complex required for transport (ESCRT) complexes that catalyze the scission of necks of membranes and vesicles during vesicular budding (Wollert et al., 2009). After ESCRT proteins are assembled onto a membrane and scission has occurred, VPS4a is an ATPase that controls the release of the complex

from membranes. This process is used in vesicle budding, cytokinesis, and human immunodeficiency virus-1 (HIV-1) budding. WNK1 binds to VPS4 inside cells and colocalizes with a small fraction on immunofluorescence.

Association of WNK with ion handling

Another mechanism for management of membrane proteins affected by WNK proteins is revealed in the regulation of the renal outer medullary potassium (ROMK) channel. WNK4 and WNK1 inhibit ROMK in the distal nephron (see below) in a mechanism dependent on clathrin coated vesicle endocytosis (Kahle et al., 2003). Intersectin is a key player in endocytosis and vesicle trafficking and links to the actin cytoskeleton. It interacts with dynamin, a protein involved in clathrin-coated vesicle endocytosis; synaptosomal-associated protein 25 (SNAP-25), a member of the SNARE complex; neuronal Wiskott-Aldrich syndrome protein (N-WASP), a signal transducer to the actin network; and Rho family proteins, guanine triphosphatases (GTPases) that regulate actin (Ma et al., 2008). Intersectin contains five SH3 domains, which bind PxxP motifs, abundant in WNK proteins. The first 120 residues of WNK1 precede the kinase domain and contain three PxxP motifs, one or more of which bind the SH3C domain of intersectin. Residues 473-584 of WNK4 contain six PxxP motifs which binds to intersectin SH3A, SH3B, and SH3C (He et al., 2007). The binding of WNK1 1-119 can be prevented by another fragment, residues 120-220; binding is aided in the full length protein by a structurally but not catalytically intact kinase domain. Knockdown of intersectin or overexpression of intersectin SH3 domains increases basal ROMK activity and prevents the inhibition of ROMK by WNK1 or WNK4. Mutation of the PxxP motifs

in WNK4 abolished the interaction with intersectin and the inhibition of ROMK. Those motifs are adjacent to some of the WNK4 disease-causing mutations. These mutant WNK4 proteins show enhanced binding to the intersectin SH3 domains and cause decreased ROMK surface expression and activity, in a manner that depends on the neighboring PxxP motifs. Thus another transport protein regulated by WNKs is shown to be affected by interaction with a membrane trafficking protein.

Another interactor discovered by yeast two-hybrid screen with WNK1 is SGK1 (Xu et al., 2005a). SGK1 signaling is also dependent on PI3K, and is known to regulate ENaC via the E3 ligase Nedd4-2. This is relevant to the effects of WNK1 on hypertension, as ENaC is a target of aldosterone signaling in the cortical collecting duct of the distal nephron. Aldosterone-mediated modulation of sodium reabsorption is the body's main mechanism of regulation of sodium and fluid balance. As an electrogenic form of transport, the reabsorption of Na^+ through ENaC creates the electrochemical gradient that drives the secretion of K^+ and H^+, regulating the body's potassium level and acid-base balance. WNK1 can co-immunoprecipitate with SGK1 from HeLa cells, and WNK1 expression leads to increased activity of SGK1 and increased Na^+ current through ENaC in *Xenopus* oocytes and CHO cells. Knockdown of WNK1 prevents the stimulation of SGK1 by IGF-1 or hydrogen peroxide (Chen et al., 2009; Xu et al., 2005b). The stimulatory effect of WNK1 is decreased by mutation of Thr60, the site of Akt phosphorylation described above. This site is found to be phosphorylated in unstimulated 293 cells, and PI3K pathway inhibitors such as wortmannin or dominant-negative Akt or mutation of Thr60 to alanine inhibit WNK1 stimulation of SGK1 (Vitari et al., 2004; Xu et al., 2005b). Interestingly, SGK1 also phosphorylates WNK1 on the

same site, creating a possible feedback mechanism (Xu et al., 2005a; Xu et al., 2005b). The activation of SGK1 and ENaC is mediated non-catalytically by the N terminus of WNK1; the first 220 residues, not including the kinase domain, are able to increase SGK1 activity and ENaC current when expressed in cells. This pathway system is another example of kinase domain-independent function of WNK1 and of WNK1 interaction with another important regulator of sodium uptake in the kidney.

Early analysis of WNK4 localization in the kidney showed that in the distal convoluted tubule, WNK4 colocalized with the tight junction marker zona occludens 1 (ZO-1) (Verissimo and Jordan, 2001). Further analysis in Madin-Darby canine kidney II (MDCK) cells examined paracellular transport of ions. Wild-type WNK4 had no significant effect on sodium or chloride transport, but the disease-causing mutation D564A caused an increase in chloride-permeability and a slight decrease in sodium permeability of the epithelium. Transcellular ion flux was not affected by inhibitors of Na^+/K^+ ATPase, NKCC1/2, NCC, ENaC, or anion exchangers. Nor did the effect saturate at higher NaCl concentrations, indicating that this transport was through a paracellular pathway. In these cells, endogenous claudins 1-4 were more highly phosphorylated when WNK4 D564A was expressed, but not wild type WNK4. While wild type WNK4 co-immunoprecipitated to some extent with endogenous claudins, WNK4 D564A interacted more strongly. Other tight junction proteins including occludin and ZO-1 were not affected by wild type or mutant WNK4. The association of increased paracellular chloride absorption with disease-causing WNK4 mutations supports the “chloride shunt” hypothesis of PHA2 pathophysiology discussed previously.

WNK and the TGFβ pathway

Another membrane protein whose regulation is tied to WNK1 is the transforming growth factor-β (TGFβ) receptor (TGFβR). TGFβR signal transduction is transmitted through the Smad protein family. Upon stimulation, TGF-βRII phosphorylates RI, which then phosphorylates R-Smads. R-Smads bind to a Co-Smad and translocate to the nucleus to regulate gene transcription (Pardali and ten Dijke, 2009). WNK1 and WNK4 have been shown via co-immunoprecipitation and yeast two-hybrid to bind to the R-Smads Smad2 and Smad3, which are downstream effectors from TGFβR I/II, but not to the bone morphogenetic protein (BMP)-receptor pathway R-Smads Smad1 or Smad4 (Lee et al., 2007). This binding is via the kinase domain of WNK1/4 and is TGFβ-stimulus independent. Both WNK1 and WNK4 can phosphorylate Smad2 *in vitro,* but there is no evidence that this is required in cells. WNK1 also affects amounts of pathway components, as knockdown of WNK1 increased TGFβ transcription and decreased Smad2 mRNA and protein, in a proteasome-independent mechanism. However, despite the lower protein levels, the amount of activated phosphorylated Smad2 was greater after WNK1 depletion, and transcription of the Smad2 target genes TGFβ and plasminogen activator inhibitor 1 (PAI-1) were enhanced. Overexpression of WNK1 inhibited Smad2 phosphorylation in cells and decreased Smad2 target gene expression. A separate study of protein signaling networks also identified a key WNK1 effector, OSR1 (see below), as interacting with the TGFβ-Smad pathway (Barrios-Rodiles et al., 2005). Thus, WNK1, and perhaps WNK4, influence the TGFβ-Smad signaling pathway by selectively suppressing Smad2-dependent transcription.

OSR1 and SPAK, important WNK effectors

Of the effector proteins of WNK1, among the most well-studied are the Ste20-related proteins OSR1 and SPAK (Fig. 2-3). These proteins are widely expressed, with greatly overlapping expression patterns such that almost all tissues studied to date express at least one member. OSR1 and SPAK are closely related, showing high identity over their length (Chen et al., 2004; Piechotta et al., 2003). The main differences are an N-terminal extension on SPAK that has an unknown function and a region in the C terminus between two conserved domains.

Fig. 2-3 Domain structure of OSR1 OSR1 consists of an N-terminal kinase domain and two C-terminal protein interaction motifs, PF1 and PF2. Chen et al., *Journal of Biological Chemistry* 2004.

These proteins consist of an N-terminal kinase domain and a C-terminal regulatory region. There are two conserved domains in the regulatory region, named PF1 and PF2 (PASK-fray domain—PASK is another name for SPAK, and fray is the *Drosophila melanogaster* homolog) (Chen et al., 2004; Johnston et al., 2000). The PF1 domain is immediately adjacent to the kinase domain, and crystal structures in the Cobb lab have shown that the proximal part lies along the kinase domain opposite from the activation loop in a position similar to the common docking (CD) region in ERK. This region allows allosteric regulation of ERK activity. The PF1 domain is required for full OSR1/SPAK kinase activity. The PF2 domain is responsible for OSR1/SPAK interaction with upstream effectors and downstream substrates by recognizing a specific RFXV motif (Piechotta et al., 2002) (Fig. 2-4). Mutation of single residues in this motif can block downstream signaling (Gagnon et al., 2007). A study of mouse proteins found 131 that contain these motifs, of which 70 motifs are conserved in humans and 23 in zebrafish

(Delpire and Gagnon, 2007). Interestingly, the PF2 domain that recognizes this motif is structurally similar to the autoinhibitory region of the WNK kinases. The structure of the kinase domain of SPAK shows that the protein exists as a homodimer (Juang, et al., submitted 2009), consistent with co-immunoprecipitation data (Anselmo et al., 2006) (Fig. 2-5). The elongated activation loop in the protein is domain-swapped between the monomers in the structure, causing a tight interaction between the opposing members. Mutation of two sites, R260S/G261L, creates a protein that no longer forms a dimer. This suggests that modification of the activation loop can act to regulate OSR1/SPAK in some manner.

Like WNK1, OSR1 and SPAK are activated by osmotic stress. The *Drosophila* homolog Fray was first identified to be required for JNK activation in response to sorbitol (Chen et al., 2004). Studies of OSR1 then showed that its kinase activity is strongly increased in response to 0.5 M sorbitol, and by 0.5 M NaCl to a lesser extent. Despite this similarity to Fray, OSR1 does not cause activation of JNK, or any of the other MAPKs. Instead, OSR1 suppresses the activation of the related Ste20-like kinase PAK1, and acts on ion transporters to increase or decrease their activities.

Key downstream effectors of the OSR1/SPAK proteins are members of the solute carrier family 12 (SLC12) family of ion transporters (Piechotta et al., 2002). These include NKCC1, NKCC2, NCC, and the potassium chloride cotransporter 3 (KCC3) (Richardson et al., 2008). These ion transporters all contain RFXV motifs and were identified as binding partners for OSR1 and SPAK (Piechotta et al., 2002). NKCC1 is ubiquitously expressed in tissues and is thought to play a role in maintaining cell volume and osmotic homeostasis, and in chloride-secreting epithelia is a key component in

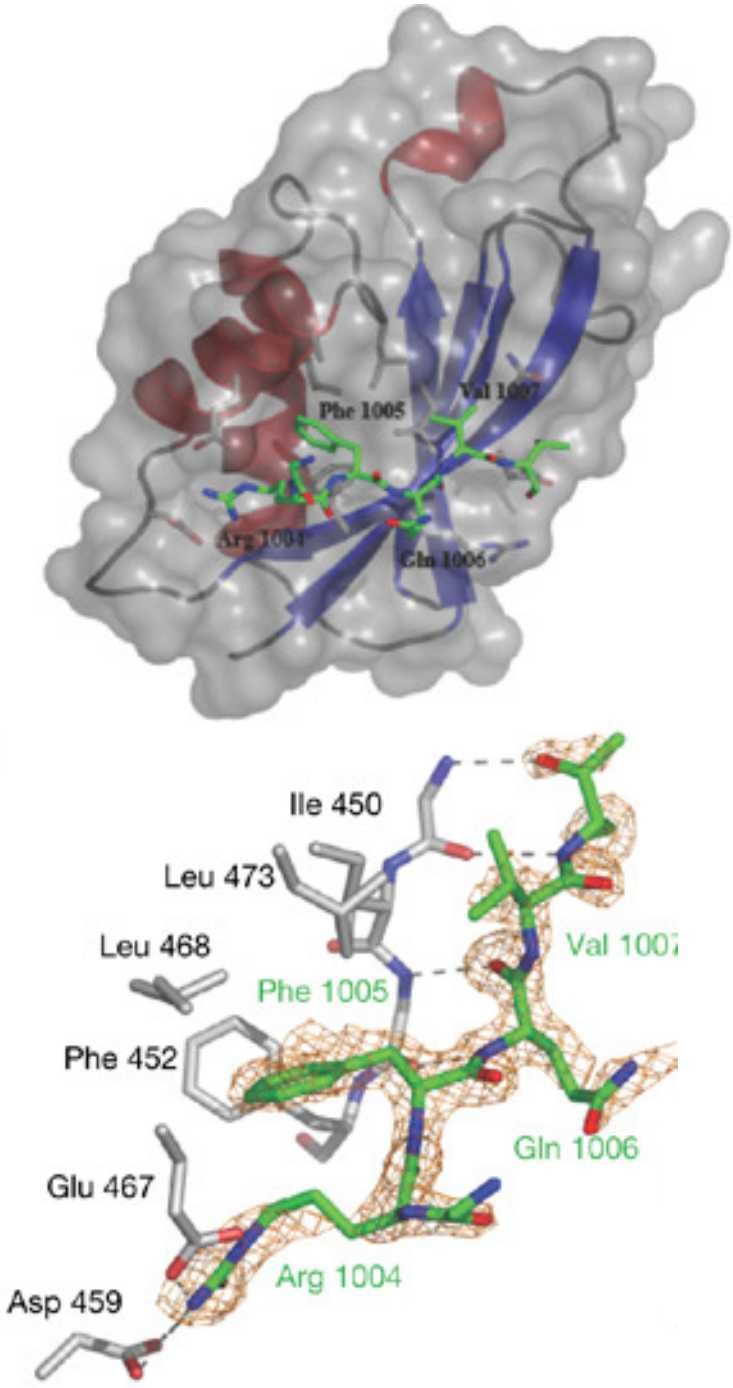


Fig. 2-4 RFXV bound to OSR1 PF2 domain Note the RFQV motif in green wireframe, bound to PF2 domain in gray space-filling model above, and wireframe below. F Villa et al., *EMBO Reports* 2007.

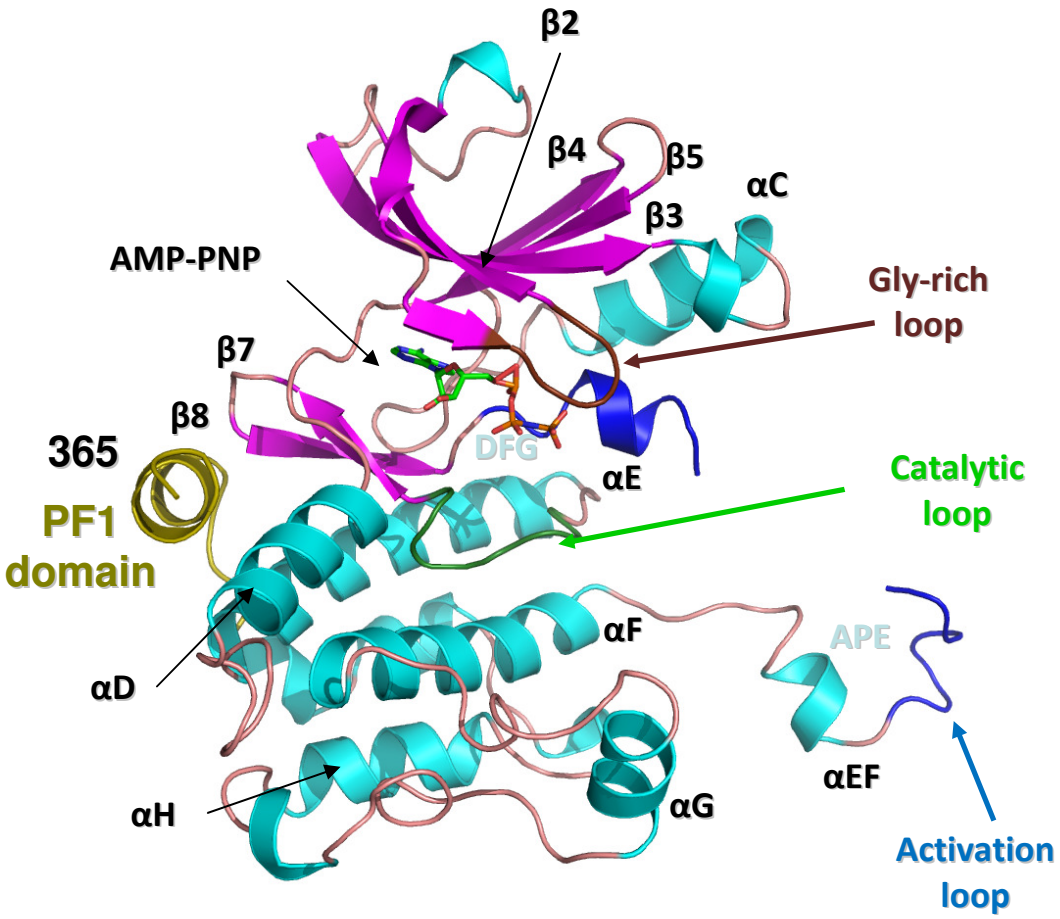


Fig. 2-5 SPAK kinase domain crystal structure Note the elongated activation loop, which sits inside the dimer partner. The beginning of the PF1 domain is apparent in yellow, opposite from the activation loop in a position of allosteric regulation in ERK2. Courtesy Yu-Chi Juang.

transport of chloride across the basolateral membrane. NKCC2 expression is restricted to the thick ascending limb of the nephron. Here it is responsible for 25% of sodium reabsorption, and is a major therapeutic target of the loop diuretics, such as bumetanide and furosemide. NCC is expressed in the distal convoluted tubule of the nephron and is responsible for a further 5% of sodium reabsorption, and can be inhibited by thiazide diuretics, including hydrochlorothiazide. SPAK and OSR1 phosphorylate residues in the N-terminal regulatory domain that are conserved between NKCC1/2 and NCC (Vitari et al., 2006).

Interactions of WNKs and OSR1/SPAK

In a further characterization of OSR1, a yeast two-hybrid screen with the PF2 substrate interaction domain identified several inserts with WNK1. All of these inserts contain the RFXV targeting motif that is recognized by PF2. WNK1 has three RFXV motifs in a 105-residue region in the C terminus. WNK1 has two other RFXV motifs, and other WNK members have at least one, in regions that are otherwise highly divergent among family members. (See fig. 2-2.)

OSR1 is activated by osmotic stress in a WNK1-dependent fashion. Knockdown of WNK1 reduces the osmotic stress-induced activation of OSR1 and influx of potassium through NKCC1, a regulator of osmotic homeostasis (Anselmo et al., 2006). The importance of the OSR1-WNK1 interaction is shown when immunoprecipitation of OSR1 from HeLa cells pulls down WNK1 as the most prominent interactor by far (Fig. 2-6). Immunofluorescent localization of WNK1 and OSR1 shows that an estimated 50%

of WNK1 colocalizes with OSR1. This localization occurs on punctae throughout the cytoplasm.

WNK1 has been shown to phosphorylate OSR1 on Thr185 in the activation loop (and SPAK on Thr243 in the corresponding location). Many kinases are phosphorylated on the activation loop to increase activity (Nolen et al., 2004). Corresponding with this, OSR1 T185A mutants have basal wild type activity and cannot be activated by WNK1. Conversely, mutation of T185 to the phosphomimetic aspartate residue creates a protein which has increased activity over wild type but cannot be further activated by WNK1. However, work on SPAK has shown the protein which has been phosphorylated by WNK1 is more active than SPAK with the T243D mutation alone. Furthermore, a second putative WNK1 phosphorylation site has been identified on OSR1. Ser325 is located in the PF1 domain immediately C-terminal to the kinase domain. This maps to an area which in the structure of the related Ste20 protein TAO1 is positioned to affect allosteric regulation. Mutation of this serine to alanine reportedly completely blocks phosphorylation of OSR1 by WNK1, including at the activation loop site T185 (Vitari et al., 2005). However, this mutation has no effect on whether WNK1 is able to stimulate OSR1 activity.

Initial work studying the physiological function of the WNK proteins followed from the observations of patients with PHA2. It was noted that the phenotype of hypertension, hyperkalemia, metabolic acidosis, and low renin is the opposite of the phenotype observed in patients with Gitelman's syndrome who have a functionally inactivated NCC (Mayan et al., 2002). In addition, many PHA2 patients were successfully treated with thiazide diuretics, which inhibit NCC. WNK4 message is

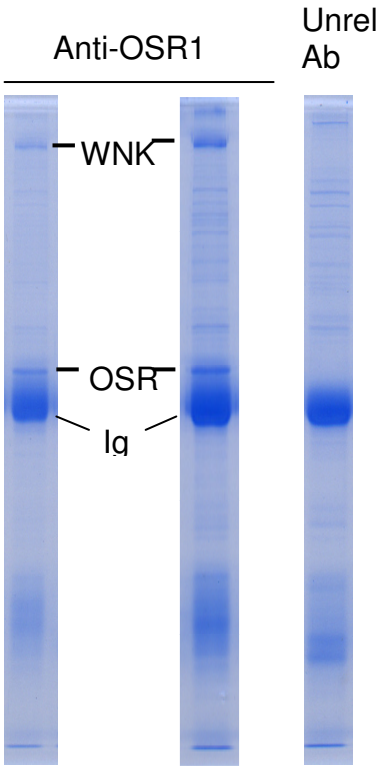


Fig. 2-6 WNK1 immunoprecipitates with OSR1 Immunoprecipitation of endogenous OSR1 reveals one primary co-precipitating band, identified by mass spectrometry to be WNK1. Lane to right is unrelated antibody for immunoprecipitation. Anselmo et al. *PNAS* 2006.

expressed in the distal convoluted tubule of the kidney, where NCC is localized, suggesting a role for WNKs in regulating this key transporter (Wilson et al., 2003). Following from this, a number of groups studied the function of these transporters in the model system of *Xenopus laevis* oocytes for their ease of microinjection and membrane transporter studies. Early studies identified that, in oocytes, WNK4 inhibited the function of NCC, but the PHA2-causing mutations abolished this inhibition (Wilson et al., 2003; Yang et al., 2003). An overactive NCC would drive increased sodium reabsorption, leading to hypertension. This inhibitory effect on NCC is kinase function-dependent, as catalytically inactivated mutants of WNK4 were also unable to suppress NCC activity. This effect was attributed to the decreased surface localization of NCC when co-expressed with wild type WNK4. In these studies, WNK1 had no effect directly on NCC, but when coexpressed with WNK4 was able to restore the full functioning of NCC. This led to the hypothesis that the disease causing mutations were a loss of inhibitory WNK4 function with the missense mutations, or a competitive inhibition by overexpressed WNK1 with the intronic mutations. The regulation of membrane trafficking was consistent with WNK actions in other membrane transporter systems. It should be noted that this work was done without the expression of OSR1/SPAK, and that some of the clinical mutations of WNK4 had little inhibitory effect on NCC in this system, leaving open other possible explanations for WNK physiologic activity in the kidney.

Regulation of WNK4 effects on potassium secretion has also been studied in oocytes. ROMK is a potassium channel expressed in the cortical collecting duct of the distal nephron, in the same cells as the sodium channel ENaC. Activity of both is stimulated by the mineralocorticoid aldosterone. The electrogenic reabsorption of sodium

through ENaC provides the driving force for K^+ secretion; therefore increased aldosterone-stimulated ENaC activity leads to increased ROMK activity. Reconstitution experiments in *Xenopus* oocytes showed that ROMK, when expressed with WNK4, has decreased activity compared to when it is expressed alone (Kahle et al., 2003). In contrast to the effect on NCC, this action seems to be kinase-independent, as catalytically inactive mutants had the same inhibitory effect on ROMK. Like NCC, the inhibitory effects were related to decreased surface localization of ROMK, in this case via a clathrin-dependent endocytosis, without affecting total protein levels of the transporter. Interestingly, the disease-causing mutations Q562E and E559K do not negate WNK4's inhibitory effect on ROMK, but rather enhance it. The N terminus of WNK1 is also able to inhibit ROMK by stimulating its clathrin-coated vesicle-mediated endocytosis in a synergistic manner with WNK4 (He et al., 2007; Lazrak et al., 2006). This is mediated by the first 119 residues of WNK1, and can be inhibited by residues 120-220. In the full protein, the kinase domain is required to be structurally intact but not catalytically active in order to allow the binding of the N-terminal residues to intersectin to affect ROMK. Additional support for this conclusion comes from a double mutant that both changes the catalytic lysine to aspartate and the salt bridge pair aspartate to lysine which is still able to inhibit ROMK without catalytic activity (He et al., 2007). The kidney also expresses the alternatively spliced form KS-WNK1, which does not contain the kinase domain but does contain the autoinhibitory region, at a concentration perhaps ten-fold or more than full-length WNK1. KS-WNK1, and specifically the autoinhibitory region, is able to bind to full-length WNK1 and reverse its inhibition of ROMK activity, although it has no effect on the channel by itself. Presumably, the autoinhibitory region of KS-WNK1 is able to

interfere with the kinase domain, thereby interfering with the effect of the N-terminal fragment of WNK1. Therefore full-length WNK1 inhibits K^+ secretion and KS-WNK1 promotes it. Interestingly, when rats are fed a K^+-deficient diet, the transcript in the kidney for full-length WNK1 is increased, and that for KS-WNK1 is decreased; the converse also holds true (Lazrak et al., 2006). Therefore, the balance between WNK1 splice forms in the kidney could help explain the regulation of K^+ handling in the kidney.

However, some of the difficulties in studying ion transport in oocytes were revealed by studies of WNK4 and NKCC1. One group reported that WNK4, similar to previous studies on ROMK and NCC, inhibited the uptake of ions by NKCC1 when expressed in oocytes by decreasing the surface expression of the transporter (Kahle et al., 2003). However, a separate group reported that WNK4 had no affect on NKCC1 activity (Gagnon et al., 2006). Importantly, when the second group added SPAK to the oocytes, a marked *increase* in activity was noted with WNK4, but not with SPAK alone. This reflects the difficulty in interpretation of oocyte experiments; missing or extra components affect the relationships among all of the interactors. Inhibition of transport and surface downregulation in oocytes was also seen with WNK4 and the chloride-formate exchanger (CFEX) (Kahle et al., 2004). Perhaps in oocytes, WNK4 is able to cause a global effect on membrane translocation. Although not all transporters are affected, as in the same study the anion exchanger Pendrin was not affected. Different transporters are regulated by different mechanisms of endocytosis and WNK4 may only affect a subset in oocytes. As an added note, most of the original studies were done without testing for effects of osmotic conditions, which are known to affect WNK and OSR/SPAK activities.

As mentioned previously, early research on disease-causing mutations of WNK1 and WNK4 focused on the role of NCC. This was due to the fact that thiazide diuretics which block NCC are effective treatments for patients with WNK4 mutations, and because increased apical localization of NCC in the distal convoluted tubule was noted in mice with disease-causing WNK4 mutations knocked in. However, the role of WNK1 was relegated to varying inhibition of WNK4 effects. The identification of the WNK1-OSR1 interaction, and the fact that RFXV motifs are present not only in NKCC2 but also in NKCC1 and NCC, suggested that other WNK proteins can directly interact with these ion transporters. Richardson et al. showed that OSR1 and SPAK phosphorylate NCC in 293 cells and can increase sodium uptake (Richardson et al., 2008). This requires the RFXV motifs to be intact. This interaction provides a direct mechanism for WNK1 to affect sodium reabsorption and therefore hypertension both in the thick ascending limb, where NKCC2 is located, and in the distal convoluted tubule, where NCC is expressed. WNK1 has been shown to phosphorylate OSR1/SPAK, and knockdown of WNK1 reduces OSR1-mediated activation of NKCC1 in response to osmotic stress (Anselmo et al., 2006). Disease-causing WNK1 mutations lead to overexpression of the protein, and these patients are not as treatable with thiazide diuretics. Also, WNK1 actions through SGK1 and ENaC affect sodium handling in the cortical collecting tubule. Effects at multiple sites of sodium reabsorption mediated by WNK1 actions on different transporters would explain why a diuretic targeting a single transporter would not be as effective. In contrast, WNK4 expression is localized to the distal nephron, and would likely only affect NCC, thereby allowing a single diuretic to correct symptoms in these patients. In addition, other minor phenotypic differences occur between patients with

mutations in WNK1 and WNK4. Patients with WNK4 mutations have hypertension as a predominant and early clinical feature, and often also have hypercalciuria (Mayan et al., 2002). However, a study of a large family with a WNK1 deletion showed that most patients presented first with hyperkalemia, years before developing hypertension, and they usually had normal urinary calcium (Achard et al., 2003). This further suggests that the early model in which WNK1 acts simply by inhibiting the actions of WNK4 on NCC is too limited to fully describe the pathophysiology.

While these studies so far have focused on WNK1 and WNK4, the presence of RFXV motifs in many diverse proteins reflect other possible downstream effectors of WNK signaling (Delpire and Gagnon, 2007). Because all four WNKs also contain RFXV motifs, it is likely that all of the WNKs are able to interact with the widely expressed OSR1 and SPAK. In fact, WNK3 has been postulated to play a role in neuronal regulation of chloride signaling. WNK3 was recently shown to activate NKCC2 and NCC function, via activation of SPAK (Ponce-Coria et al., 2008; Yang et al., 2007b). The chloride electrochemical gradient across the cell membrane changes during development. In adults, opening of GABA chloride channels in neurons leads to chloride ion influx and hyperpolarization of the cells. This is the mechanism by which anticonvulsant medications are able to inhibit neuronal excitation. But when these anticonvulsant medications are given to infants, seizures can be provoked because the intracellular chloride concentration is increased during development and early life and opening of GABA channels causes chloride efflux and depolarization. These changes in chloride distribution are hypothesized to be due to WNK3 regulation NKCC1 expression.

Studies to date have revealed the importance of two WNK family members in causing human disease, and there is supporting evidence for roles for the remaining two. While WNK1 and WNK4 have been extensively studied, the roles WNK2 and WNK3 remain relatively uncovered. This dissertation will explore the roles of the four WNK kinases in affecting common substrates OSR1/SPAK, and further elucidate the relative abilities of the WNK family members.

CHAPTER THREE

COMPARISONS OF WNK ISOFORM INTERACTIONS WITH OSR1

Introduction

Research on the WNK kinases increased significantly when mutations in WNK1 and WNK4 were identified as causing inherited forms of hypertension (Wilson et al., 2001).This discovery provided both a direction to focus research and a system in which to at least attempt a physiologically relevant functional assay. Thus, studies to date have primarily focused on WNK1 and WNK4 and their roles in the regulation of renal transporters. However, WNK1 is expressed ubiquitously, WNK2 and WNK3 in multiple tissues, and there is some WNK4 expression outside of the kidney (Delaloy et al., 2003; Holden et al., 2004; Kahle et al., 2004; Moniz et al., 2008; O'Reilly et al., 2003; Verissimo and Jordan, 2001; Verissimo et al., 2006; Wilson et al., 2001; Xie et al., 2009). It is to be expected that further research into the less well-studied family members will provide insights not only into new aspects of WNK1 and WNK4 function but also lead to novel discoveries concerning WNK2 and WNK3. Already, WNK2 and WNK3 have been identified in screens for various cancers and autism-spectrum disorders (Jun et al., 2009; Qiao et al., 2008).

One feature found in common among the WNK family members is the presence of RFXV motifs in their sequences (See fig. 3-1). These motifs provide recognition mechanisms for the OSR1/SPAK kinases to bind to upstream activators and downstream substrates. Mutation of any of the conserved residues in the motif is sufficient to block the interaction (Delpire and Gagnon, 2007; Vitari et al., 2006). Of the proteins identified

to contain RFXV motifs, the vast majority had one motif but WNK1 had five repeats (Delpire and Gagnon, 2007). All of the other WNKs have one or two RFXV motifs conserved in the C-terminal tail, a region otherwise highly divergent between proteins. WNKs 1, 2, and 3 also have an additional motif conserved in the kinase core (Fig. 3-2A).

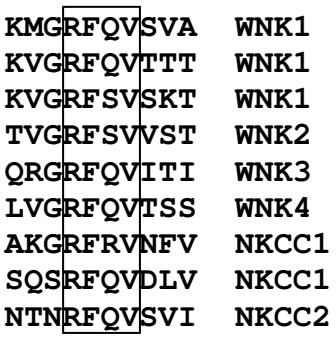


Fig. 3-1 Alignment of RFXV motifs RFXV motifs are conserved in all WNKs and in OSR1 targets NKCC1/2.

Previous work has shown that WNK1 and WNK4 are able to phosphorylate and activate OSR1 and downstream targets, particularly members of the SLC12 family of electroneutral cation-chloride coupled cotransporters (Anselmo et al., 2006; Moriguchi et al., 2005; Vitari et al., 2005). This family includes the renal transporters NKCC2, expressed in the thick ascending limb and targeted by loop diuretics, and NCC, found in the distal convoluted tubule and inhibited by thiazide diuretics. These transporters are thought to play significant roles in the pathophysiology of PHA2 caused by mutations in WNK1 and WNK4 (San-Cristobal et al., 2008). NKCC1 is related to these proteins, is expressed ubiquitously and helps regulate chloride balance and osmotic stress in cells (Haas and Forbush III, 2000). These three SLC12 family transporters contain RFXV targeting motifs. WNK1 has been shown to be required in the OSR1-mediated activation of NKCC1 in response to osmotic stress (Anselmo et al., 2006). This WNK-OSR1/SPAK interaction is dependent on the RFXV motif, as mutation of this motif blocks phosphorylation of OSR1. Increasing amounts of peptide containing the motif can competitively inhibit activation of NKCC2 by WNK1 (Anselmo et al., 2006; Vitari et al., 2006). The importance of OSR1/SPAK to WNK1 function is also

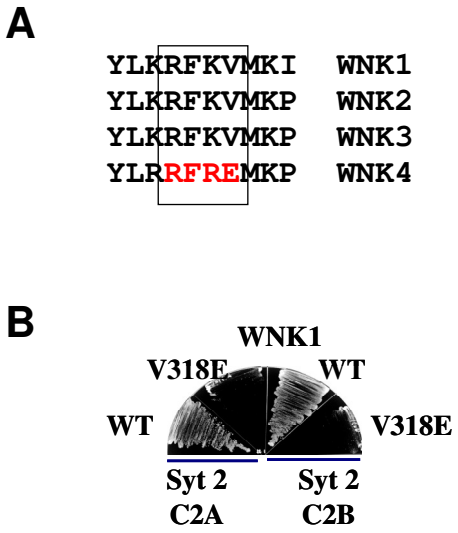


Fig. 3-2 RFXV motif in WNK kinase domain A) Alignment of RFXV motif conserved in WNK1-3 kinase domain. V318 is E in WNK4. B) Yeast two-hybrid with synaptotagmin2 C2 domains and WNK kinase domain either wild type (wt) or V318E. V318 is required for WNK1-Syt2 interaction. Lee et al. *Mol Cell* 2004.

suggested by that fact that WNK1 is by far the predominant protein co-immunoprecipitating with OSR1 from HeLa cells, and by their significant endogenous co-localization by immunofluorescence, as discussed previously.

The valine in the RFXV motif in the kinase domain conserved in WNKs 1-3 is changed to a glutamate in WNK4. While interaction of the PF2 domain of OSR1 is thought to occur at the C-terminal RFXV motifs, it is possible that interactions of the kinase domain alone with OSR1 might be affected by this motif. The valine in this motif has already been shown to play a role in binding of the WNK1 target synaptotagmin2 (Syt2). Yeast two-hybrid assays showed that while WNK1 and WNK4 kinase domains both interact with Smad2, WNK1 and not WNK4 bind to Syt2 (Lee et al., 2004). Mutation of V318 in the WNK1 kinase domain to glutamate was sufficient to block the interaction with WNK1 (Fig. 3-2B). This supports the possible role of this RFXV motif to direct interactions of the kinase domain with target substrates.

The presence of multiple RFXV motifs in WNK proteins and the demonstrated importance of OSR1/SPAK in regulating the known WNK1 and WNK4 physiological functions suggest that other WNK proteins may interact with OSR1/SPAK. Activation by all WNK isoforms was assayed and utilized to evaluate specificity. The importance of the RFXV motif located in the kinase domain in differential WNK1 and WNK4 actions on OSR1 was also evaluated.

Methods

Protein preparation

Bacterial expression constructs were created in the pGEX-KG (OSR1, NKCC2) or pHis-parallel (WNK) vectors and transformed into *Escherichia coli* Rosetta strain cells. Protein expression was induced with 40 to 400 μM isopropyl β-D1-thiogalactopyranoside (IPTG) for 4-16 hours. The bacteria were pelleted, then lysed by a liquid nitrogen freeze-thaw cycle, addition of lysozyme, and sonication. For glutathione-S-transferase (GST)-tagged proteins: lysates were then suspended in 10 mM Tris pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonylfluoride (PMSF), 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM benzamidine. GST-tagged proteins were purified by incubation of lysates with glutathione-agarose beads in 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.6, 100 mM KCl, 1 mM EDTA, 0.5% Triton X-100, 20% glycerol, 1 mM DTT, 1 mM benzamidine, 1 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM benzamidine, followed by elution with 5 mM free glutathione. Eluates were then dialyzed against 1000X volume of 20 mM Tris pH7.5, 1 mM DTT, 1 mM EGTA, 0.125 mM PMSF, 0.137 mg/L pepstatin A. For His-tagged proteins: lysates were suspended in 46.6 mM Na_2HPO_4, 3.4 mM NaH_2PO_4 pH 8.0, 0.3 M NaCl, 10 mM benzamidine, 2 μg/ml leupeptin, 1.4 μg/ml aprotinin, 1 mM PMSF, 1 μg/ml pepstatin. His-tagged proteins were purified by incubation of lysates with Qiagen nickel-nitrilotriacetic acid (Ni-NTA) agarose for 3 hours at 4°C. Resin was washed with at least 100 volumes of 20 mM imidazole in fresh buffer. Proteins were eluted with gradient of 20-125 mM imidazole and fractions collected. Samples containing target protein as

visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were pooled and concentrated in Centricon Ultracel YM-10 centrifugal filter devices.

For the WNK kinase domains, the following protein pieces were used: rat WNK1 210-483, mouse WNK2 184-457, mouse WNK3 135-408, rat WNK4 146-432. Full length rat OSR1 was used for all constructs, using wild type or single kinase inactive mutants D164A or K46R. For substrate for OSR1 activity, GST-tagged human NKCC2 1-175 was used.

Kinase assay

For assays of WNK protein activity, full length OSR1 K46R was utilized as a substrate. OSR1 activity was assayed with NKCC2 1-125 as substrate. GST-tagged enzymes and substrates were added to kinase buffer (10 mM HEPES pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, and 1 mM benzamidine), 500 μM ATP, and prepared [γ-^{32}P]ATP was added at approximately 5,000-13,000 dpm / pmol ATP specific activity. Protein amounts: WNKs – 20 pmol (0.7 μM); OSR1 – 40 pmol (1.3 μM) The reaction was incubated at 30°C for 10 minutes and terminated with addition of sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris-Cl (pH 6.8), 2% SDS, 0.1% Bromophenol blue, 100 mM β-mercaptoethanol, 10% glycerol). Reaction samples were then loaded onto 12% (29:1) polyacrylamide gels for electrophoretic separation. Gels were dried, then exposed to film to visualize bands by autoradiography. Quantification was performed by cutting protein bands from SDS-polyacrylamide gels, adding scintillation fluid and counting with a Beckman LS 3801 liquid scintillation counter. Concentration of attached phosphate was determined assuming 95% detection efficiency.

Coupled kinase assay

Activation of OSR1 activity by WNK was assayed by kinase assay as above, with the following changes. Protein amounts: WNK1 5 pmol (0.14 μM); OSR1 20 pmol (0.57 μM); NKCC2 80 pmol (2.28 μM). All reagents except [γ-^{32}P]ATP and NKCC2 were added to the initial reaction, which was performed at 30°C for 30 minutes, then transferred to ice. NKCC2 and [γ-^{32}P]ATP were then added, and the reactions were incubated 30°C for an additional 10 minutes. Reactions were then stopped with sample buffer and analyzed as above.

WNK kinetics

Assays to determine kinetic constants for WNKs toward OSR1 were performed using the same basic methods as kinase assays above. 300 μM ATP and 1 pmol WNK (31 nM) were added to a range of OSR1 K46R concentrations from 1 pmol (31 nM) to 64 pmol (2 μM). Reactions were incubated at room temperature for 45 minutes. GraphPad Prism 5 software was used to determine kinetic constants and for statistical analysis.

NKCC assay

NKCC1 activity was assayed essentially as described previously (Anselmo et al., 2006). Rat WNK1 1-490 constructs were prepared in the pCMV-myc vector. Endogenous WNK1 was knocked down using the small interfering RNA (siRNA) WNK1.1 oligonucleotide from Ambion: CAGACAGUGCAGUAUUCAC. HeLa cells were grown to 80% confluency, then subcultured at a density of 1:2 in 24-well plates. WNK1.1 or

scrambled oligonucleotide was mixed with Lipofectamine RNAiMAX in Opti-MEM for 10-20 minutes at room temperature, then added to 24-well plates at final oligonucleotide concentration of 33.3 nM. Cells were incubated overnight in 5% CO_2 at 37°C. The next day, scrambled or WNK1.1 oligonucleotide and pCMV vectors were separately mixed with Lipofectamine 2000 for 20 minutes at room temperature. Expression vectors (0.5 µg/ml final concentration) and siRNA oligonucleotides (11 nM final) were then added to 24-well plates and incubated overnight. The next day, cells were washed twice in 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES pH 7.4, 10 mM glucose, 10 mM sodium pyruvate, 0.1% bovine serum albumin, and then incubated in this solution for 30 minutes at 37°C. Thereafter the solution was aspirated and replaced with fresh solution containing 10^6 cpm/ml ^{86}Rb and 0.5 mM ouabain (Na^+/K^+ ATPase inhibitor). Half of the wells were also exposed to 10 µM bumetanide (NKCC1 inhibitor). Cells were incubated for five minutes at 37°C, then washed twice in cold 100 mM $MgCl_2$, 10 mM HEPES pH 7.4. Cells were lysed in 2% SDS and heated to aid in transfer to eppendorf tubes, then boiled for 5 min for complete lysis. Liquid scintillation counting was performed on 100 µl of each lysate. Protein concentration for normalization was determined using Pierce MicroBCA Protein Assay Kit. ^{86}Rb uptake in cells was pooled from triplicate repeats in each experiment. The difference in uptake between wells with and without bumetanide was then determined to give bumetanide-sensitive K^+ uptake.

Results

The ability of WNK1 and WNK4 to phosphorylate OSR1 and increase OSR1 activity toward NKCC2 was compared by *in vitro* kinase assays using the kinase domains

of WNK1 and WNK4 and full length OSR1 wild type and catalytically inactive D164A. As seen in figure 3-3, WNK1 or WNK4 alone without OSR1 is not able to phosphorylate NKCC2 to a significant degree. Long exposures did show faint increases, reflected on the quantification of NKCC phosphorylation in figure 3-3B. Equal amounts of WNK1 and WNK4 are both able to phosphorylate OSR1 D164A (Fig. 3-3C). Multiple experiments showed a slight increase in OSR1 D164A phosphorylation by WNK4, although not to a statistically significant degree. For comparison, an example of a second set of experiments arranged in the same manner but loading amounts of WNK1 and WNK4 to get equal phosphorylation of OSR1 D164A is shown below. While the activities of WNK1 and WNK4 kinase domains to phosphorylate OSR1 are equal, their stimulation of NKCC1 phosphorylation is not. The presence of WNK1 leads to a 58-fold increase in phosphorylation of NKCC2 by OSR1, while WNK4 only causes a 16-fold increase. This difference is even more dramatic when WNK amounts are adjusted to equal units of OSR1 phosphorylation activity; the increase in NKCC2 phosphorylation is far greater in the presence of WNK1 than WNK4. Nevertheless, the data indicate that WNK4 is also able to phosphorylate OSR1 and increase its activity toward NKCC2. It remains possible that WNK stimulation of OSR1 activity toward NKCC2 is determined by more than just phosphorylation of OSR1.

To determine the abilities of other WNK proteins to act on the OSR1-NKCC2 pathway, kinase domains of all four WNK proteins were prepared and assayed *in vitro*. We focused on early differences in activity by assaying for ten minutes. *In vitro*, all four WNK kinase domains are able to phosphorylate OSR1 (Fig. 3-4). There is no significant difference in the ability of the WNK kinases to phosphorylate OSR1 K46R at

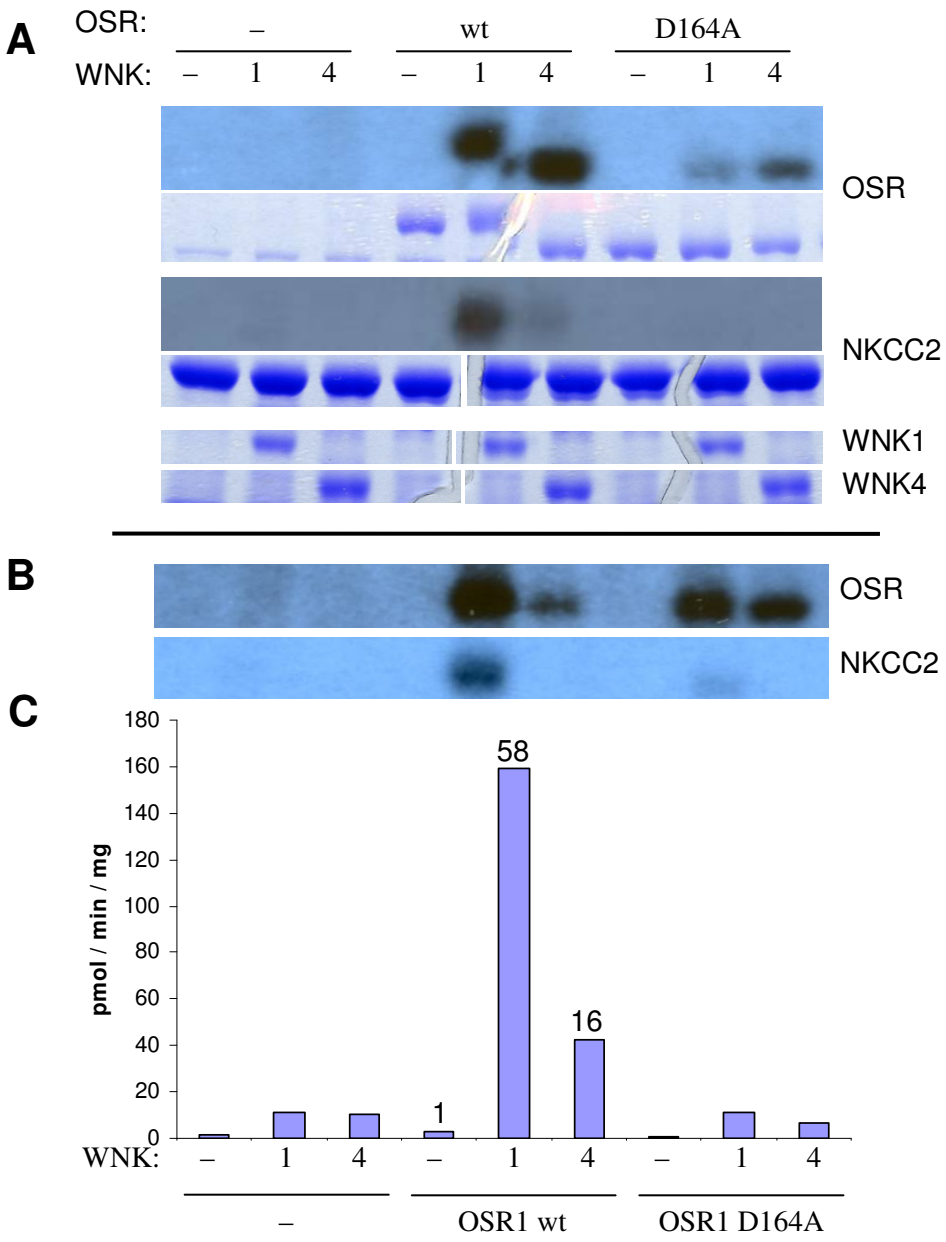


Fig. 3-3 WNK1 vs WNK4 phosphorylation of OSR1 A&C) 1 µg OSR1 wild type or D164A was incubated for 30 min with 1 µg WNK1 or WNK4, then ^{32}P-labeled ATP and NKCC2 1-125 were added for 10 minutes. Bands were visualized by autoradiography (above) and Coomassie stain (below). Quantification of incorporation in NKCC2 shown in C. Scale is pmol Pi incorporated in NKCC2 per minute per mg OSR1. Numbers represent fold activation over wild type. OSR1 in lane 5 appears to run higher due to crack in gel during drying process; not seen in other experiments, compare with B. B) Same experiment, loading WNK1 & 4 to obtain equal activities toward OSR1.

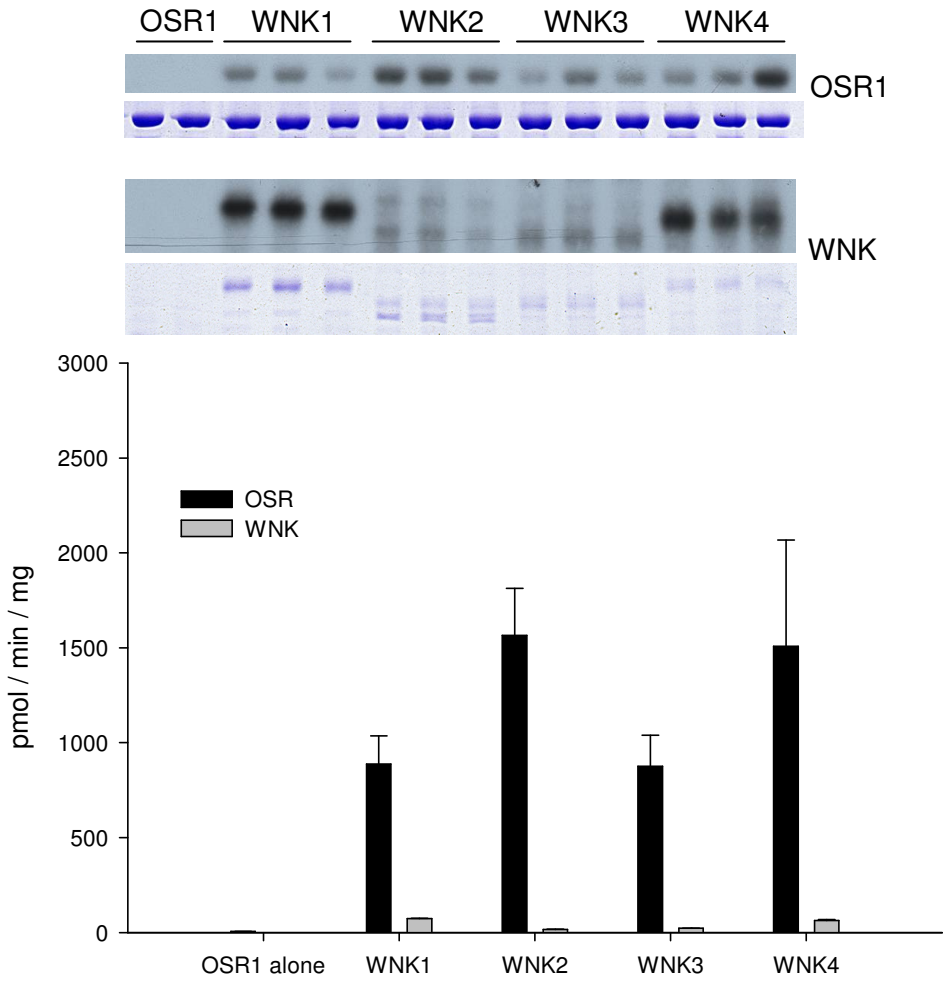


Fig. 3-4 WNK phosphorylation of OSR1 K46R *In vitro* kinase assay of WNK1-4 kinase domain activity on kinase-inactive OSR1. 14.4 pmol WNK species were incubated for 10 min. with 41.6 pmol OSR1 K46R. Bands were separated by SDS-PAGE, visualized by autoradiogram (above) and Coomassie blue (below). Quantification by scintillation counting shown below, scale is pmol Pi incorporated in to OSR1 K46R per minute per mg WNK.

ten minutes. While there is slightly more WNK1 protein in the experiment shown in figure 3-3, as well as slight trends in differences of OSR1 phosphorylation, repeated experiments showed no consistent or significant differences between the WNK kinases. All four WNK kinases are also able to stimulate OSR1 phosphorylation of the target substrate NKCC2 (Fig. 3-5). Addition of WNK kinases caused a 50- to 100-fold increase in NKCC2 phosphorylation over that by OSR1 alone. There was no significant difference between WNK1 and WNK4 in stimulating OSR1 activity. While differences can be seen in the incorporation of phosphate in the final ten minutes, this experiment does not show what degree of OSR1 or WNK phosphorylation occurred in the initial 30 minute preincubation.

Once it was determined that OSR1 functions as an *in vitro* substrate for all WNK isoforms, it was possible to determine kinetic constants for each WNK. Because all WNKs may bind to OSR1 under certain conditions, it was useful to determine how well each might phosphorylate this substrate. The various WNK kinase domains were incubated with increasing amounts of OSR1 K46R catalytically inactive protein for 45 minutes at 25°C. The results are shown in figure 3-6 and table 3-1. From inspection of the auto-radiograms, the plots, and the

Isoform	**K_m (μM)**	**Vmax (nM/min)**	**k_{cat} (min^{-1})**
WNK1	1.5 ± 0.2	19.6 ± 1.7	0.63 ± 0.05
WNK2	0.2 ± 0.1	1.7 ± 0.4	0.05 ± 0.01
WNK3	2.6 ± 0.3	27.2 ± 2.0	0.88 ± 0.07
WNK4	4.3 ± 1.2	36.6 ± 7.9	1.18 ± 0.25

Table 3-1 Catalytic constants for WNK isoforms

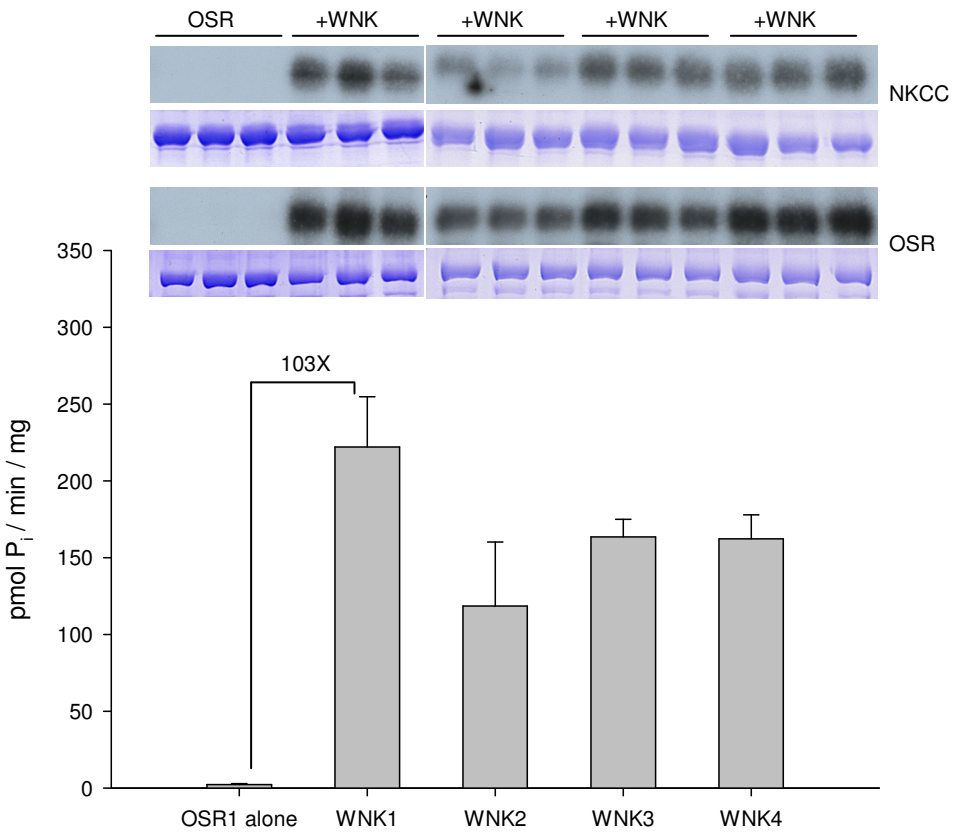


Fig. 3-5 WNK isoform activation of OSR1 20 pmol of OSR1 wild type and 5 pmol of each WNK isoform kinase domain were preincubated with cold ATP for 30 min. Then NKCC2 1-125 and ^{32}P-labeled ATP were added for 10 min. Bands were separated by SDS-PAGE, visualized by autoradiography (above) and Coomassie blue (below) WNK bands were too faint to see by Coomassie stain. Quantification by scintillation counting below. Scale is pmol P_i incorporated in to NKCC2 per minute per mg OSR1.

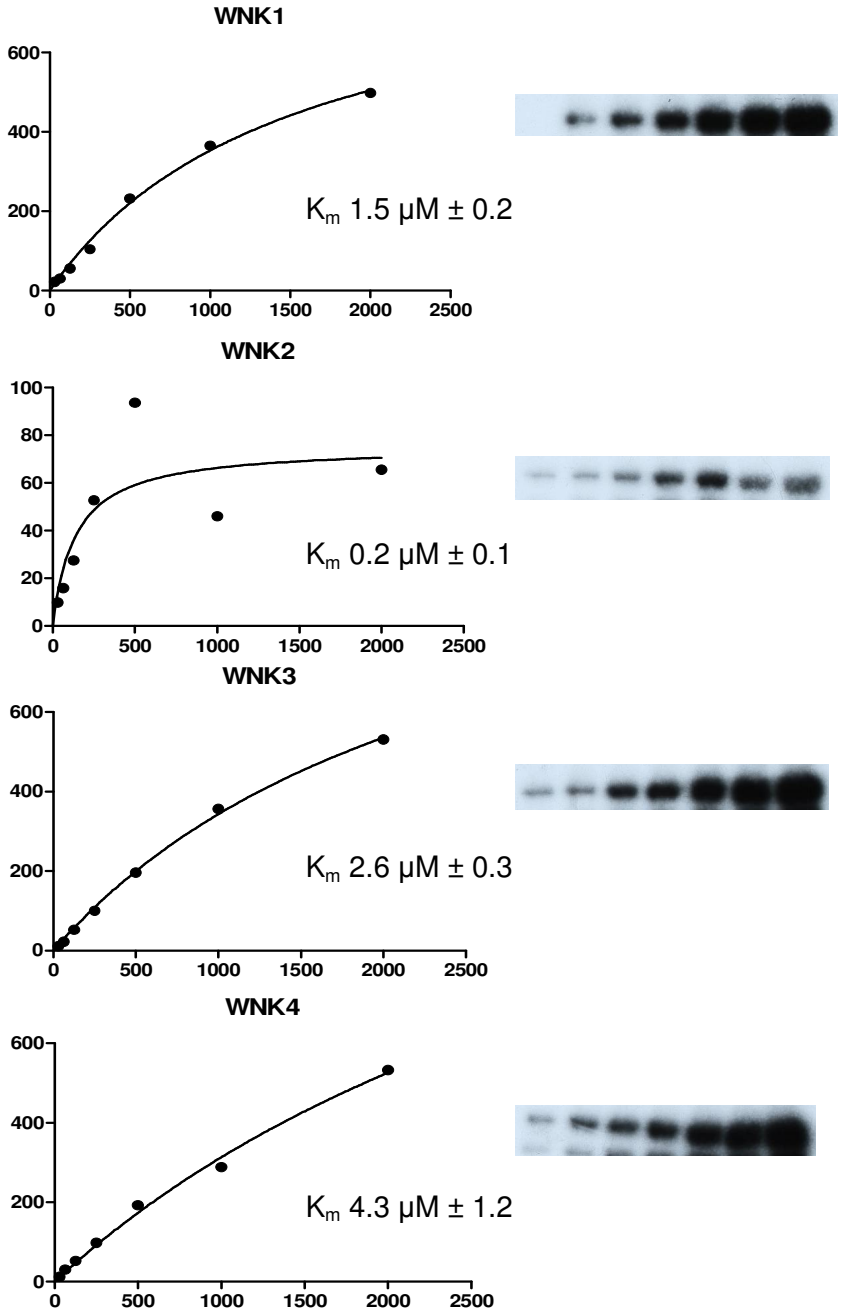


Fig 3-6 Determination of kinetics constants of WNKs toward OSR1 1 pmol WNK kinase domains were incubated with 1-64 pmol OSR1 K46R for 45 minutes at room temperature. Bands were separated by SDS-PAGE and visualized by autoradiography (insets). Total incorporation for each substrate concentration is plotted (nM ^{32}P vs. nM OSR1 K46R).

statistics associated with the calculated values, data for WNK2 seem the least reliable. Kinetic parameters for the other three WNKs were quite similar. K_m values ranged from 1.5-4.3 μM, with standard errors less than 15% for WNKs 1 and 3, and k_{cat} values ranged from 0.63 to 1.18 min^{-1} with standard errors less than 10% for WNKs 1 and 3. An additional set of data will help to resolve questions about the quality of these determinations.

I considered the idea that the presence of an RFXV motif in the kinase domain of WNK1-3 but not found in WNK4 might influence activity Therefore I assayed the ability of the kinase domain of WNK1 V318E to phosphorylate and activate OSR1 *in vitro*. As seen in figure 3-7, while there is a significant difference in autophosphorylation between WNK1 wild type and V318E, there is no difference in the extent of OSR1 phosphorylation. No difference was found from the kinase domain alone in affecting the *in vitro* activity of OSR1 (Fig. 3-8).

As WNK1 V318E was less effective than wild type in binding Syt2, we attempted to examine effects of the mutation in affecting WNK1 activation on NKCC1 activity. As an initial assay, we expressed the kinase domain to see if it was sufficient to replace full length WNK1 function. NKCC1 activity was measured as the bumetanide-sensitive uptake of ^{86}Rb, a potassium-mimetic. Bumetanide is an NKCC2 specific inhibitor. Na^+/K^+ ATPase activity was also inhibited by ouabain for the short time period of ^{86}Rb uptake. Knockdown of WNK1 by siRNA accomplished approximately 50% reduction in ^{86}Rb uptake, consistent with results that were previously published (Anselmo et al., 2006). However, attempted replacement by overexpression of WNK1 1-490,

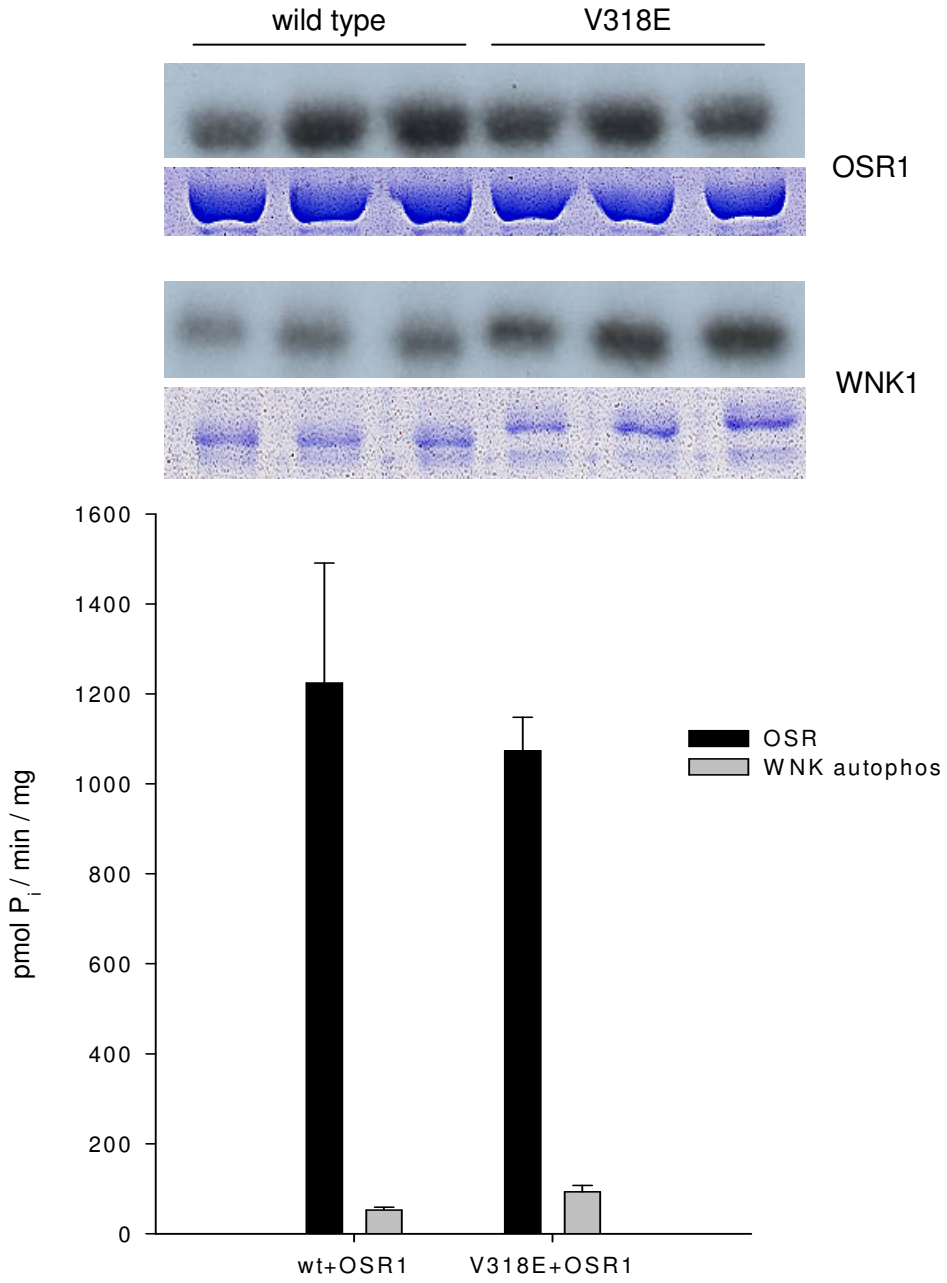


Fig. 3-7 WNK1 wild type vs. V318E phosphorylation of OSR1 K46R *In vitro* kinase assay of 20 pmol of WNK1 wild type and V318E kinase domains on 40 pmol OSR1 K46R, incubated for 15 minutes. Bands were separated by SDS-PAGE, visualized by autoradiography (above) or Coomassie blue (below). Quantification by scintillation counting below. Scale is pmol P_i incorporated in OSR1 K46R per minute per mg WNK1

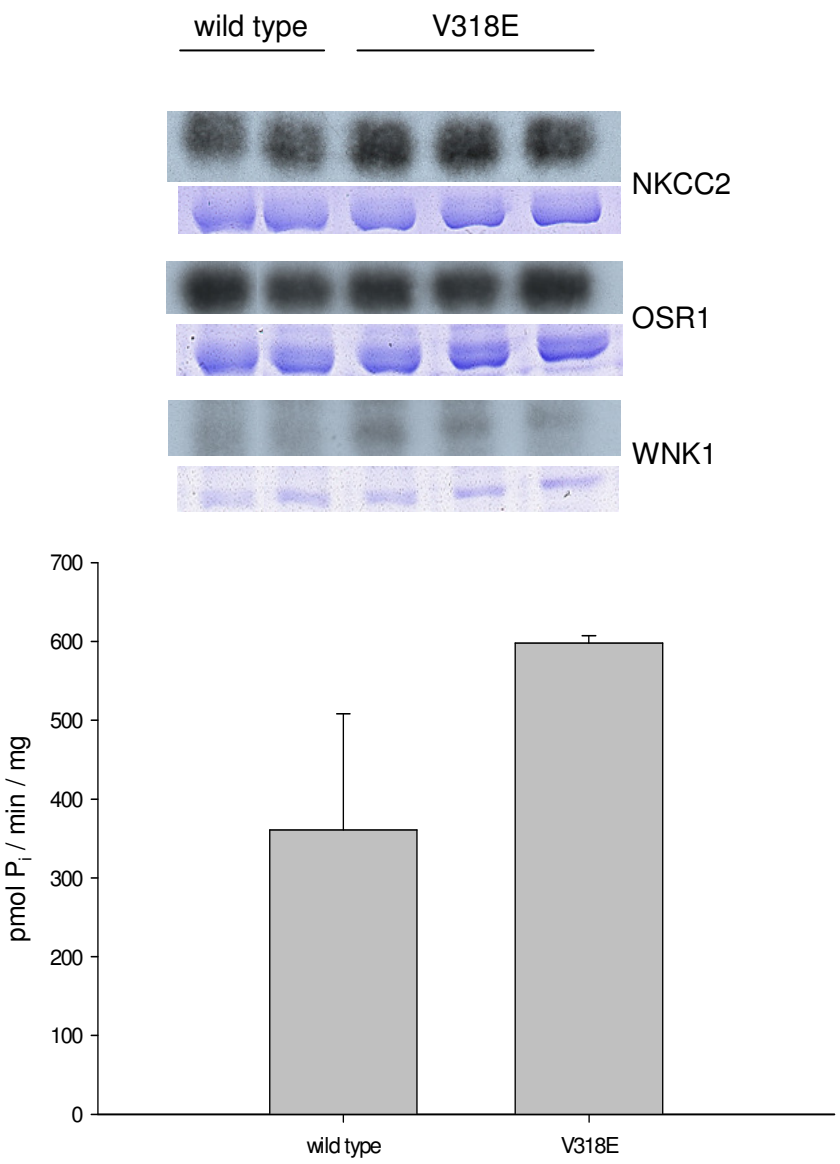


Fig. 3-8 WNK1 wild type vs. V318E activation of OSR1 Coupled kinase assay of WNK1 210-483 either wild type or V318E incubated for 30 min with OSR1, and then addition of NKCC2 1-125 and ^{32}P-labeled ATP for 10 min. Bands separated by SDS-PAGE, visualized by autoradiography (above) or Commassie blue stain (below). Incorporation of ^{32}P on to NKCC2 substrate quantification shown below. Scale is pmol P_i incorporated per minute per mg OSR1.

consisting of the N terminus to the end of the kinase domain, was insufficient to restore NKCC1 function (Fig. 3-9). Neither wild type nor V318E was able to increase activity of NKCC1 over that of the knocked-down cells. We have not yet rescued with full length protein, although those experiments are being undertaken now. Until that time, conclusions cannot be definitively made.

Discussion

We undertook 1) to determine if the different WNK species were able to act on OSR1 and the downstream effector NKCC2; 2) to attempt to explain some initially observed differences in our hands, and 3) to resolve specificity questions raised in the literature. The results provide some answers and suggest some relatively simple additional experiments to clarify some issues further. All four WNK kinases are able to phosphorylate OSR1 and increase its activity towards a recombinant N-terminal fragment of a validated substrate, NKCC2. The relative abilities of the WNK isoforms in stimulating this activity in vitro are roughly equal. This suggests that there may be a role for each WNK isoform to act through the OSR1/SPAK pathway in other physiological processes outside of the kidney.

While the kinase domains are all able to phosphorylate OSR1, differences in activation between WNK1 and WNK4 suggest that phosphorylation of OSR1 may not fully explain WNK effects. This is consistent with data from other known interactors and effectors of WNKs. WNK kinase activity is not required for the effects seen on MEKK3, procaspase-3 and apoptosis, Munc18c, and various renal ion transporters when studied in *Xenopus* oocytes (Oh et al., 2007; Verissimo et al., 2006; Xu et al., 2004). WNKs are

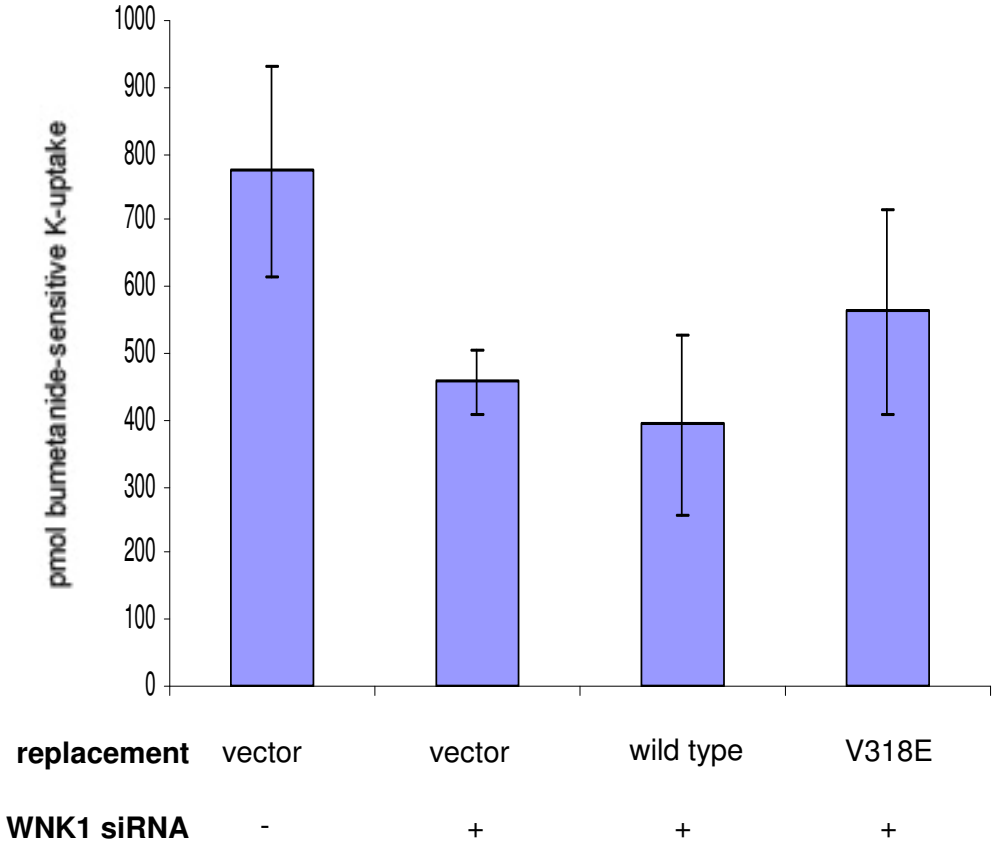


Fig. 3-9 WNK1 kinase domain replacement of WNK1 shows no difference
Endogenous WNK1 in HeLa cells was knocked down in two rounds, and replaced with either vector, WNK1 1-490 wild type or WNK1 1-490 V318E. NKCC1 activity was then assayed as bumetanide-sensitive uptake of ^{86}Rb.

large proteins with generally unknown function outside of their kinase domains but containing multiple protein-protein interaction motifs such as PxxP and coiled-coils. WNKs could act as scaffolding proteins to bring proteins together and enhance their interaction. Follow up studies comparing WNK isoforms *in vivo* could evaluate if their interactions with OSR1/SPAK are relevant, but may not be directly comparable due to differences in likely functional contexts. Expression of WNK2, WNK3, and WNK4 in HeLa cells and measurement of NKCC1 activity via rubidium-uptake assay would show if other WNKs are able to activate OSR1 in cells as well as *in vitro*. While NKCC2 is not likely to be a target outside of the kidney, NKCC1 is expressed ubiquitously. For example, WNK3 has been suggested to regulate NKCC1 activity in different brain tissues to affect Cl^- gradients and neuronal excitability (Kahle et al., 2005).

As mentioned, many of the known functions of WNKs do not require catalytic activity. This is despite the fact that some of the known interactors were discovered by yeast two-hybrid screens using the WNK kinase domain as bait. The K_m determined for the WNK isoforms were in the micromolar range. Interestingly, WNK1 and OSR1 interact closely in the cell, as WNK1 from HeLa cells predominantly immunoprecipitates with OSR1. This is likely due to the multiple RFXV motifs present in the C terminus and might explain why a relatively inefficient WNK1 is still required for the OSR1-NKCC1 response to osmotic stress. The affinity of the RFXV motif for OSR1 may be more important physiologically than the K_m. As expected from more qualitative experiments, WNKs are seemingly poor enzymes, as reflected by the determined k_{cat} of one per minute or worse. However this might not be physiologically relevant, as in a 1:1 complex in cells, one turnover is all that would be required. Attempts to model substrates for WNK

have been hampered by its poor activity. This could reflect poor inherent catalysis, or it could be that we have yet to discover conditions that would increase catalytic activity to its maximum. The only kinases known to phosphorylate WNK1 are Akt and SGK1, but phosphorylation of the N-terminal threonine 60 does not seem to affect kinase activity (Vitari et al., 2004).

A separate group recently assayed the catalytic activity of GST-WNK1 1-491 toward an unspecified OSR1 peptide using a mobility shift assay (Yagi et al., 2009). They determined a K_m of WNK1 toward OSR1 peptide of 68 μM and a k_{cat} toward ATP of 4.6 min^{-1}. In contrast to our experiments, they utilized a longer WNK1 piece, an OSR1 peptide fragment, and a different assay system. Our catalytic values are limited by the fact that the scale of protein production was not sufficient to create enough protein to greatly exceed the determined K_m. Additional experiments may reveal higher K_m values than those determined here. One caveat is that these reactions took place over 45 minutes at room temperature, with a maximal substrate:enzyme ratio of 64:1. The activity assays of the WNKs showed that with ratios of 2:1 to 4:1, incubation for 30 minutes gave 100-fold or greater increases in activity of OSR1. While it is possible that still further activation of OSR1 is possible, this extent of activation suggests that the concentration of unphosphorylated OSR1 T185 is decreasing by the end of the reaction. Thus, it could be that the primary site for phosphorylation has already been filled by 45 minutes and I was assaying WNK phosphorylation of secondary sites. As mentioned previously, many kinases are phosphorylated on multiple activation loop sites. This would explain how, even at 45 minutes, WNK4 is still apparently phosphorylating at an increased rate. Shorter time points should be investigated in the catalytic constant determination.

Interestingly, while WNK2 phosphorylation of OSR1 was on par with other WNKs, it had a non-statistically significant trend toward decreased activation of OSR1 relative to the other isoforms. Perhaps WNK2 does not phosphorylate sites that promote greater activation of OSR1, such as those sites seen in the following chapter. Experiments at shorter time points, with more precise measurements, and ones that include labeled ATP for the pre-incubation period would illuminate these findings. Also, it would be worthwhile to compare the values obtained for WNK activity toward OSR1 with those toward MBP as a reference for specificity comparisons.

Mutation of V318E had no apparent effect on WNK1 activity towards OSR1. However, it is still intriguing that V318, important for WNK1 interaction with a completely different class of proteins, is found in a known interaction motif for OSR1/SPAK. Also of note, the similar motif RVXF has been shown to direct targeting of protein phosphatase-1 to regulators and substrates (Wakula et al., 2003). It is possible that WNK interactions with other proteins might also be mediated by this RFXV motif. The V318E mutant should be studied in other known WNK-interacting systems.

While we were not able to determine any effect of replacement of knocked-down WNK1 with the kinase domain alone, we still have been unable to produce a complete rescue with full-length WNK. Further experiments are currently underway to attempt that reconstitution. This will then allow us to investigate the minimal necessary components required for full activity and the effect of specific mutations such as V318E. The V318E mutant can be re-examined in the context of the full length protein. It would be expected that the C-terminal RFXV motifs would be important in mediating this pathway; however a role for the kinase domain motif is still possible. Previous work has shown that

increasing amounts of a fragment containing those motifs can competitively inhibit the co-immunoprecipitation of OSR1 and WNK1 from HeLa cells. This will also be examined to see if this competition affects NKCC activity.

The OSR1/SPAK proteins are possible targets of all of the different WNK isoforms, as they can phosphorylate OSR1/SPAK and contain RFXV targeting motifs. OSR1/SPAK are ubiquitously expressed in the body and can function as mediators of WNK isoform signaling in multiple different tissues. Further study will reveal the role of this signaling axis in physiologic processes outside of the kidney.

CHAPTER FOUR

FUNCTIONAL ANALYSIS OF PUTATIVE PHOSPHORYLATION SITES ON OSR1/SPAK

Introduction

The closely related sterile20 family OSR1 and SPAK are known effectors of WNK1 signaling pathways (Anselmo et al., 2006; Chen et al., 2004; Vitari et al., 2006). Their sequences are highly similar, except for an extra N-terminal tail on SPAK of unknown function and a segment in the C terminus between the PF1 and PF2 domains. OSR1/SPAK are upstream activators of the cation-coupled cotransporters NKCC1, NKCC2, and NCC, of which the latter two are expressed in the kidney and implicated in the pathophysiology of the WNK1- and WNK4-associated PHA2 (Huang et al., 2008; Wilson et al., 2001). NKCC1 is expressed ubiquitously and regulates cell volume maintenance and chloride trafficking in neuronal systems (Kahle et al., 2005). These proteins interact with activators and substrates by binding via their PF2 domain to RFXV motifs (Delpire and Gagnon, 2007; Vitari et al., 2006). All WNK isoforms contain these motifs and are able to phosphorylate and activate OSR1. WNK1 and OSR1 co-localize and are prominent co-immunoprecipitants from HeLa cells.

WNK1 has been shown to phosphorylate OSR1 on the activation loop at threonine 185 (Vitari et al., 2005). The T185A mutant has catalytic activity roughly equal to the activity of wild type OSR1 alone, but cannot be activated. A phosphomimetic T185D mutant has approximately ten-fold greater activity than wild type protein, but cannot be further activated by WNK1. Many protein kinases are activated by multiple

phosphorylation events along the activation loop, whether by activating enzymes or autophosphorylation (Nolen et al., 2004). There are a number of possible serine/threonine phosphorylation sites in the OSR1 activation loop (Fig. 4-1). Little is known about the effects of these sites on OSR1 activity. Interestingly, recent work in the lab on the crystal structure of the kinase domains of OSR1 and SPAK reveal that the proteins exist as dimers with the activation loop swapped between the domains. It is not known if this swapped configuration is required for full activity, or if phosphorylation events along the activation loop affect the domain swap or dimerization.

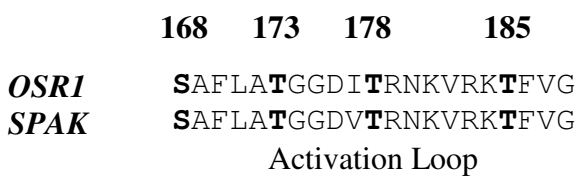


Fig. 4-1 Phosphorylation site on OSR1/SPAK activation loop

A second site of phosphorylation on OSR1 by WNK1 has been identified as serine 325 (Vitari et al., 2005). This site has unknown function, but is located in the PF1 domain of the protein, just C-terminal to the kinase domain. The crystal structure of the kinase domain includes the proximal end of the PF1 domain and shows that it lies along the side of the kinase domain opposite the activation loop, in a position similar to the CD region of ERK. The CD region is important in the binding of ERK regulators to cause allosteric shifts in the kinase active site. In this chapter I have investigated possible roles of this site in WNK1 phosphorylation and activation of OSR1.

Methods

Protein preparation

Bacterial expression constructs were created in the pGEX-KG (OSR1, NKCC2) or pHis-parallel (WNK) vectors and transformed into *E. coli* Rosetta strain cells. Protein expression was induced with 40 to 400 μM IPTG for 4-16 hours. The bacteria were pelleted, then lysed by a liquid nitrogen freeze-thaw cycle, addition of lysozyme, and sonication. For GST-tagged proteins: lysates were then suspended in 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM benzamidine. GST-tagged proteins were purified by incubation of lysates with glutathione-agarose beads in 10 mM HEPES pH 7.6, 100 mM KCl, 1 mM EDTA, 0.5% Triton X-100, 20% glycerol, 1 mM DTT, 1 mM benzamidine, 1 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM benzamidine, followed by elution with 5 mM free glutathione. Eluates were then dialyzed against 1000X volume of 20 mM Tris pH7.5, 1 mM DTT, 1 mM EGTA, 0.125 mM PMSF, 0.137 mg/L pepstatin A. For His-tagged proteins: lysates were suspended in 46.6 mM Na_2HPO_4, 3.4 mM NaH_2PO_4 pH 8.0, 0.3 M NaCl, 10 mM benzamidine, 2 μg/ml leupeptin, 1.4 μg/ml aprotinin, 1 mM PMSF, 1 μg/ml pepstatin. His-tagged proteins were purified by incubation of lysates with Qiagen Ni-NTA resin for 3 hours at 4°C. Resin was washed with at least 100 volumes of 20 mM imidazole in fresh buffer. Proteins were eluted with gradient of 20-125 mM imidazole and fractions collected. Samples containing target protein as visualized by SDS-PAGE were pooled and concentrated in Centricon Ultracel YM-10 centrifugal filter devices.

For the WNK kinase domains, the following protein pieces were used: rat WNK1 210-483, and rat WNK4 146-432. Full length rat OSR1 was used for all constructs. The substrate for OSR1 activity, GST-tagged human NKCC2 1-175 was used.

Kinase assay

For assays of OSR1 activation loop mutants, NKCC2 1-125 was used as a substrate. GST-tagged enzymes and substrates were added to kinase buffer (10 mM HEPES pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, and 1 mM benzamidine), 500 μM ATP, and prepared [γ-^{32}P]ATP was added at approximately 5,000-13,000 dpm / pmol ATP specific activity. Protein amounts: OSR1 – 11.9 pmol (0.3 μM); NKCC2 – 110 pmol (2.8 μM) The reaction was incubated at 30°C for 10 minutes and terminated with addition of SDS sample buffer (50 mM Tris-Cl (pH 6.8), 2% SDS, 0.1% Bromophenol blue, 100 mM β-mercaptoethanol, 10% glycerol). Reaction samples were then loaded onto 12% (29:1) polyacrylamide gels for electrophoretic separation. Gels were dried, then exposed to film to visualize bands by autoradiography. Quantification was performed by cutting protein bands from SDS-polyacrylamide gels, adding scintillation fluid and counting with a Beckman LS 3801 liquid scintillation counter. Concentration of attached phosphate was determined assuming 95% counting efficiency.

Coupled kinase assay

Activation of OSR1 activity by WNK was assayed by kinase assay as above, with the following changes. Protein amounts: WNK1 5 pmol (0.14 μM); OSR1 20 pmol (0.57 μM); NKCC2 80 pmol (2.28 μM). All reagents except [γ-^{32}P]ATP and NKCC2

were added to the initial reaction, which was performed at 30°C for 30 minutes, then transferred to ice. NKCC2 and [γ-^{32}P]ATP were then added, and the reactions were incubated 30°C for an additional 10 minutes. Reactions were then stopped with sample buffer and analyzed as above.

Results

Mutation of the catalytic aspartate residue D164A, reduces basal activity of OSR1 (Fig. 4-2A). Several activation loop mutants show significantly increased activity compared to wild type protein. The phosphomimetic T185D mutant of the known WNK1 phosphorylation site causes a 6.5-fold increase in OSR1 activity over wild type. Minimal but reproducible increases in activity are seen with the single T173D and T178D mutants in proximity to T185. Mutation of S168 to D had no effect on OSR1 activity (data not shown). When combined, the T173D/T178D double mutant had no greater increase in activity than the single mutants alone. However, when combined with a negative charge at T185, mutation of either site to D resulted in a synergistic increase in activity to 21-fold over that of wild type. As seen with the T173D/T178D double mutant, the T173D/T178D/T185D triple mutant had no further effect compared to either T185D double mutant. Nevertheless, I noted 100-fold or greater increases in activity stimulated by WNK1, as shown in figure 3-5. Similar work by Yu-Chi Juang in the lab with the closely related SPAK protein showed that the equivalent mutation (T243D) was more active than wild type protein alone (Fig. 4-2B). The activity of the SPAK mutant was also less than that of wild type SPAK phosphorylated by WNK1. The T243D mutant also could not be activated further by WNK1. These findings confirm that T185 in OSR1 and

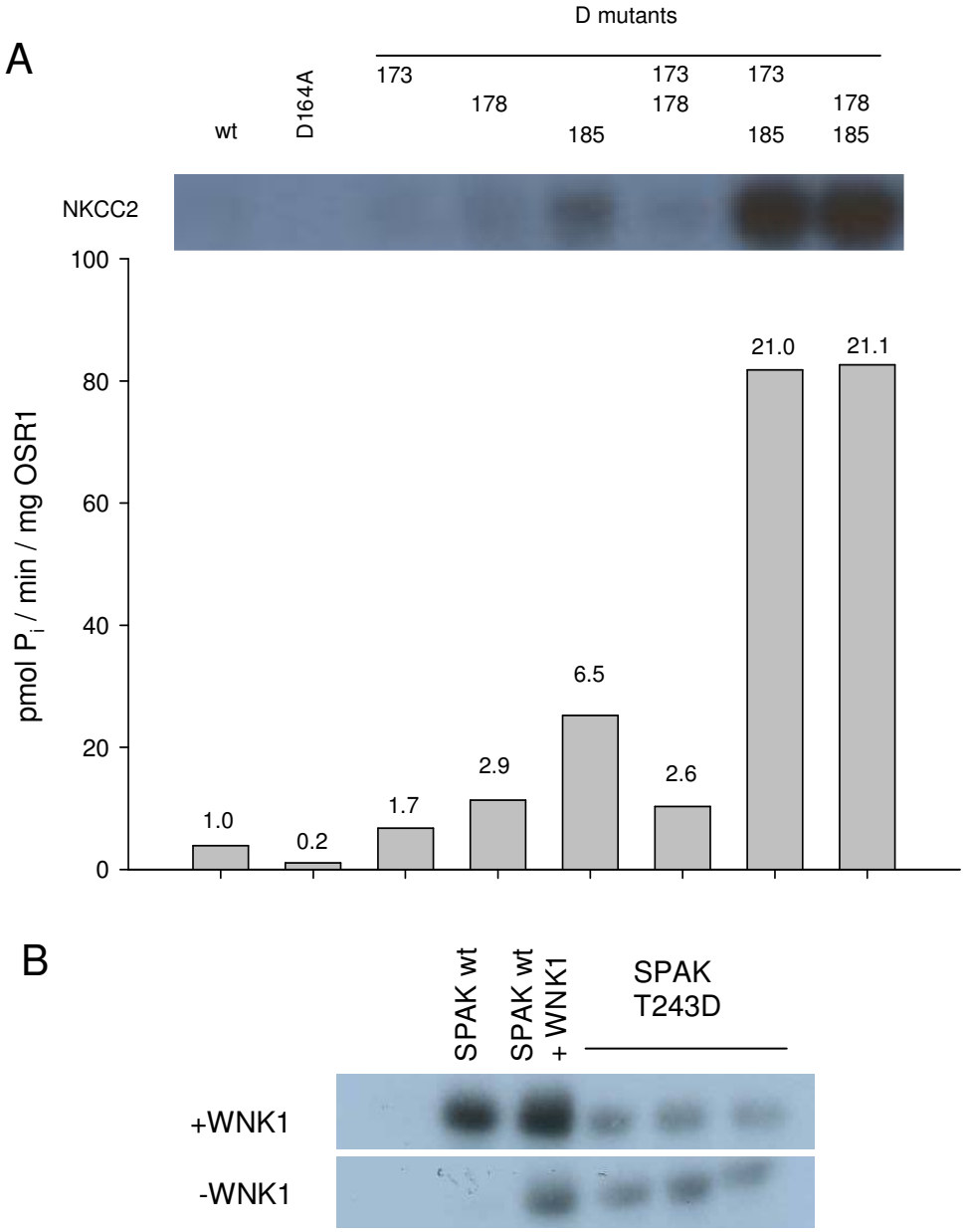


Fig. 4-2 T-D mutations of activation loop A) Phosphomimetic aspartate mutants were made in possible phosphorylation sites along the OSR1 activation loop, and activity measured by *in vitro* kinase assay of 1 µg OSR1 on 5 µg GST-NKCC2 1-125 for 10 min. Bands were separated by SDS-PAGE, and visualized by autoradiography (above). Below is quantification by scintillation counting. Numbers refer to fold activity over wild type. B) Autoradiogram of SPAK T243D or wild type activity on NKCC2, +/- WNK1 phosphorylation. Part B courtesy Yu-Chi Juang.

T243 in SPAK as the major activating sites.

The role of Ser325 in OSR1 phosphorylation and activation was assayed with coupled kinase assays. After preincubation with either WNK1 or WNK4, the activities of OSR1 wild type, S325A, and S325E mutants on N-terminal residues 1-125 of NKCC2 were examined. The OSR1 S325A mutant showed no significant difference from wild type activity, and S325E had only two-fold increased activity (Fig. 4-3). When incubated with WNK1 or WNK4, wild type showed over 200-fold increases in activity compared to unphosphorylated. This increase was unaffected by the S325A mutation, which showed the same behavior as the wild type. However, WNK1 and WNK4 stimulated OSR1 S325E to activities roughly twice as great as that of wild type. Intriguingly, the presence of OSR1 S325E also caused greater WNK1 and WNK4 autophosphorylation. Thus, while OSR1 S325A behaves similarly to wild type without affecting its phosphorylation state, OSR1 S325E shows enhanced activation in response to WNK1, and enhanced WNK1 phosphorylation.

Discussion

We investigated the role of phosphorylation in the activation of OSR1/SPAK. The typical kinase activation loop is phosphorylated by WNK1, as determined by mass spectrometry analysis (Vitari et al., 2005). Phosphomimetic mutation of the T185 site is the most effective single mutation in increasing OSR1 activity. The other activation loop sites shown to increase OSR1 activity when a negative charge is present were not identified by the mass spectrometry study. There are several possible explanations; for

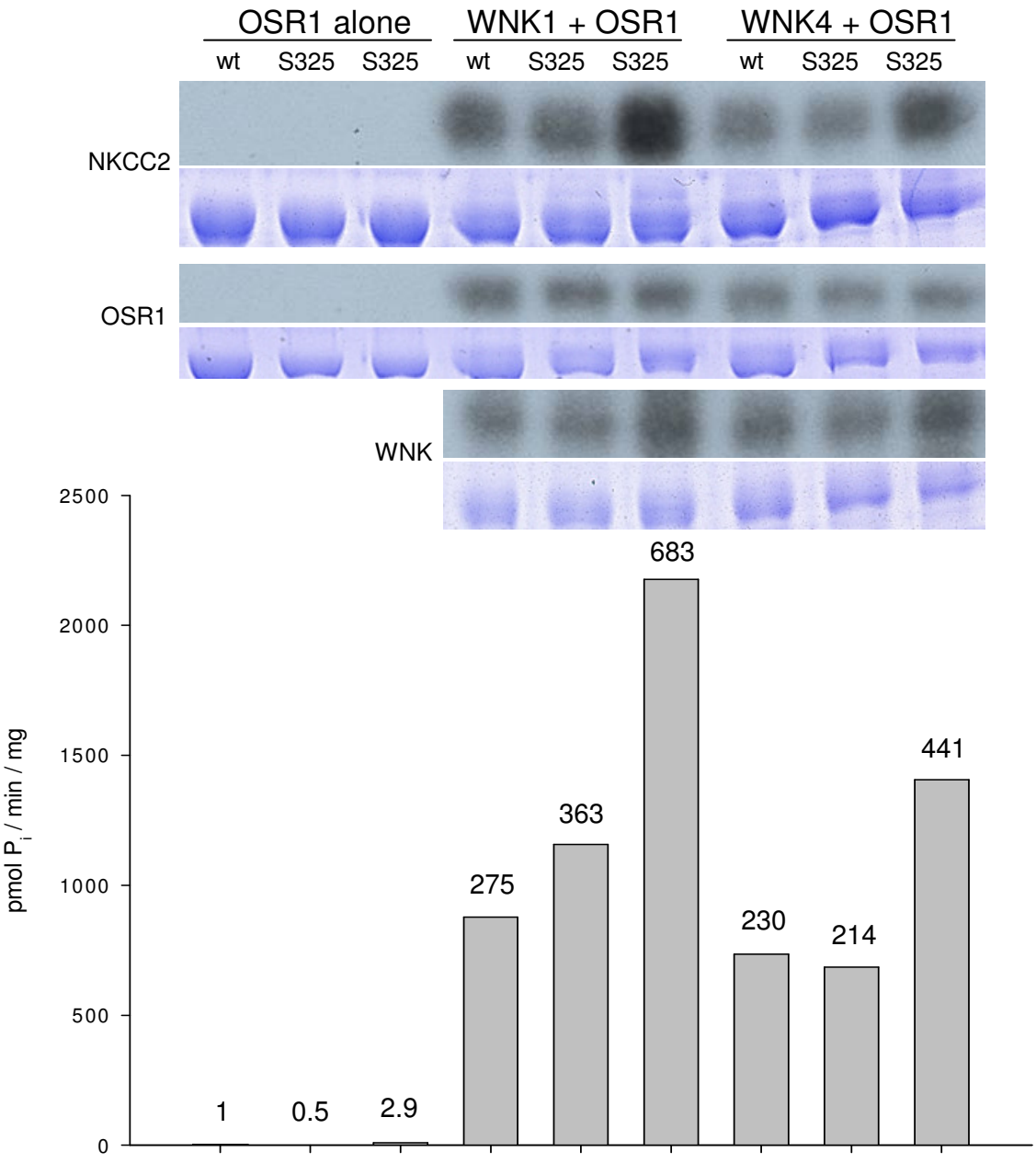


Fig. 4-3 Role of S325 in affecting activity of OSR1 *In vitro* kinase assays of OSR1 wild type, S325A, and S325E, preincubated with WNK1 or WNK4 kinase domains for 30 min. OSR1 activity was then assayed for 10 min against NKCC2 1-125 with ^{32}P-labeled ATP. Bands were separated by SDS-PAGE and visualized by autoradiography (above) or Coomassie blue stain (below). Quantification by scintillation counting shown below. Scale is pmol P_i incorporated into NKCC2 per minute per mg OSR1. Numbers refer to fold activity relative to OSR1 wild type without WNK.

example, autophosphorylation may be responsible for further increasing activity, or these could be irrelevant physiologically. The T185D mutant is not able to fully recapitulate the effect of phosphorylation by WNK1. The S325A mutation has no effect on WNK1-stimulated OSR1 activity. And the T185A mutant is not activated further by WNK1.

However, while a negative charge on S325 is not required for OSR1 activation, as shown by the S325A data, mutation of S325 to E causes an increase in WNK1-stimulated OSR1 activity. This is consistent with previous data (Vitari et al., 2005). However, the S325 experiments have only a limited ability to describe the effects of PF1 mutations on the relative phosphorylation of WNK and OSR1. These proteins were incubated for 30 minutes without labeled ATP, in order to study the maximal effects of OSR1 on NKCC2. Further study should be done with labeled ATP present for the entire reaction to study earlier events in WNK1 phosphorylation.

Together, the data suggest that a strict coupling of phosphorylation and activation may not fully explain the WNK-OSR relationship. As mentioned in previous chapters, WNK activation of downstream effectors is rarely dependent on its kinase activity, even when the kinase domain itself is required. WNK catalytic activity is required for activation of OSR1, as the WNK1 S382A mutant does not increase OSR1 activity (Anselmo et al., 2006). These *in vitro* reactions are with the WNK kinase domains alone, so the role of other parts of the protein, most notably the RFXV motifs in the C terminus, is not being investigated. Instead, non-phosphorylating interactions between WNK and OSR1 are suggested. A key fact is that the S325A mutant, while blocking any phosphorylation of OSR1 by WNK (Vitari et al., 2005), is fully activated by WNK. Previous work in the lab has also shown that while the PF1 domain is required for OSR1

activity, an OSR1 truncation consisting of residues 1-323 that omits the S325 site is still activated by WNK1 (Anselmo et al., 2006; Chen et al., 2004).

One model can be hypothesized, wherein the WNK kinase domain needs to bind to OSR1 in a mechanism that utilizes the structure of the kinase domain but does not actually require catalysis. WNK, at least in the absence of its C-terminal RFXV motifs, interacts via the active site of its kinase domain with the activation loop of OSR1. In such a model the interaction causes an allosteric activation of OSR1. As a perhaps ancillary consequence, T185 on OSR1 gets phosphorylated. OSR1 T185A has the same activity as the wild type without WNK bound, as OSR1 is not in the catalytically-favorable conformation. WNK then cannot increase OSR1 activity. This is not directly because WNK cannot place a negative charge on the activation loop, but because the binding of WNK to the activation site is not maintained by the interaction with T185, and thus is not able to allosterically stimulate OSR1 activity. The previous chapter showed that WNK is a slow enzyme with a low k_{cat}, perhaps allowing for longer interaction with substrate targets. The T185D activation loop also does not interact with WNK and cannot bind it to stimulate full activity. However, the accumulation of negative charges on the activation loop is able to provoke enough change to get a partial increase in activation.

The role of the S325 on the back side of the kinase domain in the PF1 domain is analogous to the allosteric regulation provided by the CD region of ERK. Phosphorylation of S325 provides greater access to the OSR1 active site, but is not required for WNK to bind. Thus, the S325A mutant cannot be phosphorylated in the PF1 domain, so the activation loop is not as accessible and T185 also is not phosphorylated. However, WNK is still able to bind the activation loop and increase OSR1 activity, but

does not have access to T185. (Alternatively, T185 provides stability for another part of OSR1 to bind to WNK, which positioning is abrogated by substitution with alanine. Then phosphorylation of T185, or a T185D mutant, acts to terminate the binding of WNK1 and the increased activity, but leaving the protein is a partially activated state.) S325E, mimicking the greater access caused by S325 phosphorylation, is more open even before WNK1 is present, allowing faster access and increased activation. The intriguing finding of increased WNK1 phosphorylation with the S325E mutant present could represent a reciprocal allosteric opening of a WNK1 autophosphorylation site.

This model might be tested via a number of experiments. Different mutations of T185 could be tested for direct binding affinity for WNK1. The K_m of WNK1 for OSR1 wild type and T185D can be compared, as well as the k_{cat} in the presence of S325 mutants. The binding of the WNK1 SA inactive mutant can be assayed with different OSR1 T185 and S325 mutants to see if binding is increased without WNK1 phosphorylation. A T185A/S325E double mutant will allow exploration of the possibilities of a primed PF1 domain in the presence of a functional but not phosphorylated activation loop. The physiologic role of the C-terminal RFXV motifs would also need to be examined. The initial screen identifying OSR1 and WNK1 as interactors pulled out the fragment of WNK1 containing three motifs. However, while the interaction between the OSR1 C-terminal PF2 domain and the WNK C-terminal RFXV motifs is responsible for bringing the proteins together, it is unlikely to explain changes in the kinase domain functions. Instead, like other WNK proteins, a role for non-catalytic WNK-OSR1 interactions should be investigated.

CHAPTER FIVE

INITIAL CHARACTERIZATION OF WNK2

Introduction

WNK1 was originally cloned from a rat forebrain cDNA library in a screen for novel MEK family proteins (Xu et al., 2000). This same screen produced a fragment of a second family member, defined as WNK2. By homology with WNK1, this protein contained the kinase domain and N- and C-terminal flanking regions of 62 and 136 residues, respectively. At the time, this was all that was known of WNK2, but since then, a predicted protein sequence based on genome analysis has been placed in GenBank and the full length protein has begun to be characterized. Based on this sequence, the fragment of WNK2 cloned from the library consists of rat WNK2 341-806. Outside of the 91% conserved kinase domain, WNK2 also shows a region of 60% conservation just C-terminal to the kinase domain that in WNK1 was shown to be an auto-inhibitory region. I performed the initial characterization of WNK2 at that time.

Methods

Constructs/Proteins

Constructs were created by polymerase chain reaction (PCR) amplification of the indicated regions in figure 5-1 with flanking restriction enzyme sites from the clone obtained from the original screen of rat forebrain library. Note that the numbers representing the kinase domain in the rat sequence are significantly higher than those of

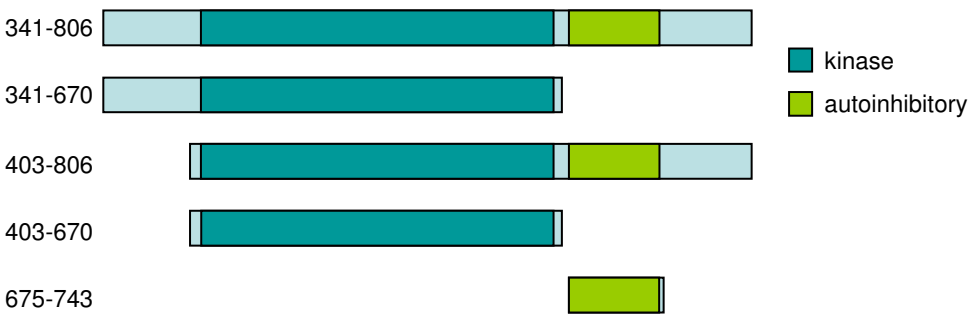


Fig. 5-1 WNK2 fragments used in assays Kinase domain present in dark green, autoinhibitory domain in light green.

the mouse sequence used in previous chapters. The rat WNK2 sequence in the database has an extra 210 residues on the N-terminus. These constructs were then ligated into the pGEX-KG bacterial expression vector or pCMV5-myc mammalian expression vector. Final constructs were verified by restriction enzyme analysis. GST-tagged pGEX-KG constructs were transformed into *E. coli* BL21 cells, and proteins induced with 40 μM IPTG for 4 hours at 30°C. The bacteria were pelleted, and then lysed by a liquid nitrogen freeze-thaw cycle, addition of lysozyme, and sonication. Lysates were then suspended in 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM benzamidine. GST-tagged proteins were purified by incubation of lysates with glutathione-agarose beads in 10 mM HEPES pH 7.6, 100 mM KCl, 1 mM EDTA, 0.5% Triton X-100, 20% glycerol, 1 mM DTT, 1 mM benzamidine, 1 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM benzamidine. This was followed by elution with 5 mM glutathione, then dialyzed against 1000X volume of 20 mM Tris pH7.5, 1 mM DTT, 1 mM EGTA, 0.125 mM PMSF, 0.137 mg/L pepstatin A.

Immunoprecipitation

Constructs in myc-tagged (WNK2) or hemagglutinin (HA)-tagged (ERK5) mammalian expression vectors were transfected via calcium phosphate into human embryonic kidney 293 cells plated the previous day at 60-80% confluency. Cells were incubated at 37°C overnight in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) for one night, and then overnight in serum-free medium. Cells were washed in phosphate-buffered saline (PBS) (130 mM NaCl, 3 mM NaH_2PO_4, 70 mM Na_2HPO_4, pH 7.4). Cells were then placed in lysis buffer (0.5% Triton X-100, 50

mM HEPES pH 7.6, 150 mM NaCl, 10% glycerol, 10 μM Na_3VO_4, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM benzamidine) and cells were snap frozen in liquid nitrogen. For Western blotting, lysates in sample buffer (50 mM Tris-Cl (pH 6.8), 2% SDS, 0.1% Bromophenol blue, 100 mM β-mercaptoethanol, 10% glycerol) were loaded onto 8 or 12% (29:1) polyacrylamide gels for separation, then transferred to nitrocellulose. Filters were then blocked in 5% milk in 100 mM Tris-Cl pH 8.0, 150 mM NaCl, 0.05% Tween-20 (TBST). Primary antibodies included anti-myc and anti-HA from Sigma. For WNK2 blots, rabbit polyclonal antisera were raised using standard methods against the WNK2 peptide LATLKRASFAKSVIGTPEF, between subdomains VII and VIII of the kinase domain. Proteins were visualized by chemiluminescence and exposed to film. Tagged proteins were immunoprecipitated from lysates with either anti-Myc (WNK2) or anti-HA (ERK5) antibodies, collected with protein A-Sepharose beads, and washed with 200 volumes of 20 mM Tris-Cl pH7.4 , 1 M NaCl. Beads with proteins attached were utilized in kinase reactions.

Kinase assay

GST-tagged proteins or Sepharose-bound proteins were suspended in 10 mM HEPES pH 8.0, 10 mM $MgCl_2$, 50 μM ATP (~70 dpm/fmol [γ-^{32}P]ATP), 1 mM DTT, and 1 mM benzamidine, plus or minus 0.3 mg/ml myelin basic protein. The reaction was incubated at 30°C for 60 minutes and terminated with addition of sample buffer. Reaction samples were then loaded on to 8 to 12% (29:1) polyacrylamide gels for electrophoretic separation, dried, then exposed to film to visualize bands by autoradiography.

Site-directed mutagenesis

The WNK2 D553A mutant was generated by site-directed mutagenesis using overlapping primers at the mutation site and PCR over the entire plasmid. The original plasmid was digested with DpnI and mutants were selected by transformation into *E. coli* cells and sequencing.

Results

WNK2 is a protein kinase

Constructs were created that expressed the WNK2 kinase domain with and without the flanking regions, and with an N-terminal myc tag. These proteins were expressed in and immunoprecipitated from 293 cells. Consistently, the proteins encoded by constructs beginning at the kinase domain (403-806 and 403-670) did not express well, either in 293 cells or in *E. coli* but were easily recognized by an anti-WNK2 serum (Fig. 5-2). However, in spite of poor expression, all of the immunoprecipitated WNK2 proteins were able to phosphorylate the model protein kinase substrate MBP in kinase assays (Fig. 5-2). These proteins also autophosphorylated, although phosphorylation was difficult to detect for the weaker expressing proteins. These results showed the first demonstration of protein kinase activity of WNK2. Like WNK1, WNK2 is a protein kinase, capable of phosphorylating substrates and itself.

Protein kinases contain some conserved residues vital for catalytic activity. As described above, WNK1 has its catalytic lysine in a different location from other protein kinases. Like WNK1, WNK2 lacks the lysine in β strand 3 and contains the same

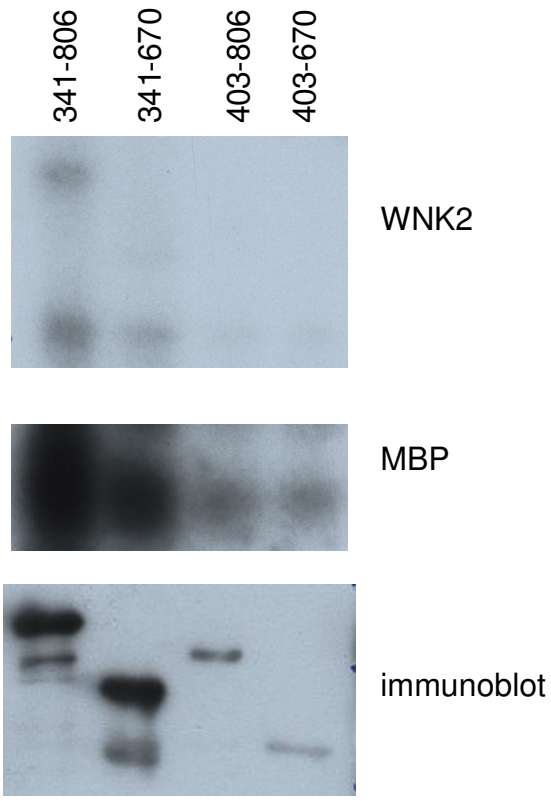


Fig. 5-2 Kinase activity of WNK2 fragments The indicated WNK2 fragments were transfected in to HEK 293 cells, then immunoprecipitated with anti-myc antibody. Beads were incubated with 10 µg MBP for one hour. Bands were separated by SDS-PAGE and visualized by autoradiography (top autophosphorylation, middle MBP). Bottom: Immunoblot of transfected 293 cell lysates with anti-Myc. Note low expression of 403-806 and 403-670, still slight activity on MBP.

cysteine in its place. Instead, this essential lysine that is required for WNK1 catalytic activity is found in β strand 2. Multiple attempts were made to mutate this lysine to analyze its essential function in WNK2 catalytic activity, but all were unsuccessful. However, another essential residue is the subdomain VII aspartate residue that is often mutated to create catalytically-inactive kinase mutants. This residue in WNK2, D553, was mutated to alanine, and GST-tagged wild type and mutant proteins were expressed in bacteria. As shown in figure 5-3, the WNK2 wild type fragment is able to phosphorylate MBP and itself in an *in vitro* reaction. However, the D553A mutant is completely inactive, unable to phosphorylate either itself or MBP. Thus, like other protein kinases, WNK2 requires the conserved aspartate residue for kinase function. This result also argues against the observed protein kinase activity being due to a protein co-immunoprecipitating with WNK2. Therefore, the WNK2 kinase domain is an active kinase, dependent on the presence of D553.

WNK2 has an autoinhibitory region

Many protein kinases have an autoinhibitory region that acts as a regulatory mechanism. In some cases, this region is in a regulatory subunit (e.g. protein kinase A) and in some it is contained within the same protein chain (e.g. protein kinase C). WNK1 has been shown to contain an autoinhibitory region just C-terminal to the kinase domain, and this region is conserved in WNK2 (Xu et al., 2002a). The fragment of WNK1 containing the autoinhibitory region, 490-555, and fragments of WNK2 were expressed in bacteria and purified for *in vitro* kinase assays. As seen in figure 5-4, addition of the WNK1 autoinhibitory region decreased WNK2 kinase activity by 40-50%. This was

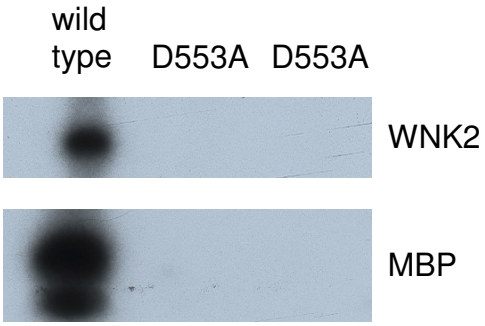


Fig. 5-3 WNK2 requires conserved catalytic aspartate for activity *In vitro* kinase assay of 2 µg WNK2 341-806 wild type or D553A on 10 µg myelin basic protein, incubated for one hour. Bands were separated by SDS-PAGE and visualized by autoradiography. D553A retains no activity.

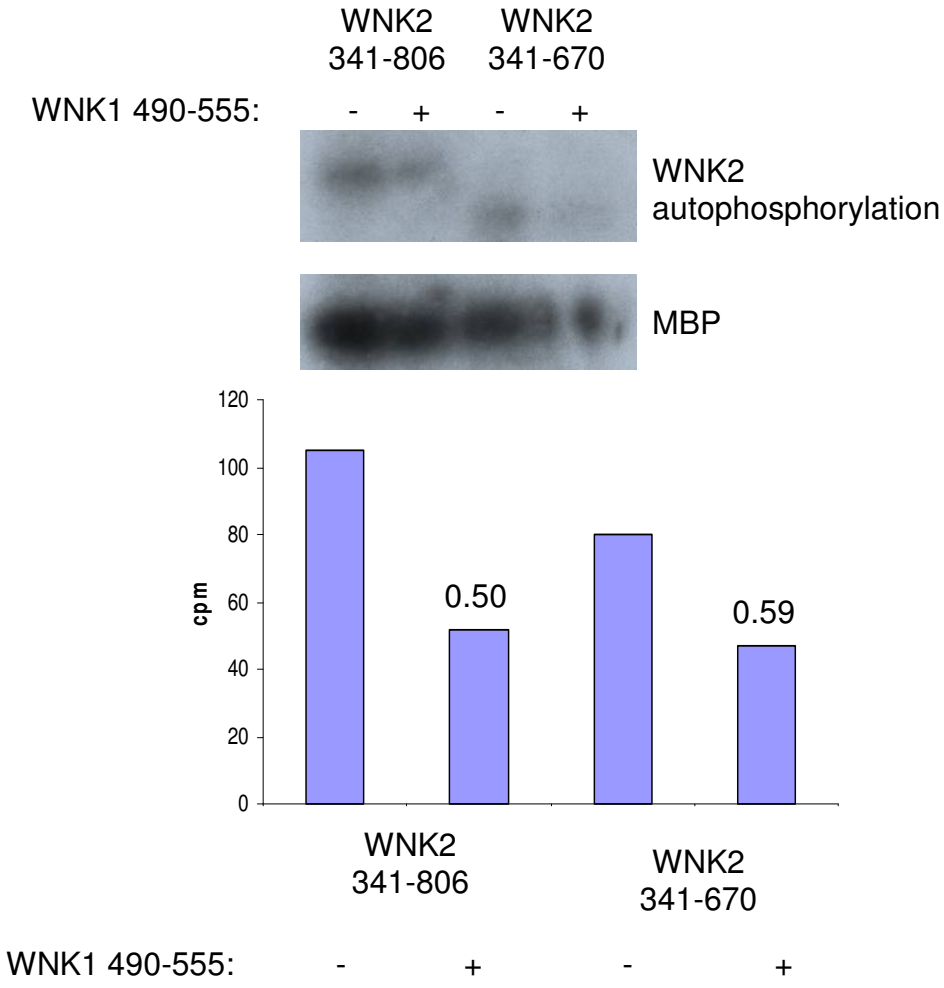


Fig. 5-4 WNK1 autoinhibitory region reduces WNK2 kinase domain activity *In vitro* kinase assay of 2 µg purified GST-tagged WNK2 fragments +/- 2 µg WNK1 autoinhibitory region, assayed on 10 µg MBP. Bands were separated by SDS-PAGE and visualized by autoradiography. Quantification of MBP incorporation below.

followed by further work in the lab that showed increasing amounts of WNK1 autoinhibitory fragment reduced WNK2 activity up to 50% (Fig. 5-5). WNK4 is also inhibited, to a greater extent than WNK2. This shows that the WNK1 autoinhibitory region can affect the WNK2 kinase domain.

To investigate the possible autoinhibitory region in WNK2 further, a construct was transformed in bacteria that encoded the putative inhibitory region, WNK2 675-743. When assayed with MBP alone, this WNK2 fragment had no activity. However, when combined with WNK2 constructs that have activity, the presence of WNK2 675-743 reduced kinase activity of the catalytic domain by approximately 50% (Fig. 5-6). The WNK2 autoinhibitory region also inhibited the kinase activity of WNK1 1-490, albeit to a slightly lesser extent than its effect on WNK2. Thus, the WNK2 region 675-743 just C-terminal to the kinase domain also contains an autoinhibitory region, and the WNK1 and WNK2 autoinhibitory regions are able to inhibit both family members. It should be noted that WNK2 proteins containing both the kinase domain and the autoinhibitory region do not consistently show decreased activity relative to the WNK2 kinase domain alone. The reason for this is unknown but may include proteolysis or misfolding due to nonoptimum fragment boundaries.

WNK2 activation of the ERK5 pathway

At the time, little was known about downstream effectors of the WNK proteins. However, WNK1 was able to activate the ERK5 MAP kinase pathway when overexpressed in 293 cells (Xu et al., 2004). We overexpressed WNK2 fragments in serum-starved 293 cells along with ERK5, immunoprecipitated ERK5 and measured

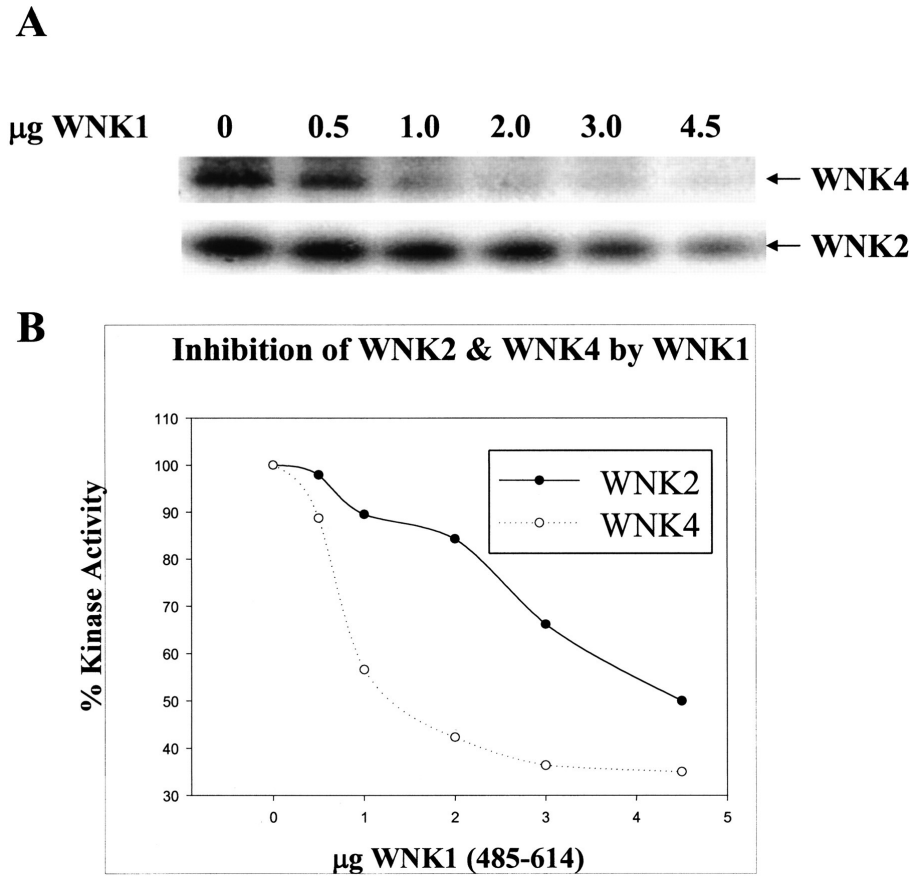
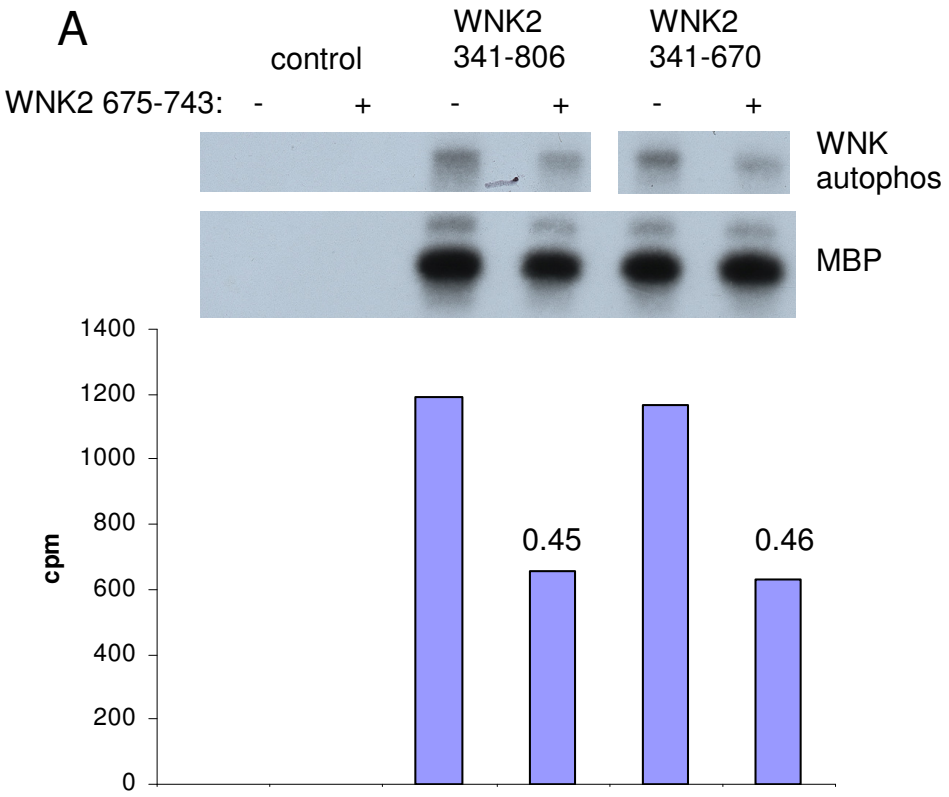


Fig. 5-5 WNK1 autoinhibitory region inhibition of WNK2 and WNK4 1 μg of the kinase domains of WNK2 or WNK4 was incubated with between 1.0 and 4.5 μg of WNK1 485-615, containing the autoinhibitory region in *in vitro* kinase assays for 20-30 min.WNK2 inhibition ranges from 10% (1.0 μg WNK1) to 50% (4.5 μg WNK1). WNK4 inhibition ranges from 44% (1.0 μg WNK1) to 65% (4.5 μg WNK1). *A* autoradiogram *B* Quantification of three experiments plotted. Lenertz et al. *J Biol Chem* 2005.



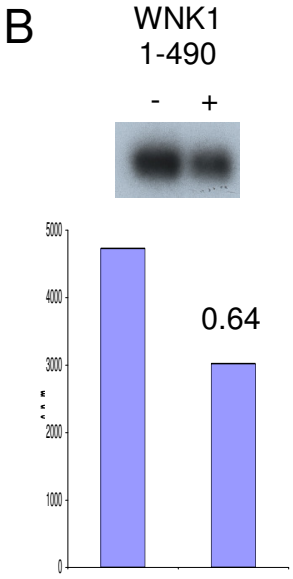


Fig. 5-6 WNK2 675-743 contains an autoinhibitory effect A) *In vitro* kinase assay: 2 µg purified GST-WNK2 kinase domain fragments were incubated +/- 2 µg WNK2 675-743 then assayed for activity against 10 µg MBP, incubated for one hour. Bands were separated by SDS-PAGE and visualized by autoradiography (above) and quantified by scintillation counting (below). Numbers reflect percent activity relative to no inhibitor. B) Assay of autophosphorylation of 0.5 µg His_6-WNK1 1-490 +/- 0.2 µg GST-WNK2 675-743 for one hour. Bands separated by SDS-PAGE and visualized by autoradiography. Quantification by scintillation counting below. Number reflects percent activity relative to no inhibitor.

kinase activity using auto-phosphorylation of ERK5, which has been shown to be an acceptable measure of ERK5 activity. While ERK5 without any overexpressed WNK has activity, coexpression with WNK2 341-806 doubles that activity (Fig. 5-7). In addition, WNK2 341-670, which lacked the autoinhibitory region, appeared to be slightly more effective in activating ERK5, although the effect was not statistically significant. Activation stimulated by WNK2 was comparable to the increase caused by WNK1 1-490. Interestingly, a WNK1 protein that contained the kinase domain plus the autoinhibitory region was unable to increase ERK5 activity. Thus, like WNK1, WNK2 in 293 cells was able to activate the ERK5 pathway, and removal of its autoinhibitory region marginally increased that activation.

Discussion

At the time of the cloning of WNK1 and the discovery of its unusual kinase domain architecture, it was surprising that the protein was catalytically active. Identification of the catalytic lysine and solving of the crystal structure of the kinase domain showed how this was possible. This unusual kinase domain was found in three mammalian proteins closely related over the kinase domains, but widely divergent over the remaining sequence. Confirming that another WNK family member is also catalytically active further supported the *in vivo* kinase activity of WNK1 while other evidence was being developed, as well as showed that the role of WNK2 *in vivo* might require its kinase activity. Experiments here confirmed the ability of WNK2 to phosphorylate itself and model substrates, and work in previous sections demonstrated it is also able to act on the WNK1 substrate OSR1. It should be noted that these types of

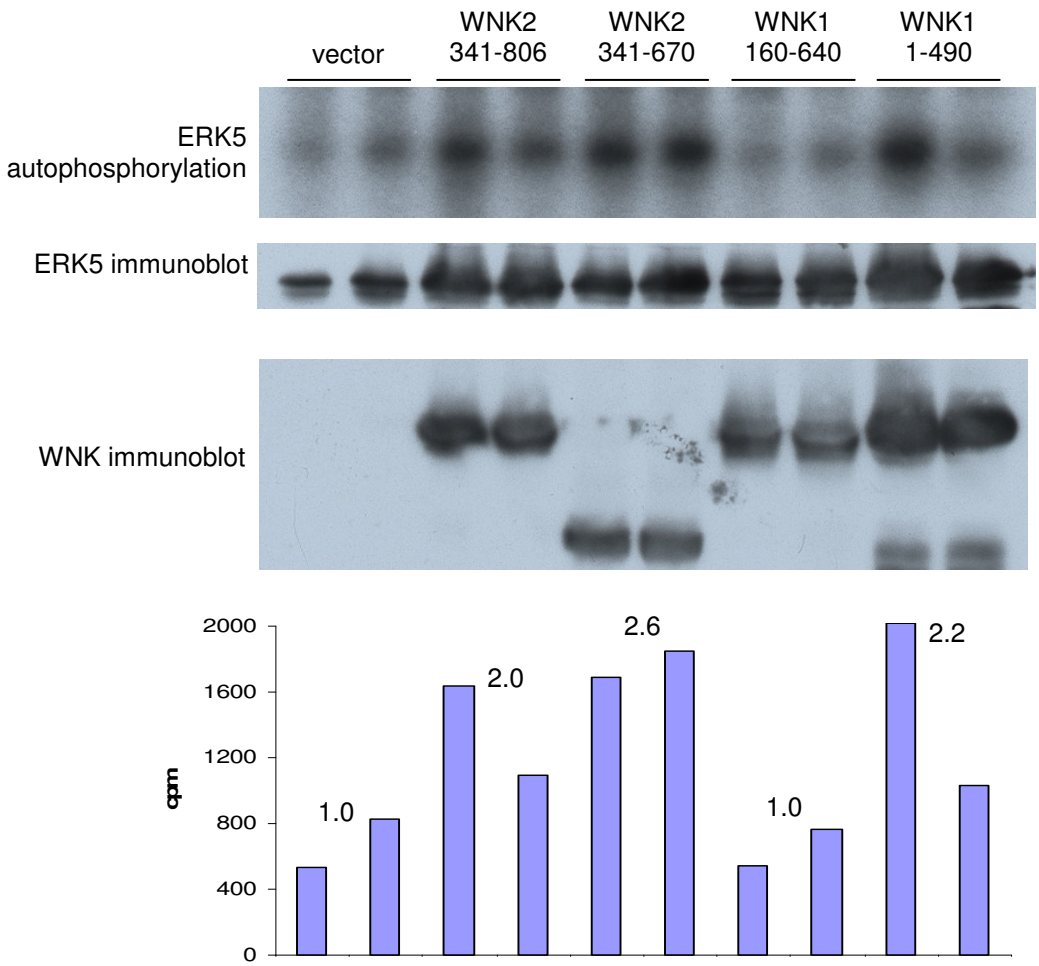


Fig 5-7 WNK2 activates ERK5 pathway Indicated WNK2 and WNK1 fragments were expressed in 293 cells along with HA-tagged full-length ERK5. ERK5 was then immunoprecipitated from cell lysates and beads were assayed for autophosphorylation for one hour. Bands were separated by SDS-PAGE and visualized by autoradiography (above). Middle, ERK5 and WNK immunoblot from lysates. Bottom, quantification of ERK5 autophosphorylation by scintillation counting. Numbers reflect average fold increase over ERK5 without WNK transfection.

experiments, however, have been done with purified proteins containing the kinase domain alone in *in vitro* kinase assays. It has not been shown that WNK2, or any other WNK family member besides WNK1, is required for *in vivo* OSR1-NKCC1/2 signaling. Expression patterns suggest that WNK2 is not expressed in the kidney and is not found endogenously in the HEK293 and murine Distal Convoluted Tubule cell model systems which have been used to study the NKCC2 pathway. If these studies are correct, WNK2 is not involved in regulating NKCC2 *in vivo*. However, OSR1 is widely expressed in the body, and WNK2 could function through OSR1 on NKCC1, the ubiquitous counterpart, or different downstream targets (Chen et al., 2004; Piechotta et al., 2003).

It is also not known what role the remaining sequence of WNK2 may play in targeting it to substrates. It should be pointed out that the interactions of WNK1 with OSR1 are mediated by a RFXV motif, of which three are located in WNK1 over a thousand residues distant in primary sequence from the kinase domain. This shows that targeting and substrate identification may rely greatly on regions outside of the kinase domain. The unusual architecture of the WNK kinase domain with the catalytic lysine in β strand 2 places the WNK active site in a more solvent-exposed position on the surface of the kinase domain (Min et al., 2004). This might explain the fact that a number of WNK substrates have been identified but all are poorly phosphorylated. If substrates of this sort are typical, that may further suggest the hypothesis that WNK kinase substrates *in vivo* are determined by binding interactions outside the kinase domain. WNK2, like the other WNKs, contains numerous PxxP motifs and at least two coiled-coil regions that promote protein-protein interactions. WNK2 also, like WNK1, contains an RFXV motif determining OSR1/SPAK interaction in its C-terminal region. While WNK2 and WNK1

are 90% conserved over their kinase domains, they can be expected to have divergent functions *in vivo* due to substantial sequence differences. Both proteins are long, 2382 residues and 2217 residues apiece, and their similarity does not extend much beyond a 330 residue region containing the kinase domain and autoinhibitory region. Thus, it may be that WNK2 acts through a common mediator, such as OSR1, but on different downstream targets via their interactions with regions outside of the kinase domain.

The presence of a WNK2 autoinhibitory region with a function conserved with other WNKs helps to confirm the importance of this region in the functioning of WNK proteins. It is also interesting that the autoinhibitory regions of the different WNKs are able to cross-inhibit. This might not be suggested by the limited conservation over this region. In fact, only a few residues are identical among the four proteins. The capacity of the autoinhibitory regions to recognize any WNK family member may play a role *in vivo*, as hypotheses have been generated about the interactions between WNK1 splice forms and WNK4 in the kidney in the regulation of renal transporters. Early work showed that, in *Xenopus* oocytes, WNK4 overexpression inhibited NCC transporter activity while WNK1 co-expression reversed the inhibition without affecting NCC on its own (Yang et al., 2003). This work was limited, and later work that included expression of OSR1/SPAK caused a change in interpretation of the results. However, in the model system, WNK1 and WNK4 seemed to have inhibitory effects on each other. This would not be explained by competition between the RFXV binding sites for OSR1/SPAK in a system that lacked these proteins. It is possible that competition and other protein interactions may be involved, but it is also possible that the proteins are able to use their autoinhibitory regions to regulate each other's activity. It has been shown that the kidney-

specific splice form of WNK1, KS-WNK1, that lacks the kinase domain but does contain the autoinhibitory region, is able to inhibit the activity of WNK1 in oocytes (Lazrak et al., 2006). While this could also be due to the competition between protein-protein interaction sites over the long C-terminal conserved region between the two splice forms, it is possible that the conserved autoinhibitory region is important in this interaction. In WNK1, the autoinhibitory effect is dependent on two phenylalanine residues that are conserved between the WNK family members (Xu et al., 2002a). If those same mutations affected cross-inhibition between WNK proteins, it would be interesting to investigate if phenylalanine-mutant constructs in oocytes also showed the same degree of WNK1-WNK4 and KS-WNK1-full-length-WNK1 inhibitory effects. While effects of interactions with OSR1/SPAK might be predominant in the NKCC signaling system, interactions between WNK proteins could be relevant in other systems. Because WNKs are expressed throughout the body, and WNK1 is expressed ubiquitously, it is likely that many tissues express more than one family member and splice variant.

In 293 cells, WNK2 overexpression is able to drive activation of overexpressed ERK5, similar to WNK1. Other work has shown that WNK1 does not directly activate ERK5, showing that some intermediate pathway components are provided in 293 cells. This is stronger evidence for a relevant role for WNKs in ERK5 signaling *in vivo* than is provided by purified protein interactions. Knockdown of WNK1 decreases ERK5 activation in response to EGF (Xu et al., 2004). It should be noted that WNK2 is deleted or underexpressed in a number of gliomas (Hong et al., 2007). Work done more recently has shown WNK2 to negatively regulate ERK1/2 (Moniz et al., 2008; Moniz et al.,

2007). Further exploration of the role of WNK2 in regulating MAP kinases, known modulators of cell cycle progression, might elucidate direct roles in oncogenesis.

WNK2 is a large protein with mostly unknown function. It is expressed in multiple different tissues throughout the body, and is at least putatively able to interact with the widely expressed OSR1 and SPAK (Moniz et al., 2008; Verissimo et al., 2006). Further studies will reflect activities both similar and divergent to its more well-studied family members WNK1 and WNK4.

CHAPTER FIVE

A VIEW TOWARD THE FUTURE

The field of research into WNK kinases is still in its infancy. Cloning of the first WNK gene was announced only nine years ago, and the linkage of WNK1 and WNK4 to PHA2 was discovered a year later. Since then, research has been stymied by a lack of easy approaches to study this unusual family. Aside from the kinase domain, WNKs lack known functional folding domains. No other proteins show similar sequence over any significant length. The distinctiveness of the WNKs, while inviting opportunities for novel discoveries, precludes easy avenues of analysis. The biggest insight to date came from comparisons of PHA2 to Gitelman's syndrome and the co-immunoprecipitation or yeast two-hybrid interaction of WNKs with OSR1/SPAK. Beyond the interaction of this important set of WNK effectors, most known interactors have been identified by yeast two-hybrid screens and have not been assorted into a cohesive mechanism.

Some effects of WNKs are mediated by the OSR1/SPAK proteins. The various WNK family members *in vitro* act on OSR1 with similar kinetics. Both the WNKs and OSR1/SPAK are widely expressed. A physiologic role for OSR1/SPAK signaling in regards to WNK4 has already been demonstrated by recent work in mice. It has been previously shown that mice with the WNK4 D561A PHA2-causing mutation knocked in show increased phosphorylation of OSR1/SPAK and increased apical localization of NCC (Yang et al., 2007c). Recently the same group studied a mouse with loss of exon 7

of WNK4, causing partial loss of autophosphorylation and phosphorylation of SPAK (Ohta et al., 2009). This mouse had reduced NCC phosphorylation, reduced Na retention, and lower blood pressure. A search for the more selective OSR1/SPAK-interacting motif [S/G/V]RFx[V/I]xx[V/I/T/S] found possible targets that could regulate membrane sorting or receptor/transporter function, including polyductin, taste and olfactory receptors, the interleukin-12 receptor, and the tumor necrosis factor (TNF) receptor superfamily member (Delpire and Gagnon, 2007). There could also be WNK tie-ins to the cytoskeleton to help direct membrane trafficking, via intersectin interactions with N-WASP and through OSR1/SPAK binding to the RFXV-containing actin-binding protein gelsolin. OSR1/SPAK signaling could provide mechanisms of WNK regulation of membrane trafficking,

The functioning of WNKs as scaffolds, while not yet proven, is tempting as a hypothesis. The size of the proteins and the presence of PxxP and coiled-coil protein-protein interaction motifs point toward a scaffolding function. WNK1 is also thought to exist endogenously as a tetramer, allowing interactions with yet more numerous binding partners (Lenertz et al., 2005). Beyond that, WNKs have been shown to have effects on multiple proteins, many through direct interactions and via different segments of the protein. For example, PxxP motifs in the N-terminal 119 residues of WNK1 have been shown to be critical for ROMK regulation and binding to intersectin (He et al., 2007; Wang et al., 2008); mutation of a kinase domain residue prevents Syt2 binding (Lee et al., 2004), RFXV motifs in the C-terminus mediate OSR1/SPAK binding; the autoinhibitory region shows intermolecular inhibition; and the C-terminal coiled-coil

motif appears to be required for regulation of transient receptor potential cation channel V4 (TRPV4) (Fu et al., 2006). These show that different proteins could interact simultaneously with either a WNK monomer or oligomer and mediate local interactions and downstream effects.

Many of the known WNK interactors play a role in membrane trafficking and vesicle regulation. It is likely that, rather than specific regulation of a set of ion transporters in the kidney, WNKs function in a fundamental and common role in membrane transport. WNKs have been associated with a number of transporters outside of the kidney, including CFEX, cystic fibrosis transmembrane conductance regulator (CFTR), TRPV6, the excitatory amino acid transporter 1 (EAAT1) glutamate transporter, and the chloride transporter solute carrier family 26 member 9 (SLC26A9) (Dorwart et al., 2007; Kahle et al., 2004; Yang et al., 2007a; Zhang et al., 2008). Interactions with fundamental regulators of membrane trafficking such as Munc18c, VPS4, and Syt2 point to WNKs' general role in vesicle regulation. WNKs also affect systems besides solute transport across membranes. TGFβR signaling and induction of transcription is altered by WNKs (Lee et al., 2007). WNK1 interactivity is required for glucose-stimulated insulin secretion from pancreatic beta cells (Oh et al., 2007). Inhibition of neurite extension in cortical neurons is mediated by WNK1 interactions with leucine-rich repeat and immunoglobulin domain-containing Nogo receptor-interacting protein 1 (LINGO-1) (Zhang et al., 2009). The $WNK1^{-/-}$ mouse reveals the role of WNK1 in endothelial cells and angiogenesis during development. No pathways known to be affected by WNK have been shown to play a role in arterial-venous specification, but a PI3K inhibitor and

dominant negative Akt have been shown in one study to promote angioblasts to develop an arterial fate (Hong et al., 2006; Swift and Weinstein, 2009). It can be hypothesized that WNKs are playing a role in these various systems via a common pathway. As suggested, this is likely to be one of membrane regulation. LINGO1 acts as a component of a tripartite receptor to mediate recognition of various myelin-associated inhibitors of nerve growth cones. And the VEGF receptor is a key regulator of arterial-venous fate determination. Regulation of solute transporters, mediation of vesicle fusion, and modification of membrane signaling events further tie WNKs to membrane and receptor regulation. Studies should be undertaken to evaluate how WNKs affect general mechanisms of cell biology and membrane trafficking. Likely other model systems of membrane regulation will be affected by the presence or absence of WNK and will reveal new targets of WNK signaling.

Besides cellular functions, the physiologic role of WNKs needs to be further explored. While the phenotypic characteristics of PHA2 directed investigations for WNKs' role in causing hypertension toward renal ion transporters, blood pressure maintenance also is regulated by the cardiovascular system, particularly in the acute state. $WNK1^{+/-}$ mice demonstrate hypotension, but show no abnormalities in urinary electrolytes and respond properly to a low-salt-diet challenge, suggesting that renal changes are not likely to be the main cause of their phenotype (Zambrowicz et al., 2003). WNK1 has already been shown to play a critical role in endothelial cells to direct angiogenesis during development (Xie et al., 2009). WNKs could play further roles in endothelial cells and small vessels to regulate blood pressure in adults. Further assays

could look at vascular smooth muscle cells in culture with endothelial cells expressing or knocking down WNK1 to see changes in function. Extra-renal roles of WNK1 are also suggested by analysis of a transgenic mouse with a lacZ reporter fused to the end of the full-length WNK1 gene (Delaloy et al., 2006). Consistent with the role in endothelial cells in directing angiogenesis, during development the WNK1-lacZ reporter was expressed in the heart as early as days 8-9 of development and in the vasculature by day 10.5. Expression was also specifically noted in the granular layer and the Purkinje cells of the cerebellum. It is not known what role WNK1 plays in the brain, although it is highly expressed. In the adult, the reporter was seen ubiquitously through all tissues. There was especially high expression in the heart, suggesting another possible route of blood pressure mediation by modification of cardiac output.

Physiologic roles for other WNKs have also been suggested. WNK3 is known to be highly expressed in γ-aminobutyric acid (GABA) receptor-positive neurons and has been suggested to regulate chloride flux through NKCC1 and KCCs (Kahle et al., 2005). It has been suggested that by shifting chloride gradients, WNK3 can affect neuronal excitability. WNK3 has also been linked to neural defects by linkage to a deletion associated with autism-spectrum disorder (Qiao et al., 2008). However WNK3 is not the only deleted gene and no direct associations of WNK3 mutations or effects with autism have been made. WNK2 has been identified in many studies of cancer as a possible tumor suppressor, as mentioned previously. WNK4 has also been shown to be expressed outside of the kidney in chloride-transporting polarized epithelia (Kahle et al., 2004). One group has shown that PHA2 patients with mutations in WNK4 also have increased

sodium transport in their airways, in an amiloride-dependent mechanism (Farfel et al., 2005). This suggests a role for WNK4 in regulation of solute transfer in areas outside of the kidney. Further work exploring the role of WNKs outside of the kidney will refine our understanding their function in diverse polarized epithelia.

Studies also need to be performed to better understand the roles of expression patterns and splice variation in the regulation of WNK function. WNK1 is known to have multiple splice variants. One of the most prominent is the KS-WNK1 form found predominantly in the kidney (Xu et al., 2002b). KS-WNK1 has been shown to antagonize the effects of full-length protein on ROMK and NCC in the kidney (Lazrak et al., 2006; Subramanya et al., 2006; Wade et al., 2006). The expression pattern of WNKs also changes based on physiologic responses. Studies of mRNA levels by *in situ* hybridization suggest that KS-WNK1 is expressed at ten-fold higher levels than full-length in the distal nephron, although this has not been validated by protein detection (Delaloy et al., 2003). Dietary potassium restriction causes an increase in expression of full-length protein while decreasing KS-WNK1 message (Lazrak et al., 2006; Wade et al., 2006). Full-length WNK1 inhibits ROMK, thereby promoting K^+ retention in a K^+-restricted state; this is reversed by KS-WNK1. Aldosterone, a mineralocorticoid hormone that promotes K^+ secretion, causes an increase in KS-WNK1 expression without affecting full-length (Naray-Fejes-Toth et al., 2004). This shows that splice form switching in the kidney is regulated by physiologic signals and likely plays a role in responses to changes in blood pressure and serum solute concentrations. The KS-WNK1 splice variant contains an additional exon 4a that is not present in the full-length variant (Delaloy et al., 2003;

O'Reilly et al., 2003). This 30-residue unique fragment contains a cysteine-repeat sequence CCCC that is predicted to be palmitoylated by the CSS-Palm 2.0 algorithm (Ren et al., 2008). The cysteine repeats could also mediate novel interactions via disulfide bonds or binding to metal-containing moieties. The KS-WNK1 form retains all of the sequence C-terminal to the kinase domain, including the coiled-coils, many PxxP motifs, the three C-terminal RFXV motifs, and the autoinhibitory region. Expression of KS-WNK1 could act as a competitive inhibitor for multiple full-length WNK1 interactors. If KS-WNK1 is palmitoylated in a fashion distinct from the full-length protein, it could mediate the localization of WNK1 interactors to different parts of the cell to either inhibit downstream pathways or create new signaling events. Palmitoylation of KS-WNK1 should be verified, and its subcellular localization should be compared with that of full-length WNK1 in renal cell lines. If they are distinct, effects on localization of binding partners such as OSR1/SPAK should be studied when KS- or full-length WNK1 is present.

Splicing variation has also been implicated in WNK3 signaling. WNK3 shows tissue-specific alternative splicing with a longer exon 18 expressed in the brain, while exon 22 is skipped in the kidneys (Holden et al., 2004). The renally-expressed form shows increased activity of NCC, while the neurally-expressed variant shows decreased activity (Glover et al., 2009). While this study was performed in *Xenopus* oocytes, where co-expression of differing partners such as OSR1 has been shown to change effects of WNK signaling to the SLC12 transporters, it does suggest that alternative splicing can regulate WNK3 downstream events and may give rise to tissue-specific changes in WNK

function. Interestingly, WNK1 also shows neuronal-specific variation in splicing. While most forms expressed in the kidney skip exon 11, exon 11-containing transcripts were abundant in neural tissues (Delaloy et al., 2003). Exon 11 is not related to the exons alternatively spliced in WNK3, yet it does encode a region conserved between humans and rodents, suggesting its possible importance. Analysis of splicing events also revealed a rare splicing event where an exon 4B is included that creates an in-frame stop codon inside the kinase domain (Delaloy et al., 2003; O'Reilly et al., 2003). While a rare event, the inclusion of this exon provides a mechanism for the regulation of WNK1 signaling at the level of splicing. These discoveries suggest that analysis of regulation of alternative splicing and functional effects of splice variants will provide further insight into WNK function.

Tissue-specific expression also is a matter for more precise study. To date, the majority of data concerning the expression patterns of WNKs and their splice variants has been based on message levels determined by *in situ* hybridization or reverse transcriptase-PCR. While analysis of mRNA is valuable and easy, it does not always directly reflect protein levels, which can differ by regulation of translation and protein degradation. Antibodies have been developed that detect endogenous WNK proteins sufficiently for detection in immunohistological assays. Use of antibodies has already suggested that WNK expression in various tissues is restricted to the polarized epithelia of various organs (Choate et al., 2003). Also, antibodies allowed detection of WNK4 protein in tissues outside of the kidney (Kahle et al., 2004). Analysis of tissues identified to contain WNK message should be extended to protein detection now that suitable

antibodies for the WNK kinases have been developed. Precise protein levels, subcellular localization, and relative expressions of isoforms and splice variants will aid in understanding the physiological relevance of differences detected by *in vitro* and cell culture experiments.

One major change in the study of WNK function is the development of more physiologically relevant model systems. Much initial work on WNK regulation of ion transporters has been performed in *Xenopus* oocytes, due to the ease of performing microinjection of cRNAs and patch-clamp analysis of electrogenic forms of transport. These studies provided many insights into WNK regulation of NKCC1/2, NCC, and ROMK. However, these studies are limited by our understanding of WNK function. Studies of WNK4 in oocytes suggested that it acted to inhibit NCC activity and apical localization (Wilson et al., 2003; Yang et al., 2003). But study of the mouse expressing a hypomorphic WNK4 reveals that WNK4 acts to increase NCC activity and apical localization. This apparent contradiction can likely be explained by differences in co-expressed proteins. Studies of WNK4 effects on the related proteins NKCC1 and KCC2 show that the effect of WNK4 expression alone was different from that when WNK4 and SPAK were co-expressed (Gagnon et al., 2006). One method to improve on this system is to investigate more relevant models. This has been done with work in murine distal convoluted tubule (DCT), inner medullary collecting duct (IMCD), and MDCK cell lines, among others. As WNK effects in non-renal tissues are studied, appropriate model cell lines will need to be utilized. While not a guarantee against cell line-specific effects, these models are at least more relevant than oocytes. Effects can also be studied in

independent but tissue-related cell lines and compared to *in vivo* and *in situ* data to validate results.

Other *in vitro* studies can be enhanced by the development of inhibitors of WNKs. One such inhibitor has been characterized already (Yagi et al., 2009). This will allow a complement to RNAi studies in various cell culture models. An inhibitor will facilitate proteomics to find novel WNK interactors and affected pathways. While the inhibitor for WNK1 described by Yagi et al. is limited in that it acts as an ATP competitor and shows cross-inhibition with the tyrosine kinases Src and hemopoeitic cell kinase (HCK), its development supports the future identification of specific inhibitors. Studies attempting to identify mutations and SNPs in WNK genes that segregate with essential hypertension have been largely unsuccessful to date. Pharmacologically-deliverable WNK inhibitors may nevertheless be useful to manipulate blood pressure in patients.

The WNK kinases are still a relatively understudied family of proteins, but research has advanced our understanding of the roles they play in important physiological processes. We are only now beginning to understand the interplay between various complex systems regulated by the WNKs. As we discover new effects and regulators of WNKs, we will be able to both move beyond the kidney to see effects on the body as a whole and look inside the cell to understand more intimately the interactions with cellular processes.

BIBLIOGRAPHY

Achard, J.M., Warnock, D.G., Disse-Nicodeme, S., Fiquet-Kempf, B., Corvol, P., Fournier, A., and Jeunemaitre, X. (2003). Familial hyperkalemic hypertension: phenotypic analysis in a large family with the WNK1 deletion mutation. Am J Med *114*, 495-498.

Anselmo, A.N., Earnest, S., Chen, W., Juang, Y.C., Kim, S.C., Zhao, Y., and Cobb, M.H. (2006). WNK1 and OSR1 regulate the Na+, K+, 2Cl- cotransporter in HeLa cells. Proc Natl Acad Sci U S A *103*, 10883-10888.

Barrios-Rodiles, M., Brown, K.R., Ozdamar, B., Bose, R., Liu, Z., Donovan, R.S., Shinjo, F., Liu, Y., Dembowy, J., Taylor, I.W.*, et al.* (2005). High-Throughput Mapping of a Dynamic Signaling Network in Mammalian Cells. Science *307*, 1621-1625.

Chen, W., Chen, Y., Xu, B.E., Juang, Y.C., Stippec, S., Zhao, Y., and Cobb, M.H. (2009). Regulation of a third conserved phosphorylation site in SGK1. J Biol Chem *284*, 3453-3460.

Chen, W., Yazicioglu, M., and Cobb, M.H. (2004). Characterization of OSR1, a member of the mammalian Ste20p/germinal center kinase subfamily. Journal of Biological Chemistry *279*, 11129-11136.

Choate, K.A., Kahle, K.T., Wilson, F.H., Nelson-Williams, C., and Lifton, R.P. (2003). WNK1, a kinase mutated in inherited hypertension with hyperkalemia, localizes to diverse Cl- -transporting epithelia. Proc Natl Acad Sci U S A *100*, 663-668.

Davies, H., Hunter, C., Smith, R., Stephens, P., Greenman, C., Bignell, G., Teague, J., Butler, A., Edkins, S., Stevens, C.*, et al.* (2005). Somatic Mutations of the Protein Kinase Gene Family in Human Lung Cancer. Cancer Res *65*, 7591-7595.

Delaloy, C., Elvira-Matelot, E., Clemessy, M., Zhou, X.O., Imbert-Teboul, M., Houot, A.M., Jeunemaitre, X., and Hadchouel, J. (2008). Deletion of WNK1 first intron results in misregulation of both isoforms in renal and extrarenal tissues. Hypertension *52*, 1149-1154.

Delaloy, C., Hadchouel, J., Imbert-Teboul, M., Clemessy, M., Houot, A.M., and Jeunemaitre, X. (2006). Cardiovascular expression of the mouse WNK1 gene during development and adulthood revealed by a BAC reporter assay. Am J Pathol *169*, 105-118.

Delaloy, C., Lu, J., Houot, A.M., Disse-Nicodeme, S., Gasc, J.M., Corvol, P., and Jeunemaitre, X. (2003). Multiple promoters in the WNK1 gene: one controls

expression of a kidney-specific kinase-defective isoform. Mol Cell Biol *23*, 9208-9221.

Delpire, E., and Gagnon, K.B. (2007). Genome-wide analysis of SPAK/OSR1 binding motifs. Physiol Genomics *28*, 223-231.

Dorwart, M.R., Shcheynikov, N., Wang, Y., Stippec, S., and Muallem, S. (2007). SLC26A9 is a Cl(-) channel regulated by the WNK kinases. J Physiol *584*, 333-345.

Erlich, P.M., Cui, J., Chazaro, I., Farrer, L.A., Baldwin, C.T., Gavras, H., and DeStefano, A.L. (2003). Genetic variants of WNK4 in whites and African Americans with hypertension. Hypertension *41*, 1191-1195.

Farfel, Z., Mayan, H., Yaacov, Y., Mouallem, M., Shaharabany, M., Pauzner, R., Kerem, E., and Wilschanski, M. (2005). WNK4 regulates airway Na+ transport: study of familial hyperkalaemia and hypertension. Eur J Clin Invest *35*, 410-415.

Fu, Y., Subramanya, A., Rozansky, D., and Cohen, D.M. (2006). WNK kinases influence TRPV4 channel function and localization. Am J Physiol Renal Physiol *290*, F1305-1314.

Gagnon, K.B., England, R., and Delpire, E. (2006). Volume sensitivity of cation-Cl- cotransporters is modulated by the interaction of two kinases: Ste20-related proline-alanine-rich kinase and WNK4. Am J Physiol Cell Physiol *290*, C134-142.

Gagnon, K.B., England, R., and Delpire, E. (2007). A single binding motif is required for SPAK activation of the Na-K-2Cl cotransporter. Cell Physiol Biochem *20*, 131-142.

Glover, M., Zuber, A.M., and O'Shaughnessy, K.M. (2009). Renal and brain isoforms of WNK3 have opposite effects on NCCT expression. J Am Soc Nephrol *20*, 1314-1322.

Greenman, C., Stephens, P., Smith, R., Dalgliesh, G.L., Hunter, C., Bignell, G., Davies, H., Teague, J., Butler, A., Stevens, C.*, et al.* (2007). Patterns of somatic mutation in human cancer genomes. Nature *446*, 153-158.

Haas, M., and Forbush III, B. (2000). The Na-K-Cl Cotransporter of Secretory Epithelia. Annual Review of Physiology *62*, 515-534.

He, G., Wang, H.R., Huang, S.K., and Huang, C.L. (2007). Intersectin links WNK kinases to endocytosis of ROMK1. J Clin Invest *117*, 1078-1087.

Holden, S., Cox, J., and Raymond, F.L. (2004). Cloning, genomic organization, alternative splicing and expression analysis of the human gene WNK3 (PRKWNK3). Gene *335*, 109-119.

Hong, C., Moorefield, K.S., Jun, P., Aldape, K.D., Kharbanda, S., Phillips, H.S., and Costello, J.F. (2007). Epigenome scans and cancer genome sequencing converge on WNK2, a kinase-independent suppressor of cell growth. Proc Natl Acad Sci U S A *104*, 10974-10979.

Hong, C.C., Peterson, Q.P., Hong, J.-Y., and Peterson, R.T. (2006). Artery/Vein Specification Is Governed by Opposing Phosphatidylinositol-3 Kinase and MAP Kinase/ERK Signaling. Current Biology *16*, 1366-1372.

Huang, C.L., Yang, S.S., and Lin, S.H. (2008). Mechanism of regulation of renal ion transport by WNK kinases. Curr Opin Nephrol Hypertens *17*, 519-525.

Johnston, A.M., Naselli, G., Gonez, L.J., Martin, R.M., Harrison, L.C., and DeAizpurua, H.J. (2000). SPAK, a STE20/SPS1-related kinase that activates the p38 pathway. Oncogene *19*, 4290-4297.

Jun, P., Hong, C., Lal, A., Wong, J.M., McDermott, M.W., Bollen, A.W., Plass, C., Held, W.A., Smiraglia, D.J., and Costello, J.F. (2009). Epigenetic silencing of the kinase tumor suppressor WNK2 is tumor-type and tumor-grade specific. Neuro Oncol *11*, 414-422.

Kahle, K.T., Gimenez, I., Hassan, H., Wilson, F.H., Wong, R.D., Forbush, B., Aronson, P.S., and Lifton, R.P. (2004). WNK4 regulates apical and basolateral Cl- flux in extrarenal epithelia. Proc Natl Acad Sci U S A *101*, 2064-2069.

Kahle, K.T., Rinehart, J., de Los Heros, P., Louvi, A., Meade, P., Vazquez, N., Hebert, S.C., Gamba, G., Gimenez, I., and Lifton, R.P. (2005). WNK3 modulates transport of Cl- in and out of cells: implications for control of cell volume and neuronal excitability. Proc Natl Acad Sci U S A *102*, 16783-16788.

Kahle, K.T., Wilson, F.H., Leng, Q., Lalioti, M.D., O'Connell, A.D., Dong, K., Rapson, A.K., MacGregor, G.G., Giebisch, G., Hebert, S.C.*, et al.* (2003). WNK4 regulates the balance between renal NaCl reabsorption and K+ secretion. Nat Genet *35*, 372-376.

Kokubo, Y., Kamide, K., Inamoto, N., Tanaka, C., Banno, M., Takiuchi, S., Kawano, Y., Tomoike, H., and Miyata, T. (2004). Identification of 108 SNPs in TSC, WNK1, and WNK4 and their association with hypertension in a Japanese general population. J Hum Genet *49*, 507-515.

Lazrak, A., Liu, Z., and Huang, C.L. (2006). Antagonistic regulation of ROMK by long and kidney-specific WNK1 isoforms. Proc Natl Acad Sci U S A *103*, 1615-1620.

Lee, B.H., Chen, W., Stippec, S., and Cobb, M.H. (2007). Biological cross-talk between WNK1 and the transforming growth factor beta-Smad signaling pathway. J Biol Chem *282*, 17985-17996.

Lee, B.H., Min, X., Heise, C.J., Xu, B.E., Chen, S., Shu, H., Luby-Phelps, K., Goldsmith, E.J., and Cobb, M.H. (2004). WNK1 phosphorylates synaptotagmin 2 and modulates its membrane binding. Mol Cell *15*, 741-751.

Lenertz, L.Y., Lee, B.H., Min, X., Xu, B.E., Wedin, K., Earnest, S., Goldsmith, E.J., and Cobb, M.H. (2005). Properties of WNK1 and implications for other family members. J Biol Chem *280*, 26653-26658.

Ma, N., Niu, R.F., and Ma, Y.J. (2008). Intersectin 1: a molecular linker in the central nervous system. Neurosci Bull *24*, 401-405.

Mayan, H., Vered, I., Mouallem, M., Tzadok-Witkon, M., Pauzner, R., and Farfel, Z. (2002). Pseudohypoaldosteronism type II: marked sensitivity to thiazides, hypercalciuria, normomagnesemia, and low bone mineral density. J Clin Endocrinol Metab *87*, 3248-3254.

Min, X., Lee, B.H., Cobb, M.H., and Goldsmith, E.J. (2004). Crystal structure of the kinase domain of WNK1, a kinase that causes a hereditary form of hypertension. Structure *12*, 1303-1311.

Moniz, S., Matos, P., and Jordan, P. (2008). WNK2 modulates MEK1 activity through the Rho GTPase pathway. Cell Signal *20*, 1762-1768.

Moniz, S., Verissimo, F., Matos, P., Brazao, R., Silva, E., Kotelevets, L., Chastre, E., Gespach, C., and Jordan, P. (2007). Protein kinase WNK2 inhibits cell proliferation by negatively modulating the activation of MEK1/ERK1/2. Oncogene *26*, 6071-6081.

Monti, J., Zimdahl, H., Schulz, H., Plehm, R., Ganten, D., and Hubner, N. (2003). The role of Wnk4 in polygenic hypertension: a candidate gene analysis on rat chromosome 10. Hypertension *41*, 938-942.

Moriguchi, T., Urushiyama, S., Hisamoto, N., Iemura, S., Uchida, S., Natsume, T., Matsumoto, K., and Shibuya, H. (2005). WNK1 regulates phosphorylation of cation-chloride-coupled cotransporters via the STE20-related kinases, SPAK and OSR1. J Biol Chem *280*, 42685-42693.

Murakami-Kojima, M., Nakamichi, N., Yamashino, T., and Mizuno, T. (2002). The APRR3 component of the clock-associated APRR1/TOC1 quintet is phosphorylated by a novel protein kinase belonging to the WNK family, the gene for which is also transcribed rhythmically in Arabidopsis thaliana. Plant Cell Physiol *43*, 675-683.

Nakamichi, N., Murakami-Kojima, M., Sato, E., Kishi, Y., Yamashino, T., and Mizuno, T. (2002). Compilation and characterization of a novel WNK family of protein kinases in Arabiodpsis thaliana with reference to circadian rhythms. Biosci Biotechnol Biochem *66*, 2429-2436.

Naray-Fejes-Toth, A., Snyder, P.M., and Fejes-Toth, G. (2004). The kidney-specific WNK1 isoform is induced by aldosterone and stimulates epithelial sodium channel-mediated Na+ transport. Proc Natl Acad Sci U S A *101*, 17434-17439.

Nolen, B., Taylor, S., and Ghosh, G. (2004). Regulation of Protein Kinases: Controlling Activity through Activation Segment Conformation. Molecular Cell *15*, 661-675.

O'Reilly, M., Marshall, E., Speirs, H.J., and Brown, R.W. (2003). WNK1, a gene within a novel blood pressure control pathway, tissue-specifically generates radically different isoforms with and without a kinase domain. J Am Soc Nephrol *14*, 2447-2456.

Oh, E., Heise, C.J., English, J.M., Cobb, M.H., and Thurmond, D.C. (2007). WNK1 is a novel regulator of Munc18c-syntaxin 4 complex formation in soluble NSF attachment protein receptor (SNARE)-mediated vesicle exocytosis. J Biol Chem *282*, 32613-32622.

Ohta, A., Rai, T., Yui, N., Chiga, M., Yang, S.S., Lin, S.H., Sohara, E., Sasaki, S., and Uchida, S. (2009). Targeted disruption of the Wnk4 gene decreases phosphorylation of Na-Cl cotransporter, increases Na excretion and lowers blood pressure. Hum Mol Genet *18*, 3978-3986.

Pardali, E., and ten Dijke, P. (2009). Transforming growth factor-beta signaling and tumor angiogenesis. Front Biosci *14*, 4848-4861.

Piechotta, K., Garbarini, N., England, R., and Delpire, E. (2003). Characterization of the Interaction of the Stress Kinase SPAK with the Na+-K+-2Clâ€“ Cotransporter in the Nervous System. Journal of Biological Chemistry *278*, 52848-52856.

Piechotta, K., Lu, J., and Delpire, E. (2002). Cation Chloride Cotransporters Interact with the Stress-related Kinases Ste20-related Proline-Alanine-rich Kinase (SPAK) and Oxidative Stress Response 1 (OSR1). Journal of Biological Chemistry *277*, 50812-50819.

Ponce-Coria, J., San-Cristobal, P., Kahle, K.T., Vazquez, N., Pacheco-Alvarez, D., de Los Heros, P., Juarez, P., Munoz, E., Michel, G., Bobadilla, N.A.*, et al.* (2008). Regulation of NKCC2 by a chloride-sensing mechanism involving the WNK3 and SPAK kinases. Proc Natl Acad Sci U S A *105*, 8458-8463.

Qiao, Y., Liu, X., Harvard, C., Hildebrand, M.J., Rajcan-Separovic, E., Holden, J.J., and Lewis, M.E. (2008). Autism-associated familial microdeletion of Xp11.22. Clin Genet *74*, 134-144.

Ren, J., Wen, L., Gao, X., Jin, C., Xue, Y., and Yao, X. (2008). CSS-Palm 2.0: an updated software for palmitoylation sites prediction. Protein Eng Des Sel *21*, 639-644.

Richardson, C., Rafiqi, F.H., Karlsson, H.K., Moleleki, N., Vandewalle, A., Campbell, D.G., Morrice, N.A., and Alessi, D.R. (2008). Activation of the thiazide-sensitive Na+-Cl- cotransporter by the WNK-regulated kinases SPAK and OSR1. J Cell Sci *121*, 675-684.

San-Cristobal, P., de los Heros, P., Ponce-Coria, J., Moreno, E., and Gamba, G. (2008). WNK kinases, renal ion transport and hypertension. Am J Nephrol *28*, 860-870.

Sjoblom, T., Jones, S., Wood, L.D., Parsons, D.W., Lin, J., Barber, T.D., Mandelker, D., Leary, R.J., Ptak, J., Silliman, N.*, et al.* (2006). The Consensus Coding Sequences of Human Breast and Colorectal Cancers. Science *314*, 268-274.

Speirs, H.J., and Morris, B.J. (2004). WNK4 intron 10 polymorphism is not associated with hypertension. Hypertension *43*, 766-768.

Stephens, P., Edkins, S., Davies, H., Greenman, C., Cox, C., Hunter, C., Bignell, G., Teague, J., Smith, R., Stevens, C.*, et al.* (2005). A screen of the complete protein kinase gene family identifies diverse patterns of somatic mutations in human breast cancer. Nat Genet *37*, 590-592.

Subramanya, A.R., Yang, C.L., Zhu, X., and Ellison, D.H. (2006). Dominant-negative regulation of WNK1 by its kidney-specific kinase-defective isoform. Am J Physiol Renal Physiol *290*, F619-624.

Swift, M.R., and Weinstein, B.M. (2009). Arterial-Venous Specification During Development. Circ Res *104*, 576-588.

Verissimo, F., and Jordan, P. (2001). WNK kinases, a novel protein kinase subfamily in multi-cellular organisms. Oncogene *20*, 5562-5569.

Verissimo, F., Silva, E., Morris, J.D., Pepperkok, R., and Jordan, P. (2006). Protein kinase WNK3 increases cell survival in a caspase-3-dependent pathway. Oncogene *25*, 4172-4182.

Vitari, A.C., Deak, M., Collins, B.J., Morrice, N., Prescott, A.R., Phelan, A., Humphreys, S., and Alessi, D.R. (2004). WNK1, the kinase mutated in an inherited high-blood-pressure syndrome, is a novel PKB (protein kinase B)/Akt substrate. Biochem J *378*, 257-268.

Vitari, A.C., Deak, M., Morrice, N.A., and Alessi, D.R. (2005). The WNK1 and WNK4 protein kinases that are mutated in Gordon's hypertension syndrome phosphorylate and activate SPAK and OSR1 protein kinases. Biochem J *391*, 17-24.

Vitari, A.C., Thastrup, J., Rafiqi, F.H., Deak, M., Morrice, N.A., Karlsson, H.K., and Alessi, D.R. (2006). Functional interactions of the SPAK/OSR1 kinases with their upstream activator WNK1 and downstream substrate NKCC1. Biochem J *397*, 223-231.

Wade, J.B., Fang, L., Liu, J., Li, D., Yang, C.L., Subramanya, A.R., Maouyo, D., Mason, A., Ellison, D.H., and Welling, P.A. (2006). WNK1 kinase isoform switch regulates renal potassium excretion. Proc Natl Acad Sci U S A *103*, 8558-8563.

Wakula, P., Beullens, M., Ceulemans, H., Stalmans, W., and Bollen, M. (2003). Degeneracy and Function of the Ubiquitous RVXF Motif That Mediates Binding to Protein Phosphatase-1. J Biol Chem *278*, 18817-18823.

Wang, H.R., Liu, Z., and Huang, C.L. (2008). Domains of WNK1 kinase in the regulation of ROMK1. Am J Physiol Renal Physiol *295*, F438-445.

Wilson, F.H., Disse-Nicodeme, S., Choate, K.A., Ishikawa, K., Nelson-Williams, C., Desitter, I., Gunel, M., Milford, D.V., Lipkin, G.W., Achard, J.M.*, et al.* (2001). Human hypertension caused by mutations in WNK kinases. Science *293*, 1107-1112.

Wilson, F.H., Kahle, K.T., Sabath, E., Lalioti, M.D., Rapson, A.K., Hoover, R.S., Hebert, S.C., Gamba, G., and Lifton, R.P. (2003). Molecular pathogenesis of inherited hypertension with hyperkalemia: the Na-Cl cotransporter is inhibited by wild-type but not mutant WNK4. Proc Natl Acad Sci U S A *100*, 680-684.

Wollert, T., Yang, D., Ren, X., Lee, H.H., Im, Y.J., and Hurley, J.H. (2009). The ESCRT machinery at a glance. J Cell Sci *122*, 2163-2166.

Xie, J., Wu, T., Xu, K., Huang, I.K., Cleaver, O., and Huang, C.L. (2009). Endothelial-specific expression of WNK1 kinase is essential for angiogenesis and heart development in mice. Am J Pathol *175*, 1315-1327.

Xu, B., English, J.M., Wilsbacher, J.L., Stippec, S., Goldsmith, E.J., and Cobb, M.H. (2000). WNK1, a novel mammalian serine/threonine protein kinase lacking the catalytic lysine in subdomain II. Journal of Biological Chemistry *275*, 16795-16801.

Xu, B.E., Min, X., Stippec, S., Lee, B.H., Goldsmith, E.J., and Cobb, M.H. (2002a). Regulation of WNK1 by an autoinhibitory domain and autophosphorylation. J Biol Chem *277*, 48456-48462.

Xu, B.E., Stippec, S., Chu, P.Y., Lazrak, A., Li, X.J., Lee, B.H., English, J.M., Ortega, B., Huang, C.L., and Cobb, M.H. (2005a). WNK1 activates SGK1 to regulate the epithelial sodium channel. Proc Natl Acad Sci U S A *102*, 10315-10320.

Xu, B.E., Stippec, S., Lazrak, A., Huang, C.L., and Cobb, M.H. (2005b). WNK1 activates SGK1 by a phosphatidylinositol 3-kinase-dependent and non-catalytic mechanism. J Biol Chem *280*, 34218-34223.

Xu, B.E., Stippec, S., Lenertz, L., Lee, B.H., Zhang, W., Lee, Y.K., and Cobb, M.H. (2004). WNK1 activates ERK5 by an MEKK2/3-dependent mechanism. J Biol Chem *279*, 7826-7831.

Xu, Q., Modrek, B., and Lee, C. (2002b). Genome-wide detection of tissue-specific alternative splicing in the human transcriptome. Nucleic Acids Res *30*, 3754-3766.

Yagi, Y.I., Abe, K., Ikebukuro, K., and Sode, K. (2009). Kinetic mechanism and inhibitor characterization for WNK1 kinase. Biochemistry.

Yang, C.L., Angell, J., Mitchell, R., and Ellison, D.H. (2003). WNK kinases regulate thiazide-sensitive Na-Cl cotransport. J Clin Invest *111*, 1039-1045.

Yang, C.L., Liu, X., Paliege, A., Zhu, X., Bachmann, S., Dawson, D.C., and Ellison, D.H. (2007a). WNK1 and WNK4 modulate CFTR activity. Biochem Biophys Res Commun *353*, 535-540.

Yang, C.L., Zhu, X., and Ellison, D.H. (2007b). The thiazide-sensitive Na-Cl cotransporter is regulated by a WNK kinase signaling complex. J Clin Invest *117*, 3403-3411.

Yang, S.S., Morimoto, T., Rai, T., Chiga, M., Sohara, E., Ohno, M., Uchida, K., Lin, S.H., Moriguchi, T., Shibuya, H.*, et al.* (2007c). Molecular pathogenesis of pseudohypoaldosteronism type II: generation and analysis of a Wnk4(D561A/+) knockin mouse model. Cell Metab *5*, 331-344.

You, L.R., Lin, F.J., Lee, C.T., DeMayo, F.J., Tsai, M.J., and Tsai, S.Y. (2005). Suppression of Notch signalling by the COUP-TFII transcription factor regulates vein identity. Nature *435*, 98-104.

Zambrowicz, B.P., Abuin, A., Ramirez-Solis, R., Richter, L.J., Piggott, J., BeltrandelRio, H., Buxton, E.C., Edwards, J., Finch, R.A., Friddle, C.J.*, et al.* (2003). Wnk1 kinase deficiency lowers blood pressure in mice: a gene-trap screen to identify potential targets for therapeutic intervention. Proc Natl Acad Sci U S A *100*, 14109-14114.

Zhang, W., Na, T., and Peng, J.B. (2008). WNK3 positively regulates epithelial calcium channels TRPV5 and TRPV6 via a kinase-dependent pathway. Am J Physiol Renal Physiol *295*, F1472-1484.

Zhang, Z., Xu, X., Zhang, Y., Zhou, J., Yu, Z., and He, C. (2009). LINGO-1 interacts with WNK1 to regulate nogo-induced inhibition of neurite extension. J Biol Chem *284*, 15717-15728.

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