REGULATION OF ENDOCYTOSIS OF ROMK CHANNEL BY WNK KINASE FAMILY

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DEDICATION

I dedicate my work to my parents Min Wang and Yong-Hui Xia, my sister Xia-Qi Wang and my girl friend Tian-Xia Li

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By

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REGULATION OF ENDOCYTOSIS OF ROMK CHANNEL BY WNK KINASE FAMILY

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WNK kinases are members of a novel family of serine/threonine kinases with atypical placement of the catalytic lysine. Mutations in WNK1 and WNK4 cause pseudohypoaldosteronism type 2 (PHA2), an autosomal-dominant disease characterized by hypertension and hyperkalemia. Renal outer medullary potassium channel (ROMK) is responsible for constitutive K+ secretion in the kidney. WNK1 and WNK4 stimulate the clathrin-mediated endocytosis of ROMK, which contributes to the pathogenesis of hyperkalemia in PHAII patients. Intersectin (ITSN) is a multimodular endocytic scaffold protein. The proline-rich domains of WNK1 and WNK4 bind with the Src-homology domain (SH3) of intersectin, and this interaction is important for the stimulation of endocytosis of ROMK by WNKs. Intersectin will further activate the GTPase activity of dynamin and the actin polymerization of N-WASP, and thus promote endocytosis of

ROMK channel. WNK1 inhibition of ROMK is further regulated by the kinase domain conformation, which is critical for WNK1 to recruit intersectin. A shorter renal alternatively spliced form of WNK1 that lacks the kinase domain, known as kidney specific WNK1 (KS-WNK1), interacts with WNK1 kinase domain and antagonizes WNK1 inhibition of ROMK. The 4a domain and the auto-inhibitory domain in KS-WNK1 are responsible for the antagonization. The antagonism of WNK1 by 4a domain of KS-WNK1 can be abolished by 2-BP (a palmitoylation inhibitor) and hydrogen peroxide (generated during K+ deficiency). These results provide a molecular mechanism for the regulation of endocytosis of ROMK by WNK kinase family.

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PRIOR PUBLICATIONS

Liu Z, **Wang HR,** Huang CL. Regulation of ROMK channel and K+ homeostasis by kidney-specific WNK1 kinase. *J Biol Chem.* 2009 May 1;284(18):12198-206.

Wang HR, Liu Z, Huang CL. Domains of WNK1 kinase in the regulation of ROMK. *Am J Physiol Renal Physiol.* 2008 Aug;295(2):F438-45.

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LIST OF DEFINITIONS

WNK kinase – With No Lysine Kinase

KS-WNK1– Kidney Specific WNK1

ROMK – Renal Outer Medullar Potassium Channel (Kir1)

PHAII – Pseudohypoaldosteronism type II

ITSN-Intersectin

SH3 – Src Homology 3

PRD – Proline-Rich Domain

NL – N-Linker Domain

KD – Kinase Domain

AID – Auto-Inhibitory Domain

CC – Coiled-Coil domain

NCC– Na-Cl cotransporter (SLC12A3)

ENaC – Epithelial sodium Channel

DN – Dominant Negative

GAP–GTPase Activating Protein

GEF–Guanine nucleotide Exchange Factor

PAT- Palmitoyl Transferase

SGK- Serum/Glucocorticoid regulated Kinase

CHAPTER ONE: GENERAL OVERVIEW

WNK kinases are members of a novel family of serine/threonine protein kinases with an atypical placement of the catalytic lysine in the kinase domain [Xu B et all, 2000]. The study of WNK kinase family gained great momentum after mutations in WNK1 and WNK4 genes were linked to pseudohypoaldosteronism type II (PHAII)[Wilson FH, et al 2001], a rare familial form of hyperkalemic (high serum K+) hypertension[Figure 1-1] [Gordon RD, 1986]. PHAII patients developed hyperkalemia at early age due to impaired K+ secretion. The onset of hypertension in PHAII patients is often delayed till adult life due to increased renal NaCl reabsorption and possible vascular pathology [Weinstein SF 1974, Sauder SE 1987, Achard JM 2003]. Patients also developed metabolic acidosis likely secondary to hyperkalemia, and hypercalciuria in some cases [Stratton JD, 1998]. The existing knowledge of renal handling of K+ and Na+ transport based on aldosterone function cannot readily explain the unusual association between hypertension and hyperkalemia, suggesting that investigation into the molecular pathogenesis of PHAII may identify new mechanisms that govern renal electrolyte homeostasis [Kahle KT, 2008]. My study focused on how WNK kinase family regulates the endocytosis of renal outer medullary potassium channel (ROMK), the potassium channel responsible for constitutive non-flow stimulated potassium secretion in the distal nephron including the convoluted tubules (DCT), cortical connecting tubule (CNT) and cortical collecting ducts (CCD). Using electrophysiology, biochemistry and fluorescent imaging techniques, I investigated in detail how WNK1, WNK4 and Kidney Specific WNK1 (KS-WNK1) form a network to regulate endocytosis of ROMK. The knowledge gained in this study not only reveals a physiological role of WNK kinase family in regulating K homeostasis, but also suggests a mechanism for dietary potassium intake to regulate blood pressure through the WNK kinase family. In this chapter, I will briefly review the mechanism of renal K+ secretion and Na+ reabsorption and the roles of WNK kinase family in regulating them.

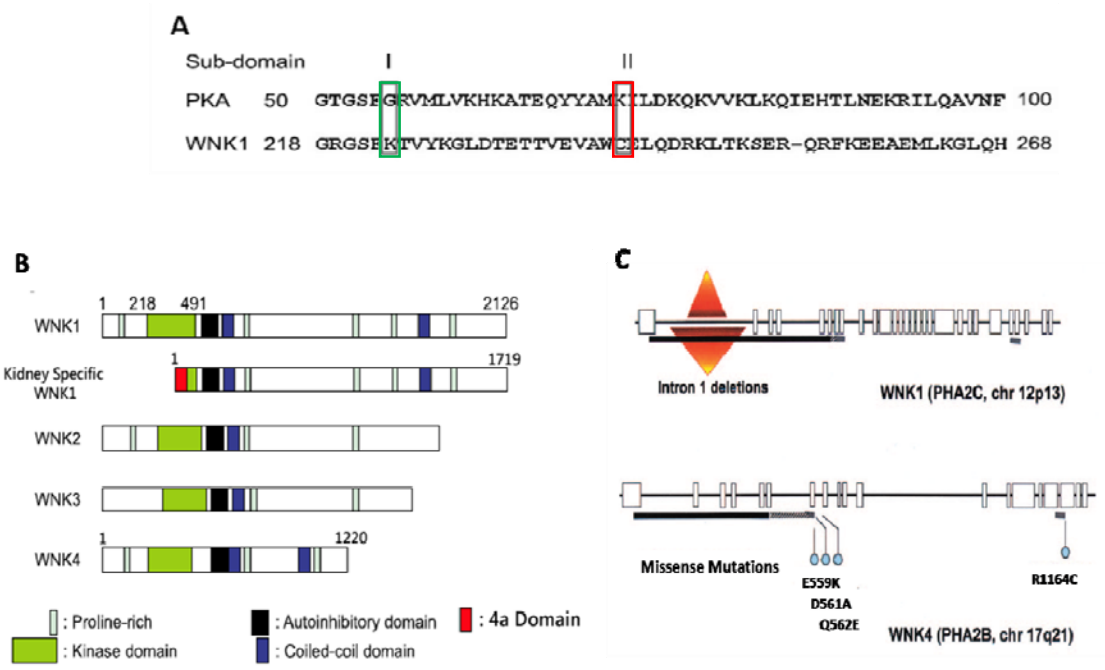

Figure 1-1. WNK kinase family and PHAII disease.

A. Partial amino acid sequence of the kinase domain of PKA and WNK1. The conserved catalytic lysine of PKA in sub-domain II corresponds to cysteine-250 in WNK1 (indicated by red box). The catalytic lysine of WNK1 (lysine-233) lies in the sub-domain I (indicated by green box).

B. Domain structure of WNK1-WNK4 and Kidney Specific WNK1. WNK1-4, shared a similar kinase domain, multiple proline-rich domains possibly interacting with SH3 domain-containing proteins , one autoinhibitory domain inhibiting the kinase activity, and 1 or 2 coiled-coil domain for oligomerization. KS- WNK lacks the kinase domain, but contain a unique 4a domain in the N-terminus.

C. Mutations in WNK1 and WNK4 lead to Pseudohypoaldosteronism type II(PHAII). Mutations in WNK1 are large intronic deletion that increases the expression of un-mutated Long-WNK1. Mutations in WNK4 are missense mutations clustering in a conserved acidic region preceded by a proline-rich domain.

Nephron: As shown in Figure 1-2, nephron is a basic structural and functional unit of the kidney, which eliminates wastes from the body, regulates blood volume and blood pressure, controls levels of electrolytes and metabolites, and regulates blood pH. Nephron can be divided into: the glomerulus, proximal tubule, henle's loop, distal convoluted tubule (DCT), connecting tubule (CNT), and collecting ducts (CCD) [Maton A 1993]. Na+ reabsorption mediated by Na–Cl cotransporter (NCC) in DCT and epithelial Na^+ channel (ENaC) in CNT and CCD, and K+ secretion mediated by Renal outer-medullary K channel or ROMK in CNT and CCD play important roles in regulating final excretion of Na+ and K+ in the urine [Palmer LG 2007].

ROMK and Potassium handling in Kidney: Potassium (K+) is the principal positively charged ion (cation) in the fluid inside of cells, while sodium is the principal cation in the fluid outside of cells. Intracellular K+ concentration is about 30 times than the extracellular K+ concentration, while intracellular Na+ concentration is more than ten times lower than extracellular Na+ concentration. Because only K+ channels remain open at negative membrane potential, the negative electric potential created by the different concentration of K+ inside and outside the cell will outweigh the contribution of sodium and chloride ions during resting state, and thus determines the resting cell membrane potential.

As the intracellular K+ is more constant, a cell's membrane potential is greatly influenced by the extracellular K+ concentration. Changes in the plasma K+ concentration can have important effects on membrane excitability. A fall in the plasma K+ concentration (hypokalemia) increases the magnitude of the cell interior-negative membrane potential. This hyperpolarizing change reduces cell excitability. Conversely, a rise in the extracellular K+ concentration depolarizes the membrane potential and increases cell excitability. Disturbances in the renal excretion of K+ via ROMK channel will alter fluid K+ levels thus cell excitability and lead to serious conditions such

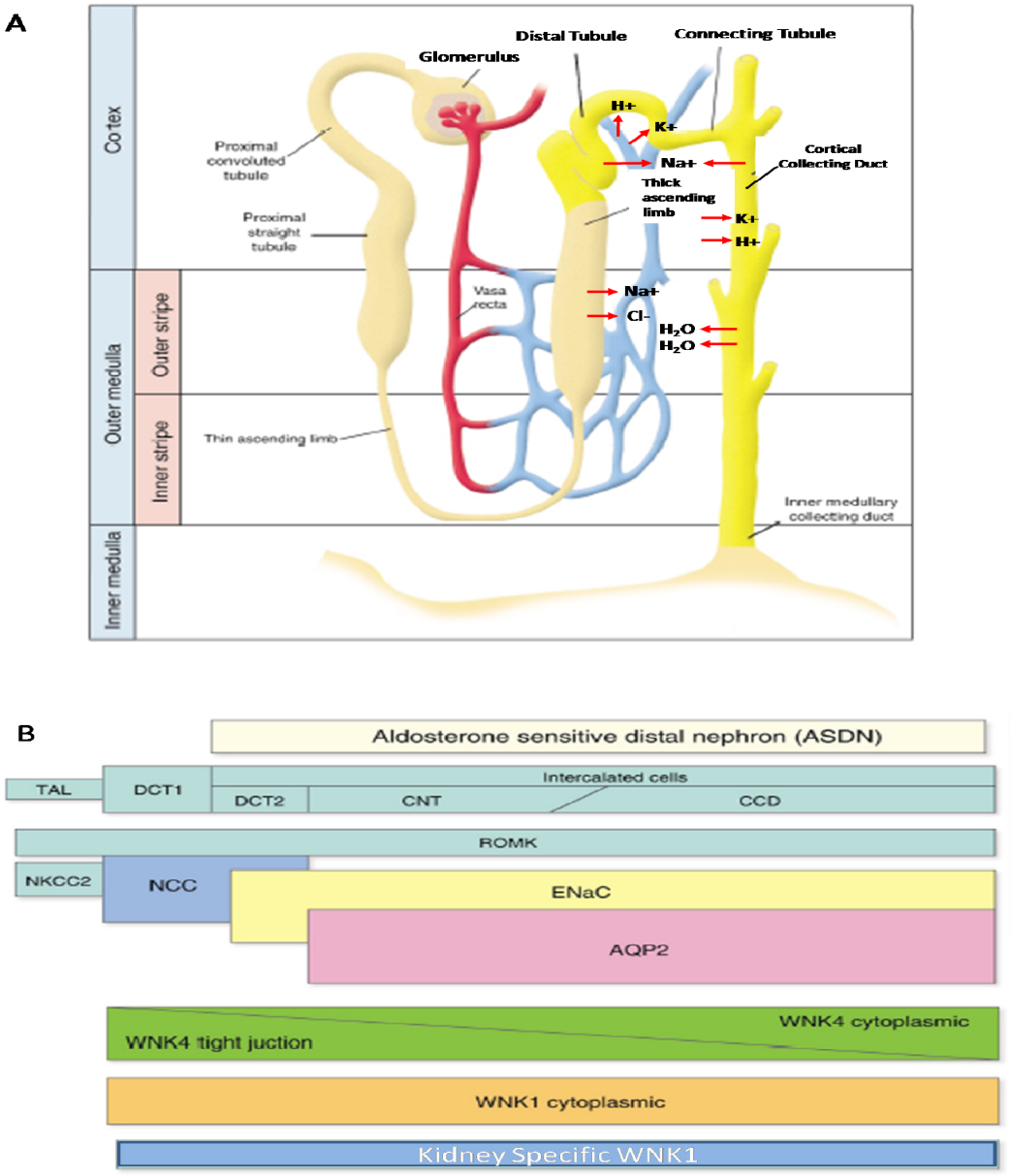

Figure 1-2. The distribution of WNK kinase family in nephron.

A. Nephron is the basic structural and functional unit of the kidney. A nephron eliminates wastes from the body, regulates blood volume and blood pressure, controls levels of electrolytes and metabolites, and regulates blood pH. A nephron can be divided into: the glomerulus, proximal tubule, loop of henle, distal tubule, connecting tubule and collecting ducts.

B. WNK1, WNK4 and kidney specific-WNK1 co-localize with renal outer medullary potassium channel (ROMK), sodium-chloride cotransporters (NCC) and epithelial sodium channel (ENaC) in different renal tubules, suggesting their functional relevance.

as muscle weakness, fatal cardiac arrhythmias and nerve impulse transmission [Lote CJ, 2000]. However, our dietary intake of K+ varies widely since K+ is the most abundant ion in fruits and vegetable. If not excreted by the kidney, the potassium in a couple of large glasses of orange juice (containing 40mEq K+) could easily double the extracellular fluid K+ concentration (60-80mEq) and lead to potentially serious symptoms or even imminent death. A major K+ secretion pathway, ROMK channel regulates K^+ secretion in CNT and CCD. To maintain K+ homeostasis, the kidneys adopt a powerful system regulating ROMK channel.

ROMK surface abundance is tightly controlled by constitutive clathrin-mediated endocytosis [Zeng WZ, 2002]. K+ secretion via ROMK is also regulated by the activity of ENaC, which provides the driving force for potassium secretion. Before the discovery of WNK kinase, a steroid hormone named aldosterone is thought to be the major hormone that regulates K+ secretion and Na^+ reabsorption. The stimulation of K+ secretion by aldosterone results from an increase in the driving force for K+ secretion via ROMK, secondary to increased Na^+ reabsorption via ENaC[Huang CL 2007]. Hypertension and hyperkalemia in PHAII patients cannot be explained by the function of aldosterone, because hypertension mediated by an increase function of aldosterone would cause hypokalemia, not hyperkalemia. The recent findings that WNK kinase family enhances renal Na+ reabsorption via ENaC and NCC, and inhibits K+ secretion via ROMK reveal an exciting novel mechanism for regulating Na+ and K+ homeostasis by kidney that can explain pathophysiology of hypertension and hyperkalemia in PHAII. Additional evidence suggests that aldosterone may crosstalk with WNK family by regulating the transcription of Kidney Specific WNK1, a shorter kinase defective WNK1 isoform expressed exclusively and at high levels in distal nephron.

Renal Sodium handling and Hypertension: Hypertension is a worldwide health problem since the industrialization. Nowadays, about one in four adults suffer from hypertension. Hypertension increases the risks of developing cardiovascular, cerebrovascular and kidney diseases. In 2001, in the USA alone, it is estimated that 54 billion dollars was spent to treat hypertension and its resulting complications [Balu S, 2006]. It is well established that salt (sodium) intake is positively correlated with the prevalence of hypertension. The first association of sodium and hypertension was proposed by Ambard and Beaujard in a paper entitled 'Causes de l'hypertension arterielle' in 1904 [Ambard L, 1904]. In the 1920s, Allen and colleagues suggested that restricting salt intake was an effective means of alleviating hypertension [Allen FM, 1922]. In the 1950s, Dahl and colleagues established the definitive connection between salt intake and hypertension in a series of careful metabolic studies [Dole VP, 1950-1953]. They first coined the term 'salt-sensitive hypertension' to emphasize the importance of salt to hypertension. Dahl and Heine showed that the genetic predisposition to salt sensitivity could be transferred via renal transplantation from susceptible to resistant inbred rats which they inbreeded [Dahl LK, 1975]. Thus, Dahl established the kidneys as the key organs responsible for conferring salt sensitivity towards hypertension.

As shown in Figure 1-3, Na+ reabsorption in the distal nephron is regulated by the thiazide-sensitive Na–Cl cotransporter (NCC) and the epithelial Na+ channel ENaC. The thiazide-sensitive Na^+-Cl^- cotransporter (NCC), a member of the SLC12 family, is mainly expressed in the apical membrane of the mammalian distal convoluted tubule (DCT) cells. NCC is responsible for cotransporting Na^+ and Cl^- from the lumen into DCT cells [Wilson FH, 2001]. The epithelial Na^+ channel (ENaC) is expressed at the apical plasma membrane in principal cells in the distal nephron of the kidney. Its activity is under the control of aldosterone, a hormone that

increases the reabsorption of sodium and the secretion of potassium in the kidneys. Na+ entry via ENaC depolarizes membrane potential and provides the driving force for K+ secretion via ROMK [Garty H, 2000] [Figure 1-3 A].

WNK kinase family in renal Na^+ reabsorption and K^+ secretion: Recently, WNK kinase family has been found to be novel regulators of renal Na+ reabsorption and K+ secretion [Figure 1-3]. WNK family has four known members at present as shown in Figure 1-1. WNK1, the first WNK kinase identified, is around 2,100 amino acids long, and contains a kinase domain in the amino terminus (amino acids 218–491 of rat WNK1). WNK2, WNK3 and WNK4 were identified later by their similarity in the kinase domain as WNK1. WNK2, WNK3 and WNK4 are products of different genes, ranging in length from 1,200 to 1,600 amino acids. WNK 1-4 all contain an N-terminal kinase domain, two putative coiled-coil domains, multiple proline-rich motifs possible binding SH3 domains [Huang CL, 2007 Oct]. A conserved auto-inhibitory domain distal to the kinase domain is also present among all WNK kinase family members, which inhibit the kinase activity of WNK. In addition, all WNK kinases have a very short acidic segment distal to their kinase domains that is conserved through species. PHAII causing mutations in WNK4 cluster within this acidic domain [Xu BE, 2000; Wilson FH, 2001; Veríssimo F, 2001].

WNK1 is ubiquitous and is expressed at high level in kidney. WNK4 is highly expressed in the kidneys and widely distributed in epithelial tissues [Wilson FH, 2001; Choate KA, 2003]. A shorter kinase defective WNK1 isoform is expressed exclusively and abundantly in the kidney and is, therefore, named kidney-specific WNK1 (KS-WNK1) [Figure 1-2 B]. Besides the identical C-terminus as WNK1, Kidney-specific WNK1 has a unique 30aa domain known as 4a domain in the N-terminus, which is encoded by exon 4A [Xu Q, 2002]. Beyond their kinase domains, WNK kinase family shares little sequence identity with each other, or any other known proteins in the

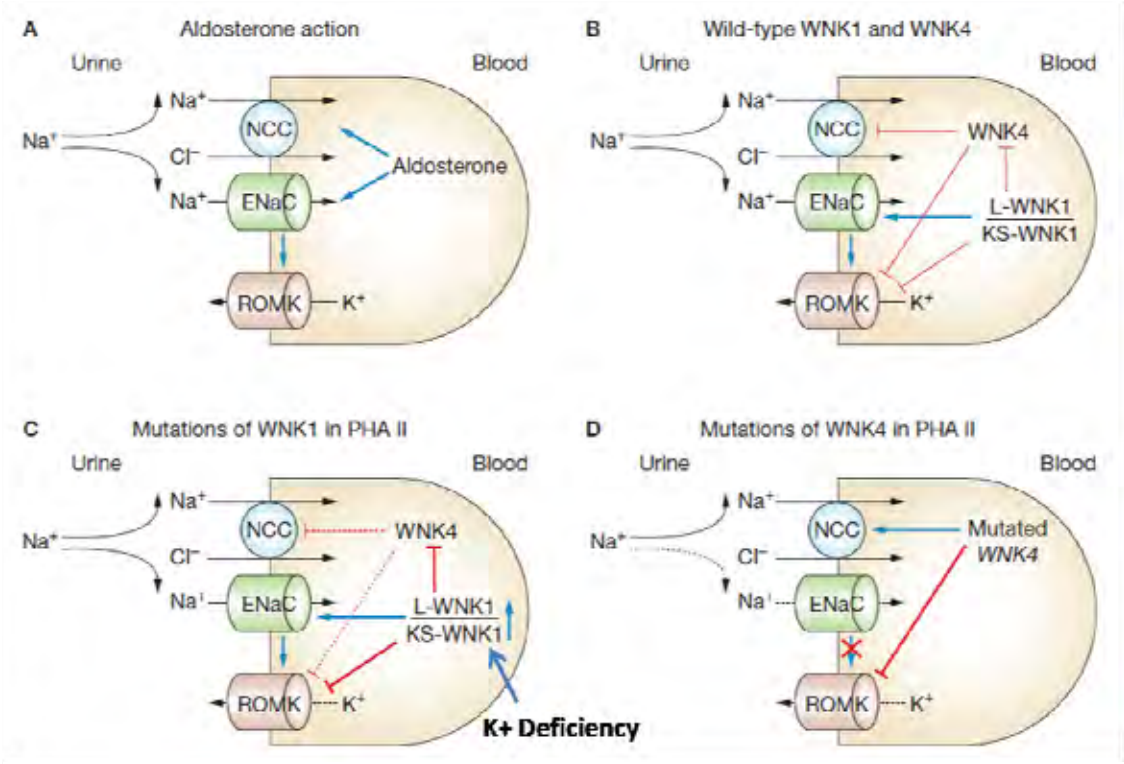

Figure 1-3. Na^+ reabsorption and K^+ secretion regulated by WNK family in the distal nephron.

A. Na+ and K+ transport regulation by aldosterone. Aldosterone stimulates Na+ reabsorption by both ENaC and NCC. Na+ reabsorption through ENaC(electrogenically) provides the driving force for K+ secretion via ROMK.

B. WNK1 and WNK4 regulation of Na+ and K+. WNK4 inhibits NCC and ROMK (red lines). An increased ratio of L-WNK1 to KS-WNK1 stimulates ENaC (blue arrow), inhibits ROMK, and antagonizes WNK4-mediated inhibition of NCC.

C. WNK1 intronic deletions in PHA II increase expression of unmutated WNK1, leading to an increased ratio of L-WNK1 to KS-WNK1, which in turn inhibited K+ secretion via ROMK and enhances Na+ reabsorption by activating ENaC and antagonizing inhibition of NCC by WNK4. K+ deficiency also increases the ratio of L-WNK1 to KS-WNK1, exerting effects on Na+ and K+ transport similar to those in PHA II.

D. WNK4 PHA II mutants inhibit ROMK more effectively than wild-type WNK4. PHAII mutations in WNK4 also impaired its inhibition of NCC, leading to increased Na+ reabsorption in DCT. It decreased the Na+ delivered to ENaC in the more distal nephron, which impaired Na+ reabsorption via ENaC and reduced driving force for K+ secretion through ROMK (red X). Thickness of lines indicates relative increases and decreases in the activity of transporters. (Figure adapted from Huang CL, 2007)

genome. As shown in Figure 1-3, WNK1 has been shown to stimulate ENaC activity, whereas WNK4 inhibits NCC. Furthermore, WNK1 antagonizes WNK4 inhibition of NCC and increase Na+ reabsorption. Both WNK1 and WNK4 inhibit ROMK. Interestingly, KS-WNK1 functions as an antagonist of WNK1 in regulation of ROMK, NCC and ENaC [Figure 1-3]. [Review, Huang CL, 2007]

WNK family and the hypotensive effect of K+ supplementation

Blood pressure and total body Na+/K+ ratio were the most tightly correlated variables among other factors [Huang CL, 2007]. Independent of Na+ intake, K+ intake is inversely correlated with the prevalence of hypertension in epidemiologic studies [Langford HG, 1983]. Potassium, not only correlated with hypertension, its supplementation proved to be an effective way to fight against salt-sensitive hypertension. Ingestion of high level of K+ (120 mmol/day) abolished salt induced hypertension, even at Na+ intake rates of 250 mmol/day [Morris RC, 1999].

Though there are plenty of studies suggesting K+ deficiency predisposed individual to hypertension by enhancing renal Na+ reabsorption [Dole VP 1950-1953], however, the mechanism by which K+ supplementation affect Na+ handling in the kidneys is not known. From works by our lab and other groups, we believe that WNK kinase family may be the long-sought "missing link" between K+ deficiency and increased renal Na+ reabsorption. High potassium level stimulates aldosterone production, which has been shown to stimulate the expression of KS-WNK1 *in vitro* [Náray-Fejes-Tóth A, 2004]. We and others also find that K+ deficiency increased the ratio of WNK1 to KS-WNK1 independent of salt intake, and K+ supplementation will do the contrary. As shown in Figure 1-4, increase of the WNK1/KS-WNK1 ratio caused by K+ deficiency not only affects K+ secretion through ROMK, but also significantly enhances Na+ reabsorption through NCC and ENaC as previously reported. In contrary, K+ supplementation

will decrease the ratio of WNK1/KS-WNK1, which will result in decreased Na+ reabsorption through NCC and ENaC. This may explain the enigmatic hypotensive effect induced by K+ supplementation.

Such a mechanism may result from millions of year's evolution. Food like vegetable and meat is high in K+, but low in salt. For ancient human beings, the source for K+ is abundant, while Na^+ is more limited. During revolution, dietary deficiency of relatively abundant K+ is often a harbinger of severe food shortage, thus a mechanism preserving both K^+ and Na^+ during starving would be crucial for survival of ancient human and animals [Eaton SB, 1985]. However, such a survival mechanism no long serves us right in modern society. Nowadays, the sodium uptake of Americans is about 7.5 times more than our Paleolithic ancestors (150 vs 20 mmol per day) [Eaton SB, 1985]. However, our K+ intake has been decreasing (50 vs 320 mmol per day) due to the decreased consumption of fruits and vegetables. The ratio of dietary Na+ to K+ intake changed from 1:16 for Paleolithic humans, to only 3:1 for modern Americans—a 50-fold increase in the ratio of dietary intake of Na+ versus K+. In modern times, K^+ deficiency no longer comes alone with Na^+ deficiency as it used to be during the evolution, but rather goes hand-in-hand with Na^+ excess. In order to conserve K^+ upon K^+ deficiency, our kidney would have absorbed too much Na+ and resulted in salt-sensitive hypertension.

In the following chapters, I will illustrate in detail about my studies on the endocytosis of ROMK channel regulated by WNK kinase family. Hopefully, this knowledge would not only contribute to our understanding the mechanism of renal K+ handling, but also be helpful to fight against salt-sensitive hypertension.

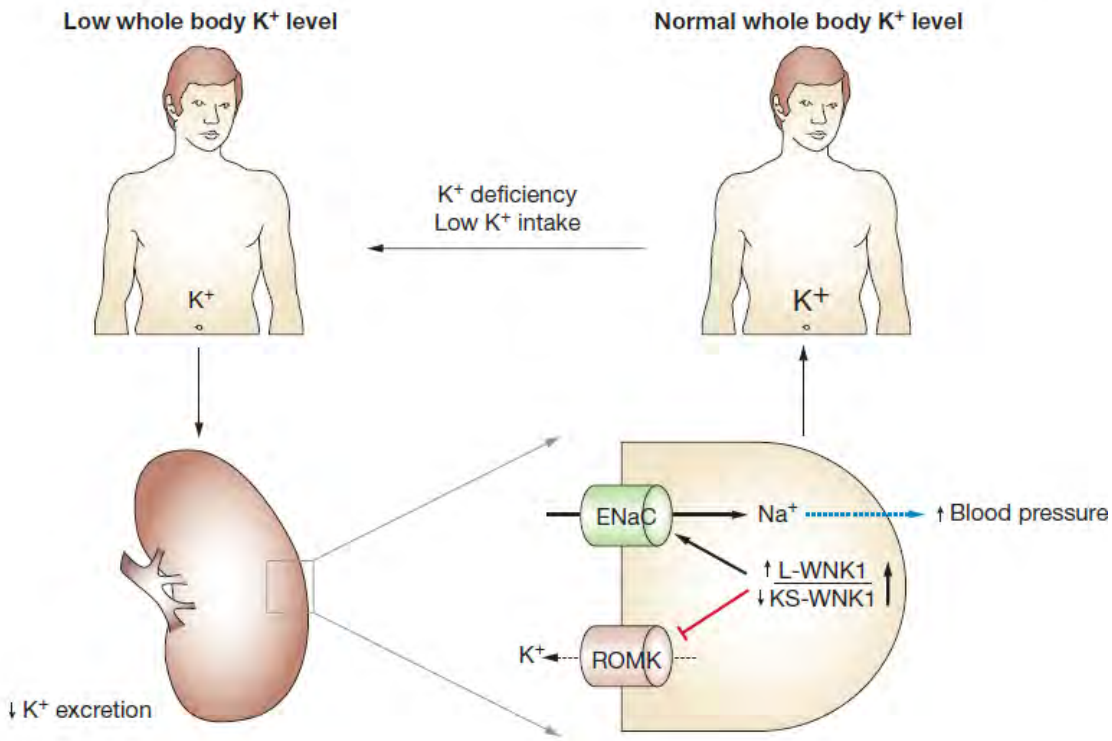

Figure 1- 4. A model of K+ deficiency induced Na+ retention and hypertension.

ROMK is responsible for K+ excretion in the distal nephron. To maintain K+ homeostasis, ROMK is downregulated during K+ deficiency; and upregulated during K+ supplement. Downregulation of ROMK is mediated by an increase in the ratio of WNK1 to KS-WNK1 during K+ deficiency in order to conserve K+; however, this also causes undesirable reabsorption of Na+ via ENaC and NCC (see in figure 3). (Figure adapted from Huang CL, 2007)

CHAPTER TWO: WNK1 and WNK4 stimulate endocytosis of ROMK through their proline-rich domains

ABSTRACT

Mutations in WNK1 and WNK4 kinase lead to PHAII disease characterized by early hyperkalemia and hypertension. ROMK is an important renal K+ secreting potassium channel tightly regulated by clathrin-mediated endocytosis in renal tubules. WNK 1 and WNK4 have been reported to stimulate endocytosis of ROMK channel in *Xenopus Oocyte*. In this study, I established human embryonic kidney cells (HEK293) expression system and utilized patch clamp technology to study the surface abundance of ROMK. In HEK293 cells, ROMK was internalized 10 times faster than in *Xenopus Oocyte,* and both WNK1 and WNK4 still inhibited ROMK by enhancing clathrin-mediated endocytosis of the channel. WNK4 inhibition of ROMK is independent of its kinase activity or kinase domain. PHAII mutations in WNK4 enhance the inhibition of ROMK. Specific proline-rich-motifs of WNK1 and WNK4 were found to be critical for inhibiting ROMK. In WNK1, three proline-rich motifs in the N-terminus are important for inhibiting ROMK. In WNK4, the three proline-rich motifs adjacent to the acidic region are critical. Disruption of the specific proline-rich motifs in WNK1 or WNK4 abolished their inhibition over ROMK.

INTRODUCTION

WNK1, the first member of WNK kinase, was first cloned in 2001 by Cobb lab in UTSW. In 2002, Mutations in WNK1 and WNK4 gene were identified as causes of PHA2, a rare autosomal dominant disease characterized by hypertension, hyperkalemia and metabolic acidosis. Those phenotypes are closely related to defects in sodium and potassium handling by the kidney, suggesting that WNKs may be previously unrecognized players in regulating potassium and sodium homeostasis. The pioneering studies on WNK kinase and renal ion channels/transporters are exciting, which spurs an explosively expansion of WNK biology in recent years.

As a critical exit pathway for K secretion, ROMK channels are regulated by both acute (such as arginine vasopressin) and long term (such as dietary K intake) factors that affects K+ secretion. To maintain K homeostasis, the density of ROMK in cortical collecting ducts increases and decreases during high and low dietary K intake, respectively. Low dietary K intake decreases ROMK, likely by stimulating clathrin-mediated endocytosis *in vivo* followed by degradation via lysosomes. Both WNK1 and WNK4 inhibit the renal outer medullary potassium channel (ROMK) by stimulating its endocytosis. PHA2-causing WNK4 mutants exhibit increased inhibition of ROMK, which potentially contributes to the pathogenesis of hyperkalemia in the affected patients. These results suggest that inhibition of K+ secretion through ROMK may contribute to hyperkalemia caused by mutations of *WNK1* and *WNK4* in PHAII.

WNK1 and WNK4 regulation of endocytosis of ROMK may represent a novel pathway for regulating K+ homeostasis. Due to the big size and the versatile functionality of WNK1 and WNK4, a study of the functional domains in WNK1 and WNK4 is particularly important for

understanding of the mechanism for regulating ROMK. WNK kinases have multiple PXXP proline-rich motifs for potential protein-protein interaction [Figure 1-1B]. Interaction between PXXP motifs and Src Homology 3 (SH3) domain plays important roles for endocytic proteins to mediate endocytosis, e.g. the proline-rich motifs in endocytic proteins like dynamin and N-WASP interacts with SH3 domain containing proteins such as intersectin and syndapin to facilitate endocytosis.

It has been speculated that the proline-rich domain (PRD) of WNK1 and WNK4 may be important for regulating endocytosis of ROMK, however, there is no work done to prove it before this study. To answer these questions, I designed the experiments in this chapter to identify the functional domain(s) in WNK1 and WNK4.

MATERIAL AND METHODS

Cell culture and transfection. HEK293 cells, Caveolin +/+ and Caveolin null cells were cultured in DMED medium supplemented with 10% FBS, 5% ampicillin and streptomycin. Cells were co-transfected with cDNAs encoding GFP-ROMK (0.3 μg per 35 mm well) and myc-tagged WNK1 or WNK4 constructs (1-1.5ug per well) using a commercial transfection kit (Fugene 6) according to the manufacturer protocol. Other constructs except WNK kinases are usually transfected at 1ug per 35mm well, with the upper limit for total DNA transfection around 2.5ug per 35mm well. In each experiment, the total amount of DNA for transfection was balanced using an empty vector. For siRNA transfection, Polyfect (Qiagen) is used instead of Fugene6 with all the other conditions unchanged.

Molecular biology. GFP-ROMK was described previously [He G and Wang HR, 2007]. Full-length cDNAs encoding rat WNK1 and WNK4 (gifts of M. Cobb and B. Xu, University of Texas Southwestern Medical Center) were cloned in pCMV5-myc vector [Wang HR, 2008]. WNK1 and WNK4 fragments were amplified by PCR and subcloned into pCMV5-myc. Point mutations were generated by site-directed mutagenesis (QuickChange kit; Stratagene) and confirmed by sequencing.

Patch-clamp recording. HEK293 cells were cotransfected with cDNAs encoding GFP-ROMK and full-length or fragments of WNK1 or WNK4 plus additional constructs as indicated. In each experiment, the total amount of DNA for transfection was balanced using an empty vector. About 36–48 hours after transfection, whole-cell currents were recorded using an Axopatch

200B amplifier (Molecular Devices) as previously described[He G, Wang HR, 2007]. Transfected cells were identified using epifluorescent microscopy. The pipette solution contained 140 mM KCl and 10 mM HEPES (pH 7.2); the bath solution contained 140 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM HEPES (pH 7.4). Capacitance and access resistance were monitored and 75% compensated. The voltage protocol consisted of 0 mV holding potential and 400-ms steps from –100 to 100 mV in 20-mV increments. K^+ current was calculated by substracting current at –100 mV (pA; measured at 25°C) with the residual current after application of $BaCl_2$ (10mM) [shown in Figure 2-1] Current density was obtained by dividing K^+ current by capacitance (pF). Results were shown as mean ± SEM (*n* = 5–10). For cell-attached single-channel recording of ROMK, the pipette solution contained 140 mM KCl, 1 mM MgCl2, 1 mM CaCl2, and 10 mM HEPES (pH 7.4). Single-channel recording analysis of single-channel conductance and open probability were performed as described previously [Lazrak A, 2006].

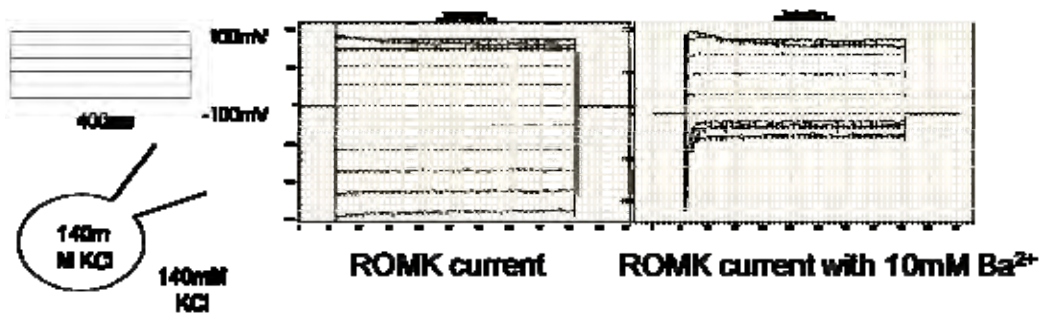

Figure 2-1. Voltage pulse protocol and ruptured whole-cell configuration.

ROMK current was measured by ruptured whole-cell patch clamp recording. The bath and pipette solution contains 140mM KCl, and the current was obtained by stepping the command voltage from -100mV to +100mV with an interval of 20mV. ROMK current for calculating current density was obtained by subtracting the current at -100mV with the residual current after Ba^{2+} treatment blocking the ROMK channel, then divided it by the cell capacity (pF).

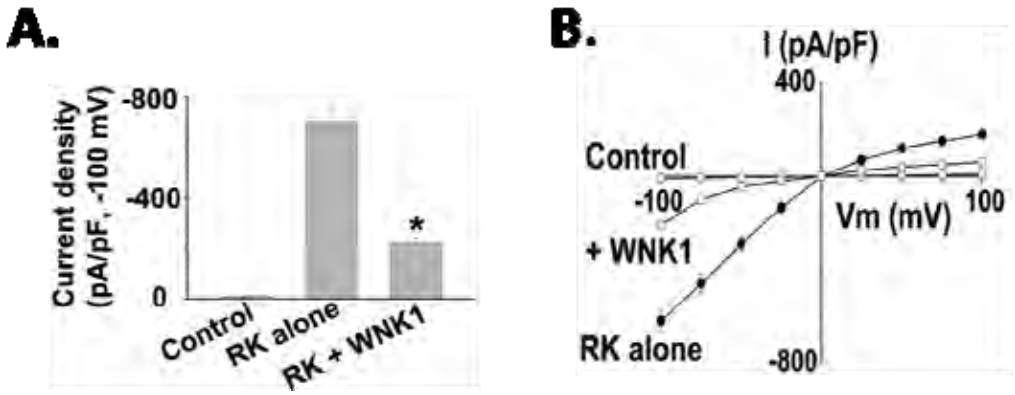

Figure 2-2. Co-expression of WNK1 decreases ROMK current amplitude and current density. A. The ROMK current density of control untransfected HEK cells(first column), HEK cells expressing ROMK (second column), and HEK cells expressing both ROMK and WNK1 full length (third column). B. The IV curve of ROMK current in control untransfected HEK cells(control), HEK cells expressing ROMK (ROMK alone) and HEK cells expressing both ROMK and WNK1 full length (+WNK1).

RESULTS

WNK1 decreases surface abundance of ROMK via a dynamin- and clathrin-dependent mechanism.

The current density of a specific ion channel can be regulated by three mechanisms: direct modulation of channel activity, forward trafficking and endocytosis. In our HEK expression system, we found that co-expression of WNK1 decreased ROMK current density [Figure 2-2]. Full length WNK1 is a large protein which makes it hard to express in HEK cell, so we made a truncated WNK1 (aa 1-491), which expressed better than the full length WNK1 (data not shown). As shown in Figure 2-2B, WNK1 (1-491) fully recapitulated the function of full length WNK1 in inhibiting ROMK current density and surface abundance. Additionally, we and another lab have previously shown that kidney specific WNK1, which has all the WNK1 C-terminal sequence after amino acid 438, failed to inhibit ROMK. These data suggests that the functional domain of WNK1 lies in the N-terminus. In the following chapters, WNK1 (1-491) will be used instead of full length WNK1, to inhibit ROMK, unless otherwise stated. For clarification of terminology, WNK1 will be used to represent the ubiquitous long WNK1 and KS-WNK1 will be used to represent kidney specific WNK1.

The inhibition of ROMK by WNK1 was prevented by co-expression of dominant negative form of dynamin but not wild-type dynamin [He G and Wang HR, 2007]. Surface biotinylation (done by He G) also suggested that WNK1 stimulated endocytosis of ROMK, since co-expression of WNK1 with ROMK decreased ROMK surface abundance, whereas dominant negative but not wild-type dynamin completely abolished WNK1 effect on ROMK. In sum, these data suggested that WNK1 inhibits ROMK by stimulating endocytosis of ROMK [He G and Wang HR, 2007].

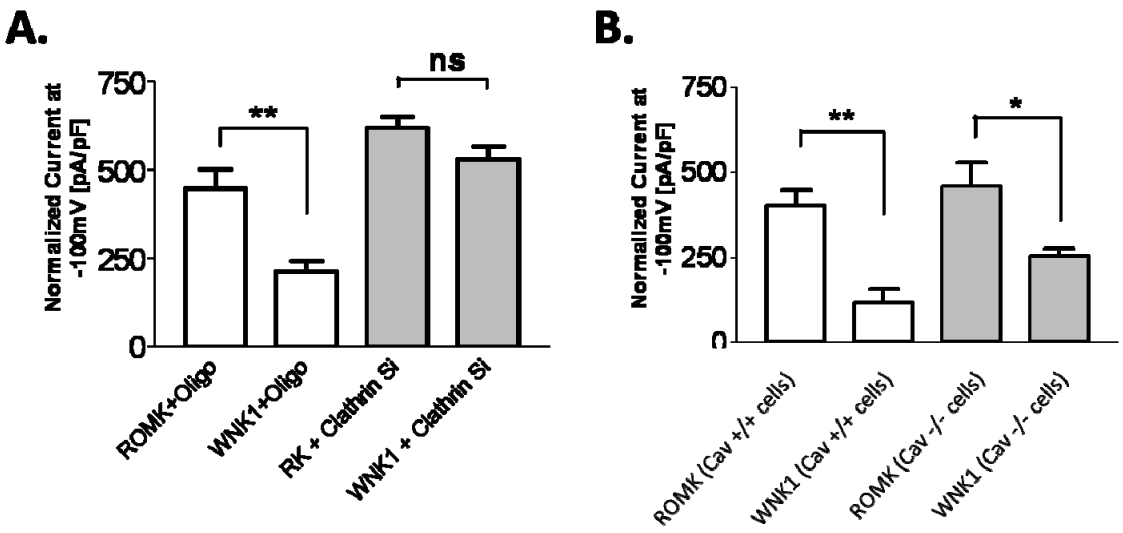

Figure 2-3. Inhibition of ROMK by WNK1 is dependent on clathrin, but not caveolin 1.

- **A.** In control group (cell cotransfected with scramble siRNA oligo,white bars) siRNA transfection does not affect WNK1 function. In clathrin knockdown group (grey bars) WNK1 failed to inhibit ROMK.
- **B.** In comparison, WNK1 still inhibit ROMK in caveolin knockout cells(Cav-/-, grey bars) as well as in the control cell line (Cav+/+, white bars)

Dynamin is involved in both clathrin and caveolin-mediated endocytosis. Clathrin knock-down by siRNA prevented WNK1 inhibition of ROMK as shown in Figure 2-3A, suggesting that WNK1 inhibits ROMK via clathrin-mediated endocytosis. Consistently, WNK1 and WNK4 can still inhibit ROMK in caveolin null cell line, suggesting that caveolin is not required for WNK kinase to inhibit ROMK [Figure 2-3B]. These data suggested WNK1 inhibit ROMK through clathrin-mediated endocytosis.

WNK4 enhances the rate of endocytosis of ROMK

Bredfeldin A (BFA) blocks forward trafficking of ROMK [Zeng WZ, 2002]. In the steady state, ROMK forward trafficking is balanced with endocytosis so that the membrane abundance of ROMK is relatively.

Acute treatment with BFA induced potent inhibition of ROMK in HEK cell, supposedly due to the endocytosis of ROMK [Figure 2-4A]. To support this, dominant-negative dynamin abolished the inhibition of ROMK by BFA [Figure 2-4C]. The half-inhibition time for BFA, defined as the time required for 50% of the maximal effect of BFA inhibition, is about 38 mins [Figure 2-4A]. This rate of endocytosis in HEK cells is 10 times higher than that in *Xenopus Oocyte*(half-inhibition time of 38min [Figure 2-4A] v.s. 360 min) [Zeng WZ et al, 2002]. Thus, clathrin-mediated endocytosis of ROMK is more active in the mammalian cell line than the previous estimated based on data obtained in *Xenopus Oocyte*. These data also suggest that HEK cell is a better model for study of endocytosis of ROMK regulated by WNK kinase family.

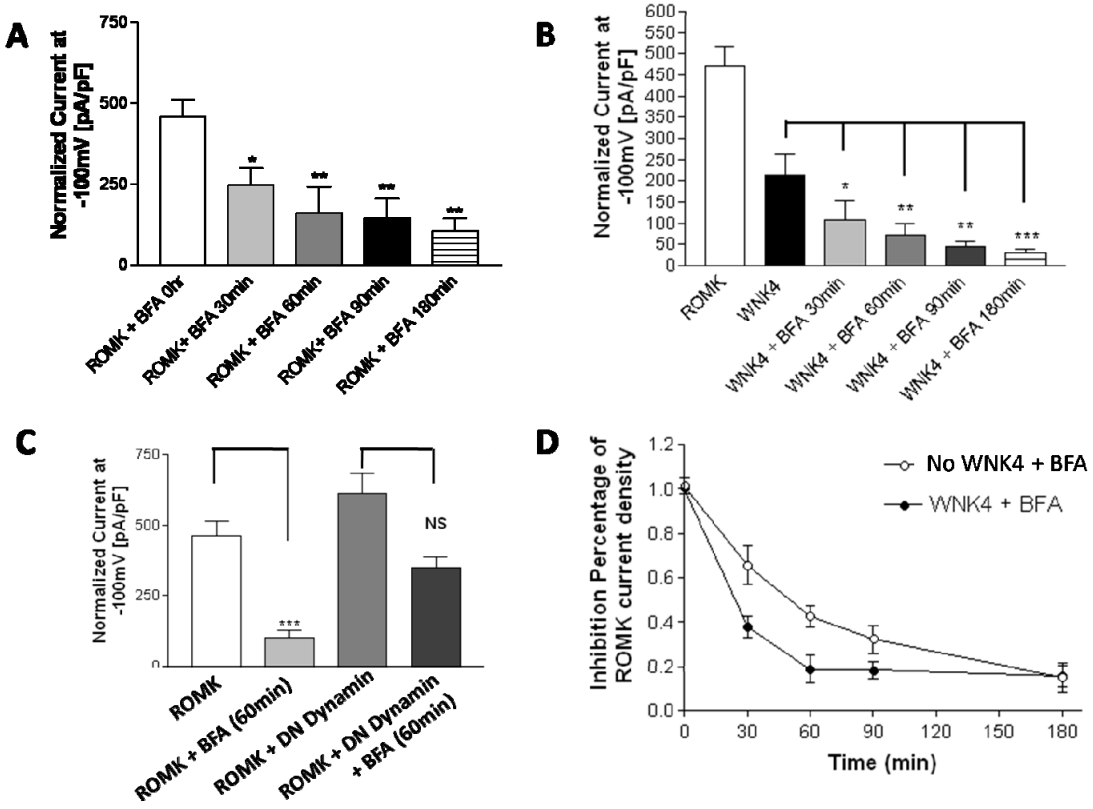

Figure 2-4. WNK4 increases the rate of endocytosis of ROMK

- **A.** The time course of ROMK current density in the presence of Bredfeldin A(BFA). As BFA inhibits export of newly synthasysed protein from Golgi, the decrease in ROMK current reflects endocytosis of the channel
- **B.** The time course of ROMK current density inhibited by BFA in WNK4 transfected cells. WNK4 accelerates the decrease of ROMK current in the presence of BFA.
- **C.** The effect of BFA can be blocked by co-expression of dominant negative dynamin (DN Dyn).
- **D.** The rate of decrease of ROMK in the presence of BFA in control group and in WNK4 group. The result showed that the WNK4 more than doubled the kinetics of Endocytosis of ROMK as indicated by half-inhibition time: 40 min in control v.s. 18min in WNK4 group.

The inhibition of ROMK by BFA was enhanced by co-expression of WNK4 [Figure 2-4B]. In WNK4 transfected cells, the percentage inhibition of ROMK by BFA was more dramatic than that of the BFA alone group. As in Figure 2-4D, within 30 min, 60% of ROMK was inhibited by BFA in WNK4 group v.s. that of 40% in control group. The calculated half-inhibition time of ROMK was 18min in WNK4 group compared to 38min in control group.

Proline-rich domain in the N-terminus of WNK1 is important for inhibiting ROMK

Rat WNK1 has 2,126 amino acids. To further identify the functional domains in WNK1 for regulation of ROMK, I compared the effects of amino acids 1–119, amino acids 1–491, amino acids 218–491, and full-length WNK1. I found that WNK1 (1–119), as well as WNK1 (1–491), and full-length WNK1 inhibited ROMK [3rd bar, Figure 2-5B]. The construct containing the kinase domain of WNK1, WNK1 (218–491), did not cause the inhibition of ROMK[5th bar, Figure 2-5B].

Amino acids 1–119 of WNK1 contain 3 PXXP proline-rich motifs at P94, P103, and P114 [Figure 2-5A]. I then examined the role of these PXXP motifs in the regulation of ROMK. Compared with wild type WNK1 (1–491), single mutants of P94A, P103A, and P114A each caused partial inhibition of ROMK currents [3rd -5th bar, Figure 2-5C]. The triple PXXP mutant of WNK1 (1–491) failed to inhibit ROMK [6th bar, Figure 2-5C]. As kidney-specific WNK1 lacks amino acids 1–119 of WNK1, the current results also support previous reports that kidney-specific WNK1 by itself has no effects on ROMK channels.

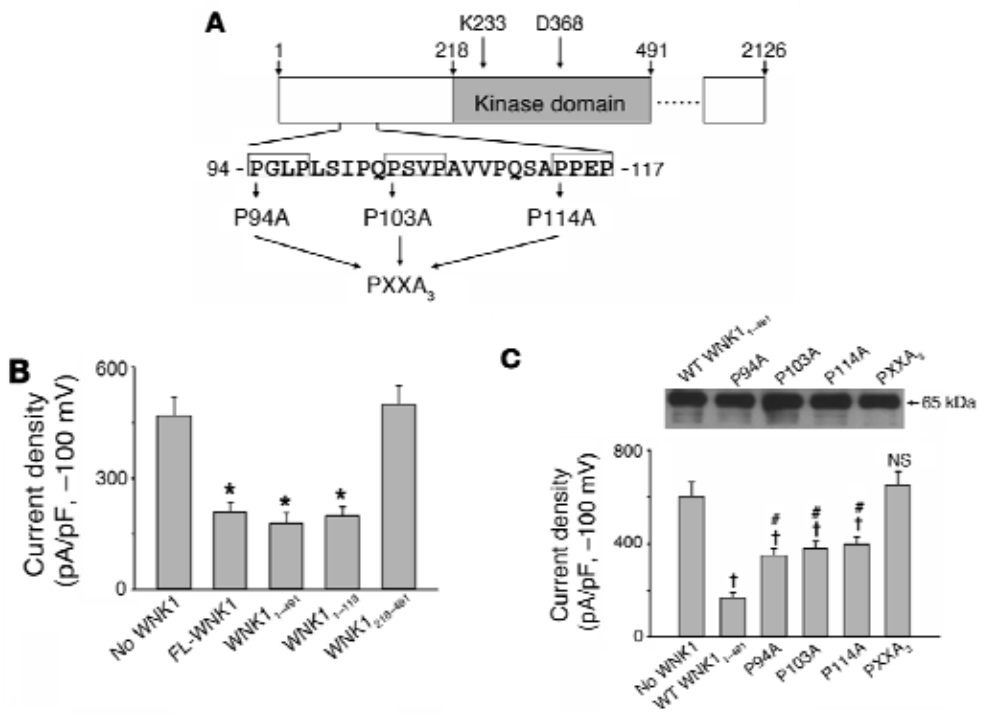

Figure 2-5. Specific proline-rich motifs in WNK1 are critical for regulation of ROMK.

A. The region of amino acids 1–119 of rat long WNK1 contains 3 PXXP motifs conserved among mouse, rat, and human proteins; the triple P94A/P103A/P114A WNK1(1–491) mutant is denoted as PXXA3. The catalytic K233 and conserved D368 of the WNK1 kinase domain are shown.

B. Effects of various N-terminal long WNK1 fragments on ROMK current density. Cells were transfected with ROMK plus indicated constructs. FL, full-length. **P* <0.05 versus ROMK alone.

C. Effects of various long WNK1 constructs on ROMK current density. The concentration that causes a maximal inhibition of ROMK current (3 μg DNA per transfection per 6-well dish) for wild-type WNK11–491 and each mutant were used. Equal levels of protein expression for each construct were confirmed by Western blot analysis using an anti-myc antibody. Modified statistics adjusted for multiple comparisons was used (see Methods). †*P* < 0.01 versus ROMK alone. #*P* < 0.01 versus ROMK plus WT WNK11–491.NS,not significantly different versus ROMK alone.

Regions of WNK4 involved in regulation of ROMK

It has been reported that WNK4 stimulates the endocytosis of ROMK in *Xenopus Oocytes*. However, whether the *Xenopus Oocytes* expression system truly represents the *in vivo* situation in human is not known. I first tested the effect of WNK4 on ROMK in mammalian cell line, HEK 293. Consistent with previous reports, full length WNK4 inhibits ROMK in HEK293 [Figure 2-6 A].

To identify region(s) of WNK4 involved in the regulation of ROMK, we made a series of truncated form of WNK4. As shown in Figure 2-6A, amino acids 1-584, which still contains the conserved acidic region and the proline-rich domain, inhibited ROMK current as well as full length WNK4. In comparison, amino acids 1–535 or amino acids 1–444 of WNK4 did not decrease ROMK currents density [Figure 2-6B]. The results were confirmed by surface biotinylation assay (done by He G [He G, and Wang HR, 2007]).

WNK4 kinase domain or kinase activity is dispensable for regulating ROMK. Kinase-dead mutations were introduced into WNK4 (1-584) construct: WNK4 (1-584) K183M, WNK4 (1-584) D318A and WNK4 (1-584) S332A, and all the kinase dead mutations did not affect the WNK4 effect of ROMK [Figure 2-6D]. Without the kinase domain, WNK4 (473-1220) [4th bar, Figure 2-6C] and WNK4 473-920 (data not shown) still inhibit ROMK. A small WNK4 peptide from aa473 to aa584, which contains the conserved acidic region and a proline-rich domain, is sufficient to inhibit ROMK [3rd bar in Figure 2-6C]. WNK4 (584-1220) also inhibit ROMK, suggesting that there are additional domains inhibiting ROMK in the C-terminus of WNK4 [4th bar in Figure 2-6C].

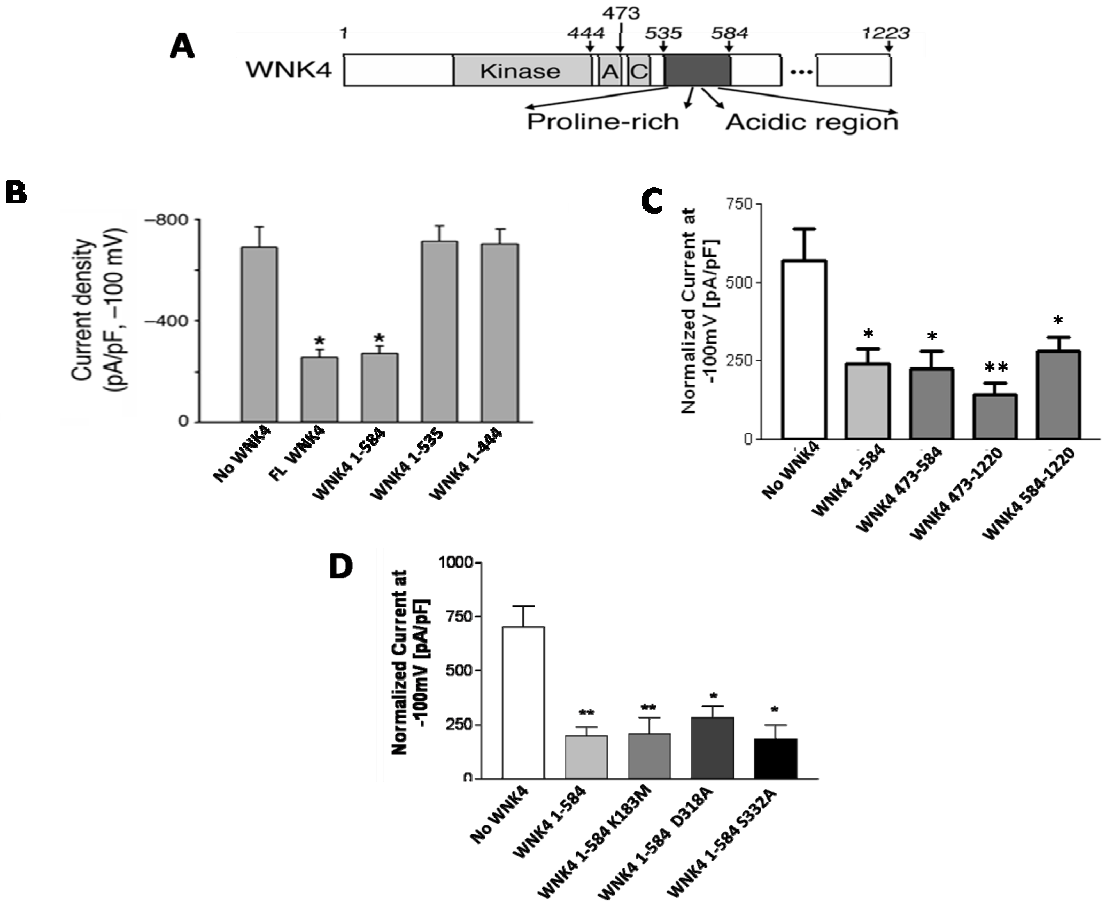

Figure 2-6. Full length WNK4 and amino acids (1-584) of WNK4 each inhibits ROMK current in HEK293 expression system.

- **A.** Domains of WNK4 involved in regulation of ROMK current density. Cells were transfected with ROMK plus indicated constructs. A, autoinhibitory domain; C, coiled-coil domain.
- **B.** WNK4(1–584) decreased ROMK surface abundance in biotinylation assay. Experiments are duplicated as shown in the figure.
- **C.** WNK4 kinase dead mutations did not affect its ability to inhibit ROMK.

Taken together, these data suggests: 1. Amino acids 473-584 of WNK4 contain a functional domain. 2. Neither WNK4 kinase domain nor WNK4 kinase activity is required for inhibiting ROMK. 3. There may be additional functional domain(s) distal to the WNK4 conserved acidic region.

PHAII mutations enhance WNK4 inhibition of ROMK

In PHAII patients, mutations of WNK4 cause hyperkalemia. To understand the mechanism, we examined the effects of WNK4 mutations on ROMK. Three PHAII mutations (E559K, D561A and Q562E) were introduced into the WNK4 1-585 constructs individually [Figure 2-7B]. With similar expression level as wild type WNK4, disease-causing WNK4 mutants E559K, D561A, and Q562E inhibited ROMK current density more than wild-type WNK4 (1–584) [Figure 2-7A]. These results were further confirmed with surface biotinylation assay done by Dr. He G in the lab [He G, and Wang HR, 2007]. Thus, the enhanced inhibition of ROMK by WNK4 mutants may contribute to hyperkalemia in the PHAII patients.

The proline-rich domain preceding WNK4 acidic domain is important for inhibiting ROMK

Amino acids 473–584 of WNK4 are sufficient for the inhibition of ROMK. This region contains 3 conserved PXXP motifs (which begin at P545, P552, and P555) adjacent to disease-causing mutations E559K, D561A, and Q562E [Figure 2-7B]. Triple mutations of PXXP motifs abolished inhibition of ROMK by WNK4 (1-584) [4th bar, Figure 2-7C] and the enhanced inhibition caused by E559K [5th bar, Figure 2-7C, data not shown for D561A and Q562E).

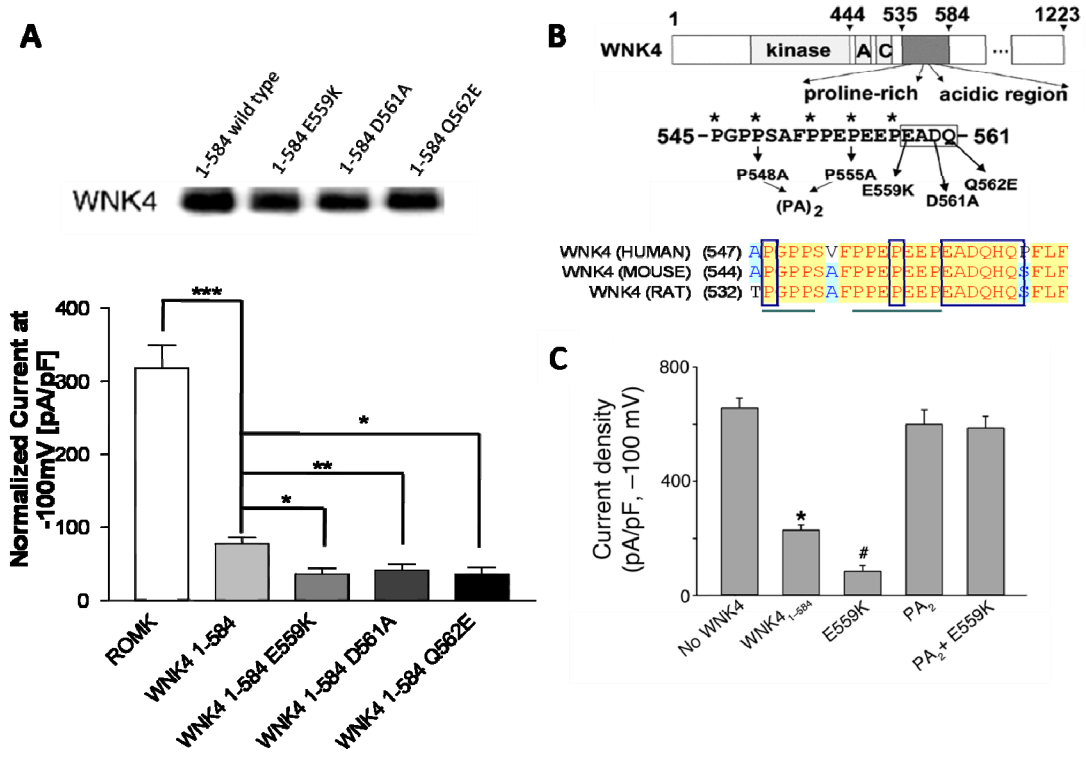

Figure 2-7. Representative experiments showing the effects of PHA2 mutations on WNK4 regulation of ROMK.

A. Lower panel, WNK4 PHAII mutations increased the ability of WNK4 to inhibit ROMK current density compared with wild-type. Upper panel,western blotting of the wild-type and mutants shows they were expressed at the similar.

B. Amino acids 545–561 of WNK4 contain 3 PXXP motifs and disease-causing mutations. Double mutations of P548A/P555A disrupted all 3 PXXP motifs (denoted PA2).

C. Triple PXXP mutations prevented the decrease of surface ROMK caused by WNK4(1–584).

D. Effects of PXXP motifs and disease-causing mutations of WNK4 on ROMK currents. Cells were transfected with ROMK plus indicated constructs. *P < 0.05 versus ROMK alone. #P < 0.05 versus ROMK plus WNK4(1–584).

Altogether, these data suggests that the proline-rich domain in the N-terminal of WNK1 and the proline-rich domain before the acidic region in WNK4 are critical for them to inhibit ROMK. The detailed mechanism will be addressed in Chapter 3.

DISCUSSION

In the first part of this study, we established a human embryonic kidney (HEK294) cell expression system, which is a better model in mimicking the *in vivo* situation in kidney than *Xenopus Oocytes*. We then established the patch clamp assay to study the surface abundance of ROMK. Using this system, we showed that: 1. WNK1 and WNK4 inhibit ROMK by increasing the rate of endocytosis of ROMK; 2. WNK4 PHAII mutations inhibit ROMK better than wild-type, and WNK4 inhibition of ROMK is independent of its kinase domain or kinase activity. 3. Proline-rich domain in both WNK1 and WNK4 are important for inhibition of ROMK.

BFA treatment inhibits ROMK by blocking ROMK forward trafficking. The decrease in ROMK current in the presence of BFA reflects endocytosis of the channel. The half-inhibition time of ROMK by BFA treatment is only 40min in HEK cell compared to that of 360min in the *Xenopus Oocytes*, representing a nearly 10 fold increase of the internalization rate of ROMK. WNK4 over-expression further reduces ROMK half-inhibition time to about 18min. HEK293 cell line is a mammalian cell line. The endocytosis rate of ROMK in HEK cells is closer to that of physiological condition than Xenopus Oocytes.

To understand the mechanism by which WNK1 and WNK4 stimulate endocytosis of ROMK, the first step is to identify the functional domain(s) in WNK1 and WNK4 for inhibiting ROMK. By using a series of truncated WNK1 and WNK4 constructs, I found the functional domain of WNK1 lie in the N-terminal proline-rich region; the functional domain of WNK4 is also a proline-rich domain, adjacent to the conserved acidic region where the PHAII mutations clustered.

Additionally, small PRD containing peptides, like WNK1 (1-119) and WNK4 (473-584), are sufficient to inhibit ROMK. So, the proline-rich domain of WNK1 and WNK4 are both required and sufficient to inhibit ROMK. However, it is unexpected that small peptide like WNK1 (1-119) and WNK4 (473-584) can inhibit ROMK independently, since they are unlikely to target specifically to ROMK without the rest of the sequences. Since WNK1 and WNK4 can form oligomers, it is possible that those small WNK peptides will take advantage of the endogenous WNK for targeting or other functions. Thus it will be interesting to study if WNK1 (1-119) and WNK4 (473-584) could still inhibit ROMK after knocking down endogenous WNK1 or WNK4 using siRNA.

The C-terminus of WNK1 does not participate in regulating ROMK, because KS-WNK1, containing all the WNK1 C-terminal sequence after kinase domain, does not inhibit ROMK. However, there may be other functional domains in the C-terminus of WNK4, because I found that WNK4 (584-1220) also inhibit ROMK [5th bar, Figure 2-6C]. Our unpublished data suggests those C-terminal functional domains in WNK4 are also proline-rich domains. With the functional domain in WNK being identified, next we will study the downstream mechanism by which WNK mediates the endocytosis of ROMK. Chapter 3 will focus on identifying the downstream players of WNK1 and WNK4 proline-rich domain, and elucidating how they team up with WNK kinases to stimulate the endocytosis of ROMK. WNK1 kinase domain participates in the regulation of ROMK, since kinase dead WNK1 failed to inhibit ROMK. However, WNK1 kinase domain did not inhibit ROMK by itself, rather, it could regulate the function of the proline-rich domain of WNK1 (1-119). The function of WNK1 kinase domain and the regulation of the proline-rich domain will be studied in detail in Chapter 4.

CHAPTER THREE: Intersectin links WNK kinases to endocytosis of ROMK

ABSTRACT

In the previous chapter, specific proline-rich domains (PRD) of WNK1 and WNK4 were found to inhibit ROMK through endocytosis. In the present study, intersectin (ITSN), an endocytic scaffold protein was identified to interact with PRD of WNK1 and WNK4. WNK1 binds with ITSN SH3-C and WNK4 binds with ITSN SH3-A, B and C. Disruption of specific PRDs in both WNK1 and WNK4 by mutations or truncations abolished their binding with intersectin. Knockdown of ITSN in HEK cell abolished the inhibition of ROMK by WNK1 and WNK4. The kinase domain of WNK4 bound specifically with ROMK, and WNK4 also bound with WNK1. The over-expression of WNK4 kinase domain prevented the inhibition of ROMK by both WNK1 and WNK4. PHAII mutations enhanced the interactions of WNK4 with both ITSN and ROMK, leading to an increase in the endocytosis of ROMK. Intersectin binds with endocytic proteins dynamin and N-WASP. WNK1 and WNK4 kinase regulation of ROMK depends on dynamin and N-WASP but not cdc42, a potent activator of N-WASP. Beyond recruitment, intersectin 1 short form, the ubiquitously expressed intersectin isoform, further activated the GTPase activity of dynamin and stimulated N-WASP-mediated actin polymerization with its SH3 domains. Finally, the localization of ITSN1 in the distal nephron supports its role in WNK-mediated endocytosis of ROMK *in vivo*. These results provide a molecular mechanism by which intersectin links WNK1 and WNK4 to endocytosis of ROMK.

INTRODUCTION

Clathrin-mediated endocytosis (CME) is the main pathway for internalization of membrane proteins, such as receptor, ion channel/membrane transporter and is essential for controlling cell signaling [Marsh M, 1999]. During CME, the clathrin coat is first assembled on the cytoplasmic face of the plasma membrane, followed by invagination into clathrin-coated pits; then vesicles were pinched off by scission and finally become free CCVs [Figure 3-1B]. In cultured cells, the assembly of a CCV takes about 1min, and several hundred to a thousand or more can form every minute. The main components of a clathrin coat are the 190 kD clathrin heavy chain (CHC) and the 25 kD clathrin light chain (CLC), which form three-legged trimers, called triskelions [Figure 3-1A]. Endocytosis via clathrin-coated vesicles (CCVs) is a complex process involving a team effort of many endocytic "accessory proteins". Complexity may also arise from the high demand for speed and efficiency required for the endocytic machinery. Furthermore, a diverse population of proteins including receptors, ion channels, and membrane transporters undergo clathrin-mediated endocytosis in both regulated and constitutive manner, which requires CME machinery to be highly specific [Marsh M, 1999].

SH3 domain containing proteins comprises a unique and important group of endocytic protein [Simpson F, 1999]. SH3 domains are conserved non-catalytic domains of 50-70 amino acids that have been shown to mediate protein-protein interactions by binding to short proline-rich domain [Figure 3-1C]. Being one of the most abundant protein modules found in eukaryotes, SH3 domain plays important roles in the cell, ranging from signal transduction to regulation of and endocytosis and cytoskeleton rearrangement [Li SS, 2005]. Proline-rich domains that interact with SH3 domains are also widely distributed in the proteomes of both prokaryotes and eukaryotes.

Why are the interaction between SH3 domain and PRDs favored for mediating CME? The answer to this intriguing question appears to lie within proline itself. Among the 20 naturally occurring amino acids, proline is a well-known breaker of regular secondary structures such as α-helices and β-sheets, which makes sure that proline-containing sequences are exposed to the solvent and be accessible as opposed to being buried within the core. Secondly, the closure of the side chain of proline in a five-member ring severely restrains the types of conformation that proline and proline-rich domain can adopt, thus ensured the specificity for interaction. The positive residues (usually R/K) flanking PXXP motifs provides additional energy and specificity for binding with SH3 domains. The interaction between the SH3 domain and PRD is fast (occur within seconds) and reversible. Besides these, the binding affinity ranges greatly with different sequence combination (*K*d 10nM-200μM) [Li SS, 2005]. Taken together, the unique features of SH3-domain and PRD provide many choices for protein interaction in terms of availability, selectivity, affinity, and reversibility. It is well suited for the diverse needs of CME and makes SH3-PRD interaction the most popular type of interaction in CME.

Ion channels and membrane transporters are abundant in kidney cells to maintain ionic homeostasis. Renal cells rely heavily on CME to regulate the abundance of membrane proteins, and expressed high level of various endocytic proteins. Among them, important endocytic proteins such as endophilin, amphiphysin, and intersectin (ITSN) all contain Src-homology 3 (SH3) domains to interact with PXXP proline-rich motifs [Figure 3-1C] [Simpson F, 1999].

Here we showed that WNK1 and WNK4 bound specifically with intersectin SH3 domains but not with those of endophilin and amphiphysin [Figure 3-2A] [He G and Wang HR, 2007]. As shown in Figure 3-3A, intersectin is a multi-modular scaffolding protein that regulates the formation of

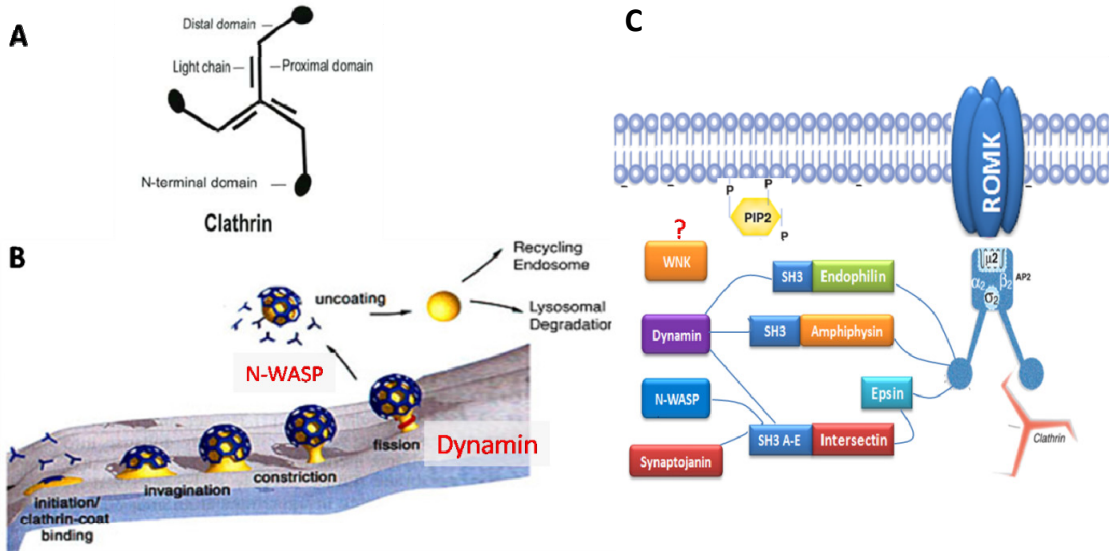

Figure 3-1. Clathrin-mediated endocytosis and the role of SH3 domain containing proteins.

Clathrin-mediated endocytosis (CME) is a major cellular pathway for internalization of membrane proteins like receptors and ion channels. During clathrin-mediated endocytosis, the clathrin coat is assembled on the cytoplasmic face of the plasma membrane, forming pits that invaginate to pinch off (scission) and become free clathrin-coated vesicles (CCVs). Clathrin-mediated endocytosis depends on two sets of proteins: those comprising the clathrin coat (such as AP2 and clathrin), and an array of other proteins often referred to as 'accessory' proteins (such as Intersectin, N-WASP and dynamin). The interaction between endocytic accessory proteins is often mediated by SH3 domains and Proline-rich domains.

clathrin-coated vesicles. Intersectin has two isoforms: a ubiquitously expressed Intersectin-short form (Intersectin-s) and a neuronal expressed intersectin-long form (intersectin-l). Both form of Intersectin have two Eps15 homology (EH), one central coiled-coil domain and five Src homology 3 (SH3) domains. EH domains target intersectin to the clathrin-coated pits. The central coiled-coil domain helps intersectin to form oligomers [Yamabhai M, 1998; Sengar AS, 1999]. Intersectin SH3 domains binds many endocytic proteins like dynamin, N-wasp and synaptotagamin to facilitate endocytosis. The neuronal intersectin-long form (intersectin-l), in addition, has a Dbl homology (DH) domain, a pleckstrin homology domain (PH) and a C2 domain. The DH domain of is a specific guanine-nucleotide exchange factor (GEF) for activating cdc42, a GTPase involved in cytoskeleton rearrangement [Hussain NK, 2001; Sengar AS, 1999]. Activated cdc42 could then induce actin polymerization by activating the N-WASP proteins that bind with intersectin SH3 domains. The PH domain and C2 domain may function to bind PIP2 and Ca++.

The Drosophila form of intersectin (Dynamin Associating Protein 160kD, DAP160) is an ortholog of mammalian short intersectin. DAP160 is required for synaptic vesicle endocytosis. Knockout of DAP160 in Drosophila greatly reduced the levels of dynamin, synaptojanin, and endophilin, causing severe defects in vesicle endocytosis [Koh TW, 2004; Marie B, 2004].

Intersectin binds tightly with dynamin, an endocytic protein essential for clathrin-mediated endocytosis, caveolae internalization, synaptic vesicle recycling etc.[De Camilli P, 1995]. Dynamin is a 100-kDa GTPase. In addition to the N-terminal GTPase domain, dynamin also contains: a pleckstrin homology domain (PH) that binds with PIP2 in the membrane, a GTPase effector domain (GED) essential for self-assembly and GTPase activation, and a proline-rich domain (PRD) containing several SH3-binding sites. The GED domain of dynamin plays the major

role in activating dynamin GTPase activity, which is further facilitated by dynamin interaction with SH3 domain containing proteins and PIP2 [Barylko B, 1998]. Upon binding with SH3 domain containing proteins, dynamin could readily self-assemble into ring like structure and hydrolyze GTP, which would cause conformation changes and generate forces. This unique property enables dynamin to wrap around the necks of budding vesicles and play a key role in vesicular fission [De Camilli P, 1995].

Neuronal Wiskott–Aldrich syndrome protein (N-WASP) is another binding partner of intersectin. N-WASP belongs to the WASP_WAVE protein family and functions as an important cytoskeleton regulator in a number of cellular processes, including endocytosis, vesicle trafficking, and actin-based motility of intracellular pathogens. N-WASP controls actin network growth by regulating Arp2/3 complex-mediated actin-filament nucleation [Snapper SB, 1999]. In its resting state, N-WASP exists in an auto-inhibited conformation, wherein the verprolin–cofilin–acidic (VCA) domain in the C terminus of N-WASP is masked by an intramolecular interaction with the N-terminal CRIB (Cdc42/Rac-interactive-binding) domain. Structural and biochemical studies have shown that upon coordinated binding of PIP2 and Cdc42-GTP to the basic domain and GBD of N-WASP, respectively, a dramatic conformational change ensues, resulting in a release of the C-terminal part of N-WASP and an activation of the bound Arp2/3 complex [Rohatgi R, 1999]. Recently, Src homology 3 (SH3)-containing proteins like Grb2 [Carlier MF, 2000] and syndapin [Kessels MM, 2006] interaction with the proline-rich region of N-WASP was also found to be activating N-WASP independent of Cdc42-GTP and PIP2.

When the N-WASP nucleates actin polymerization, these newly cell formed actin filaments could be tethered to the clathrin-coat pits and generate mechanical forces to facilitate membrane

invaginations, create vesicles, and propel newly formed endocytic vesicles away from the plasma membrane. Through the study of N-WASP and many other actin related proteins, such as intersectin, there is increasing evidences highlighting the importance of the actin cytoskeleton for clathrin-mediated endocytosis [Schafer DA, 2002]. Actin skeleton is no longer viewed as a passive barrier that must be removed to allow endocytosis to proceed. Rather, actin structures are dynamically organized to assist in the remodeling of the cell surface to facilitate endocytosis.

In this chapter, I will focus on the role of intersectin, dynamin and N-WASP in WNK-mediated endocytosis of ROMK.

MATERIAL AND METHODS

Molecular biology. Plasmids encoding GST fusion proteins containing SH3 domains of ITSN (*Xenopus laevis*), endophilin (human), and amphiphysin (human; all gifts of P. McPherson, McGill University, Montreal, Quebec, Canada) have also been described previously (26). GST- and H6-tagged bacterial fusion proteins containing the C terminus of ROMK and IRK1 have been described previously. Sense oligonucleotide for N-WASP siRNA AAGACGAGAUGCUCC AAAUGG. Sense and antisense oligonucleotide for ITSN siRNA were 5GGACAUAGU UGUACUGAAAUU and 5-PUUUCAGUACAACUAUGUCCUU, respectively.

Patch-clamp recording and cell culture as described in Chapter 2.

Pull down assays. HEK293 cells were cultured and transfected as described previously (13). For the pull down assay of WNK1 and WNK4 fragments by SH3 domains, cells transfected with myc-tagged WNK plasmids were lysed in PBS buffer (1 ml per 6-well dish) containing phosphatase and protease inhibitors. Cell lysates (500 μg) were incubated with GST fusion proteins of interest (1 μg in 0.5 ml final volume), and precipitated by glutathione-4B sepharose beads. Pulldown of WNKs was examined using an anti-myc antibody. Fusion proteins were detected by an anti-GST antibody. For pulldown of full-length ROMK by H6-tagged WNK4 fragments, cells transfected with GFP-ROMK were lysed in PBS containing 1% Triton X-100. Pulldown of GFP-ROMK was examined using anti-ROMK antibody. Pulldown of HA-tagged mouse ITSN1 SH3A–SH3E (gift of J. O'Bryan, University of Illinois at Chicago, Chicago, Illinois, USA) by H6-tagged WNK4 fragments was examined using an anti-HA antibody. WNK4 fragments were detected by an anti-H6 antibody. Endogenous WNK1 and ITSN1s were detected using anti-WNK1 antibody (gift of M.

Cobb) and anti-ITSN1 antibody (gift of J. O'Bryan), respectively. The anti-ITSN1 antibody was raised against SH3 domains of mouse ITSN1.

Immunofluorescent staining. Sprague-Dawley rats (approximately 250 g, of either sex) were anesthetized and perfused via the heart with 100 ml PBS followed by 50 ml 4% paraformaldehyde in PBS. Kidneys were harvested, fixed in 4% paraformaldehyde at 4°C for 4 hours, dehydrated by immersion in 30% sucrose in PBS at 4°C overnight, and mounted in OCT (Tissue-Tek; Sakura) for sectioning. Sections (3–5 μm) were dried at 37°C for 30 minutes, treated with 0.1% Triton for 10 minutes at room temperature, and quenched in 0.1% NaBH4 for 30 minutes at room temperature followed by extensive washing in PBS. After blocking with 10% goat or donkey serum in PBS for 2 hours, sections were incubated overnight at 4°C with primary antibodies, which included rabbit polyclonal antibodies against ITSN1 (1:200; gift of J. O'Bryan), mouse monoclonal antibodies against NCX1 (1:400; gift of K. Philipson, University of California at Los Angeles, Los Angeles, California, USA), and goat polyclonal antibodies against AQP2 (1:200; Santa Cruz Biotechnology Inc.). After washing, specimens were incubated with secondary antibodies Alexa Fluor 568– conjugated goat anti-rabbit (diluted 1:500; Invitrogen) or fluorescein-conjugated donkey anti-goat (diluted 1:500; Jackson ImmunoResearch Laboratories Inc.) for 30 minutes at room temperature. Fluorescent images were obtained using a Nikon Eclipse TE2000-U fluorescent microscope and overlaid with differential interference contrast images, as we have previously described [Chu PY, 2003]. All procedures were performed in compliance with relevant laws and institutional guidelines and were approved by the University of Texas Southwestern Medical Center at Dallas Institutional Animal Care and Use Committee. The procedure for immunostaining of Intersectin (using intersectin antibody) and myc-WNK4(using mouse myc antibody) in HEK cell are similar as described above, except that HEK cells are grown on the coverslips.

Actin Staining and quantitation. In 24 wells dish, half confluent NIH 3T3 sells was transfected with GFP, GFP and intersectin 1 short form, HA-intersectin SH3 A-E domain, and GFP-intersectin DH-PH domain. 24hrs after transfection, cells were transferred to grow a monolayer on sterile coverslips till half confluent. Coverslips were then move to a clean dish for staining. Cells were washed twice with PBS and fixed by 4% Paraformaldehyde/PBS for 10min. Cells were then washed in PBS 3 times for each 5 min followed by 0.5% Triton X-100/PBS 10min to permeablize the plasma membrane. Wash cells in PBS 3 times for each 5 min. Nonspecific binding were blocked by incubating cells in 1% BSA PBS for 30min. Alexar 564-phalloidin(sigma) were diluted to 10uM and incubated with cells for 1hour at room temperature. Then cells were washed twice with PBS and mounted to a slides. Fluorescent images were obtained using Zeiss LSM 510 confocal microscopy. The intensity of actin signal was quantitated in Adobe photoshop CS2 by circling the GFP transfected cells, and read the median intensity was read by the software. 20-60 cells were usually counted per group.

Statistics. Statistical comparisons between 2 groups of data were made using a 2-tailed unpaired Student's *t* test. Multiple comparisons were made using 1-way ANOVA followed by 2-tailed Student's *t* test adjusted for multiple comparisons. *P* values less than 0.05 and 0.01 were considered significant for single and multiple comparisons, respectively.

RESULT

WNK 1 and WNK4 recruit endocytic protein--- intersectin.

WNK 1 and WNK4 kinase both have multiple PXXP type proline-rich motifs that may interact with SH3 domains. Through the domain screening experiments done in the last chapter, I found out that the functional domains of both WNK1 and WNK4 lie in a certain proline-rich domains.

We hypothesizes that the PRDs of WNK1 and WNK4 stimulate clathrin-mediated endocytosis of ROMK by interacting with some SH3-containing endocytic proteins. We then examined interactions between WNK1/WNK4 and SH3 domains of intersectin, endophilin, and amphiphysin. Amino acids 1–491 of WNK1 and amino acids 1-584 of WNK4 are sufficient for regulation of ROMK, and they were used in GST pull down assay. The SH3 domains of amphiphysin, endophilin and ITSN1 SH3A–SH3E domains were fused to glutathione S-transferase (GST) in a bacterial expression vector respectively. The SH3 domains were then purified from *E.coli* and used to pull down myc-tagged WNK1 (1–491) and WNK4 (1-584) from lysates of transfected HEK cells. We found that only ITSN1 SH3C pulled down WNK1 1–491 [Done by He G, Figure 3-2A]. The interaction between SH3 C and WNK1 is very specific, since all the other SH3 domains tested were not able to pull down WNK1. Similarly, amino acids 1–119 of WNK1, which inhibit ROMK, also bound to ITSN1 SH3C. Dr. He G in the lab further showed that the non-functional triple PXXP mutant (P94A/P103A/P114A) of WNK1 (1–491) failed to interact with ITSN1 SH3C (data not shown [He G and Wang HR, 2007]). Thus, the N-terminal proline-rich domain of WNK1 is crucial for binding ITSN SH3-C and regulating of ROMK endocytosis.

In comparison, WNK4 (1–584) interacted with ITSN1 SH3A, SH3B, and SH3C specifically [Figure 3-2B, done by He G [He G and Wang HR, 2007]]. ITSN SH3D and SH3E and the SH3 domains of

amphiphysin and endophilin did not interact with WNK4(1–584) even at much higher concentrations. Consistently, the previously tested non-functional WNK4 triple PXXP mutants [Figure 2-7C], failed to interact with ITSN SH3A, SH3B, or SH3C(data not shown). Furthermore, WNK4 disease-causing mutant E599K, D561A and Q562E pulled down more of the SH3 domain of ITSN than did wild-type WNK4 (data not shown) (The data above was done by He G [He G and Wang HR, 2007]). Thus, the regulation of ROMK endocytosis by both WNK1 and WNK4 correlated well with their interaction with intersectin SH3 domain(s).

Analysis of intersectin family expressed in HEK cell

There are two types of intersectin: intersectin 1 and intersectin 2, and each type of intersectin has a long and a short alternative splicing form. Our previous data showed that siRNA of intersectin 1 or dominant negative form of intersectin 1 prevented WNK1 and WNK4 inhibition of ROMK. The siRNA of intersectin is designed to target the highly conserved SH3 domains present in both intersectin long and short isoforms. We then asked the question: what is the endogenous form of intersectin in HEK cells? I designed four pairs of primers targeting to the variable sequences in the SH3 domains of the short form of intersectin 1 and intersectin 2, and the variable regions in DH domain of long form of intersectin 1 and intersectin 2. The RT PCR result showed that all 4 of intersectin isoforms were present in the HEK cells [Figure 3-3B]. We also did western blotting of HEK cell lysate using an intersectin antibody. The size of endogenous intersectin in HEK cell is comparable to the exogenously expressed intersectin 1 short form [Figure 3-3C]. Quantitive-PCR also showed that intersectin 1 short form is the most abundant form of intersectin expressed in both HEK cells and mouse kidneys (data not shown).

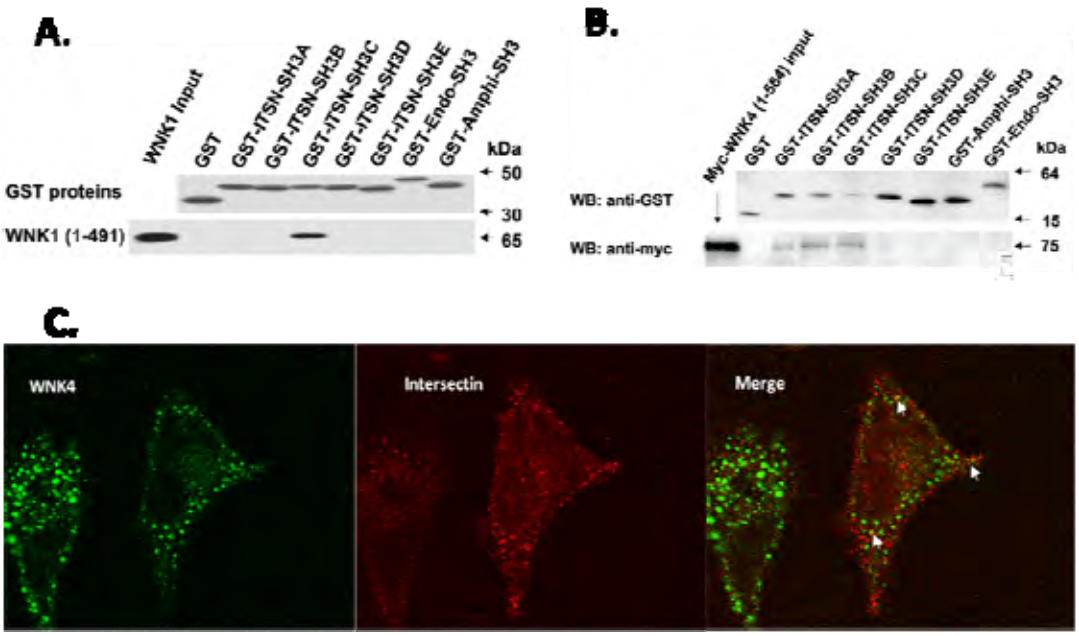

Figure 3-2. Intersectin SH3 domain(s) binds with WNK1 and WNK4.

A. Amino acids 1–491 of long WNK1 interacted specifically with the ITSN SH3 C domain. GST fusion proteins were used to pull down myc-tagged WNK11–491 from transfected cell lysates. Western blot analysis was performed with anti-GST and anti-myc antibodies,respectively. Amphi, amphiphysin; Endo, endophilin.

B. WNK4 (1–584) interacted with ITSN SH3A, SH3B, and SH3C. GST fusion proteins were used to pull down myc-tagged WNK4 (1–584) from lysates of transfected cells.

C. D. E. Immunostaining of transfected myc-WNK4 **C**, endogenous intersectin **D** and merged image **E.** White arrows indicate co-cocalization of myc-WNK4 (green) and endogenous intersectin (red)

Note: Figure A and B are done by Guocheng He [He G and Wang HR, 2007]

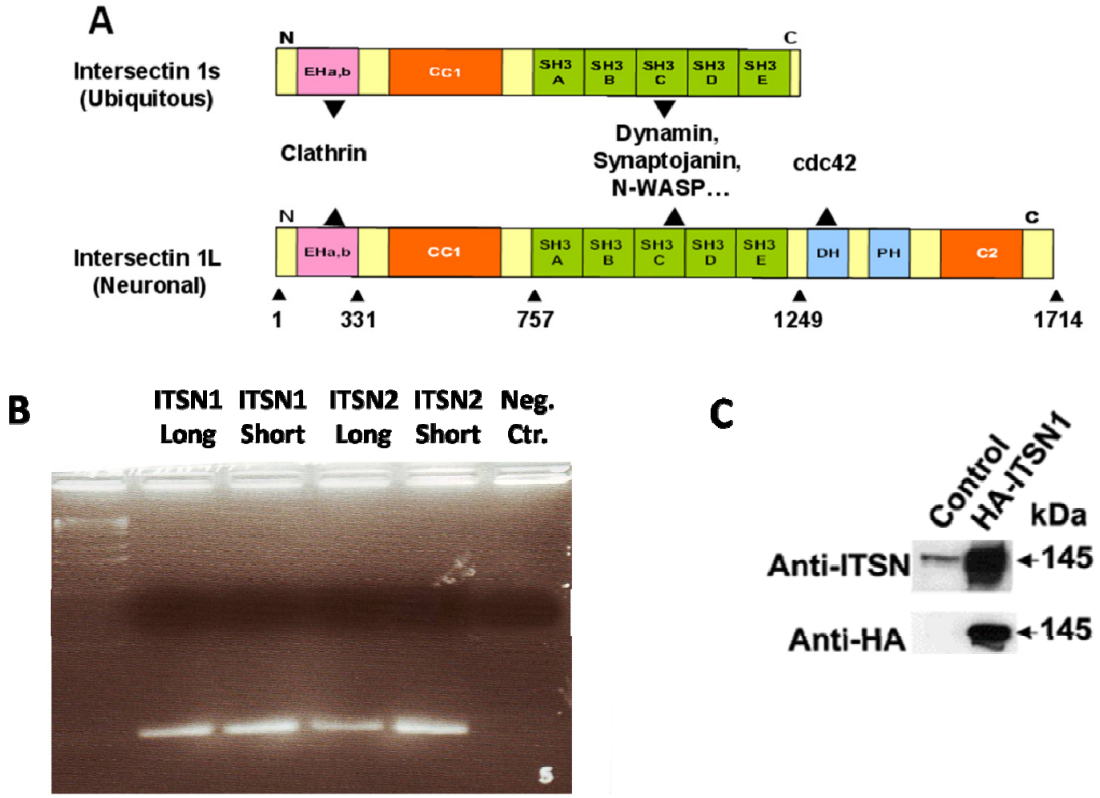

Figure 3-3. Intersectin family and their expression in HEK cells.

A. There are two intersectins, intersectin 1 and intersectin 2, each encoded by a separate gene. In addition, each intersectin has alternative spliced forms long and short forms. Both long and short isoform share identical sequences in the N-terminal: two EH domain in the N-terminus for binding with clathrin, one central coiled coil domain for intermolecule dimerization and five SH3 domains. Intersectin 1 long form also has a DH domain that function as a GEF for cdc42, a PH domain for binding with PIP2 in the membrane and a C2 domain.

B. Reverse-Transcription PCR showed that intersectin 1L, 1S and intersectin 2L, 2S are all transcripted in the HEK cells.

C. Western blotting of endogenous intersectin 1s in HEK cells compared with transfected HA-tagged intersectin 1s. (Done by He G, [He G and Wang HR, 2007])

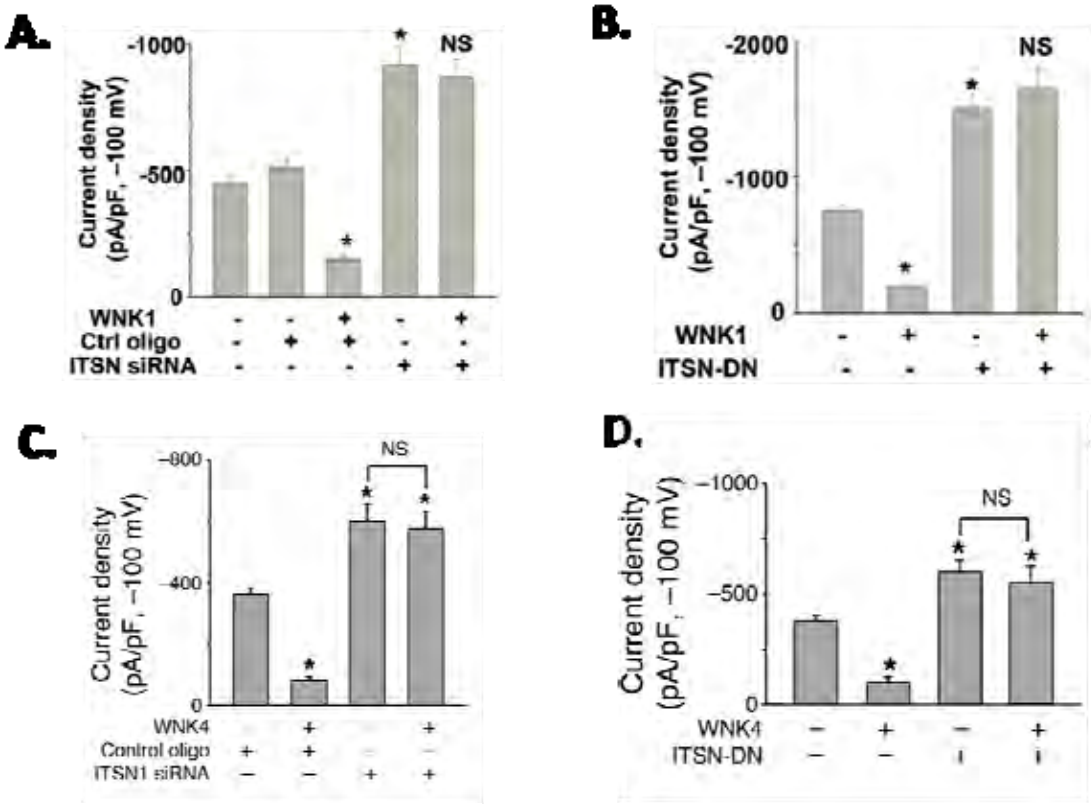

Figure 3-4. ITSN is crucial for WNK1 and WNK4 inhibition of ROMK

A. Knockdown of ITSN1 increased baseline whole-cell ROMK current density and prevented the decrease caused by WNK1(1–491). Cells were transfected with ROMK plus indicated constructs.

B. Over-expression of dominant-negative ITSN1 increased baseline whole-cell ROMK current density and prevented the decrease caused by WNK1(1–491).

C. A dominant-negative ITSN1 containing SH3A–SH3E increased baseline ROMK currents and prevented the decrease caused by WNK4 (1–584).

D. Knockdown of ITSN1 increased baseline ROMK currents and prevented the decrease caused by WNK41–584. *$P < 0.05$ versus ROMK alone.

Recruiting intersectin is crucial for inhibition of ROMK by WNK1 and WNK4

The importance of interaction with ITSN in regulating the surface abundance of ROMK by WNK1 was further examined by using siRNA to knock down endogenous ITSN. Knockdown of endogenous ITSN1s in HEK cells by siRNA was evident from Western blot analysis using an anti-ITSN antibody (data not shown). As shown in Figure 3-4A, ITSN1 siRNA coexpression increased basal ROMK current (in the absence of exogenous WNK1) and prevented the inhibition of ROMK by exogenous WNK1. The result was further confirmed by surface biotinylation of ROMK channel: co-transfection with siRNA for ITSN1 (but not control oligonucleotides) increased the surface abundance of ROMK (data not shown, done by He G) [He G and Wang HR, 2007]. Overexpression of ITSN1 SH3A–SH3E produced a dominant-negative effect that also prevented WNK1 inhibition of ROMK [Figure 3-4B]. The functional importance of WNK4-ITSN interaction in the regulation of ROMK was also demonstrated by the finding that co-expression of ITSN1 siRNA prevented WNK4 inhibition of ROMK [Figure 3-4D]. Similarly, co-transfection with ITSN1 SH3A–SH3E also abolished WNK4 inhibition of ROMK [Figure 3-4C]. These results suggest that intersectin is critical for WNK1 and WNK4 to stimulate endocytosis of ROMK.

The function of WNK4 kinase domain

Previously we found that WNK4 (1–584), but not WNK4 kinase domain (amino acids 1–444) decreased ROMK currents [Figure 2-7B]. In contrast to WNK4 (1–584), WNK4 (1–444) did not interact with ITSN (by He G). It has previously been reported that WNK4 interacts with the C terminus of ROMK [Kahle AT, 2003]. We found that WNK4 (1–444) interacted with a GST fusion protein containing the C terminus of ROMK. Disease-causing mutants of WNK4 Disease-causing interacted with ROMK much more strongly than did wild-type WNK4(1–584) (by He G, [He G and Wang HR, 2007]).

To confirm this interaction, I study the co-localization of GFP-ROMK and myc-WNK4 in HEK cells. GFP-ROMK and myc-WNK4 (1-584) were transfected into HEK cells. WNK4 was incubated with rabbit anti-myc antibody and Alexar564-labeled goat-anti-rabbit secondary antibody. As shown in Figure 3-5, the *puncta* staining of WNK4 (1-584) colocalized well with GFP tagged ROMK indicated by the bright yellow color in Figure 3-5C and F. This result suggests that ROMK and WNK4 also interact *in vivo.*

During experiments, we observed a dominant-negative-like effect of WNK4 (1-444). As shown in Figure 3-6A, the expression of WNK4 (1-444) at 1μg/35mm dish slightly increase ROMK current. Over-expressed of WNK4 (1-444) at 2ug and 3ug/35mm dish significantly increased of ROMK current density. We hypothesize that the dominant-negative effect of WNK4 (1-444) is due to competition with endogenous WNKs for binding site(s) in the C-terminus of ROMK. To support this hypothesis, overexpression of WNK4 1–444 (3μg) reversed the inhibition of ROMK caused by an exogenously expressed WNK1 (1–491) or WNK4 (1–584) [Figure 3-6B] (The control groups were all balanced with 3μg DNA vectors).

In addition, WNK4 binds specifically to ROMK, but not to IRK1 channel, which has approximately 40% amino acid sequence identical to ROMK (by He G, [He G and Wang HR, 2007]). Neither WNK4 (1–584) nor WNK1 (1-491) inhibit IRK1 currents [Figure 3-6C]. These results suggest that WNK4 binding to ROMK C-terminus is important for WNK4 and WNK1 to specifically regulate ROMK.

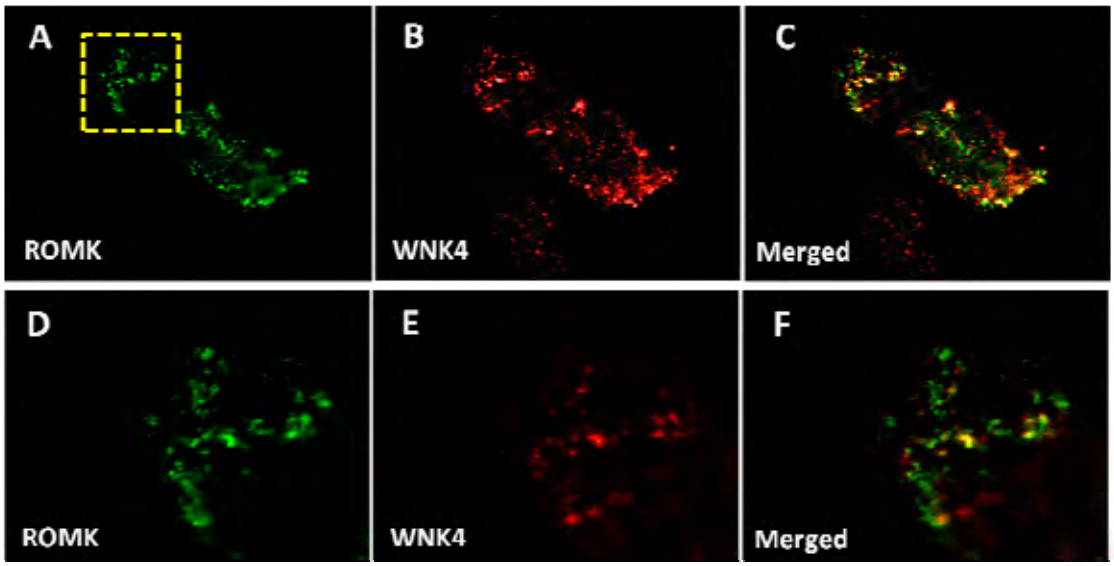

Figure 3-5. WNK4 kinase domain interacts with ROMK

B. C. Co-localization of GFP-ROMK(Green) with WNK4(Red). WNK4 was stained with rabbit anti-myc antibody and Alexar 564 labeled goat-anti-rabbit secondary antibody subsequently. merged image of ROMK and WNK4 (1-584). The bright yellow color in **F.** indicates the co-localization of ROMK and WNK4.

D . E. F. The amplified view of the yellow rectangle shown in figure **D.**

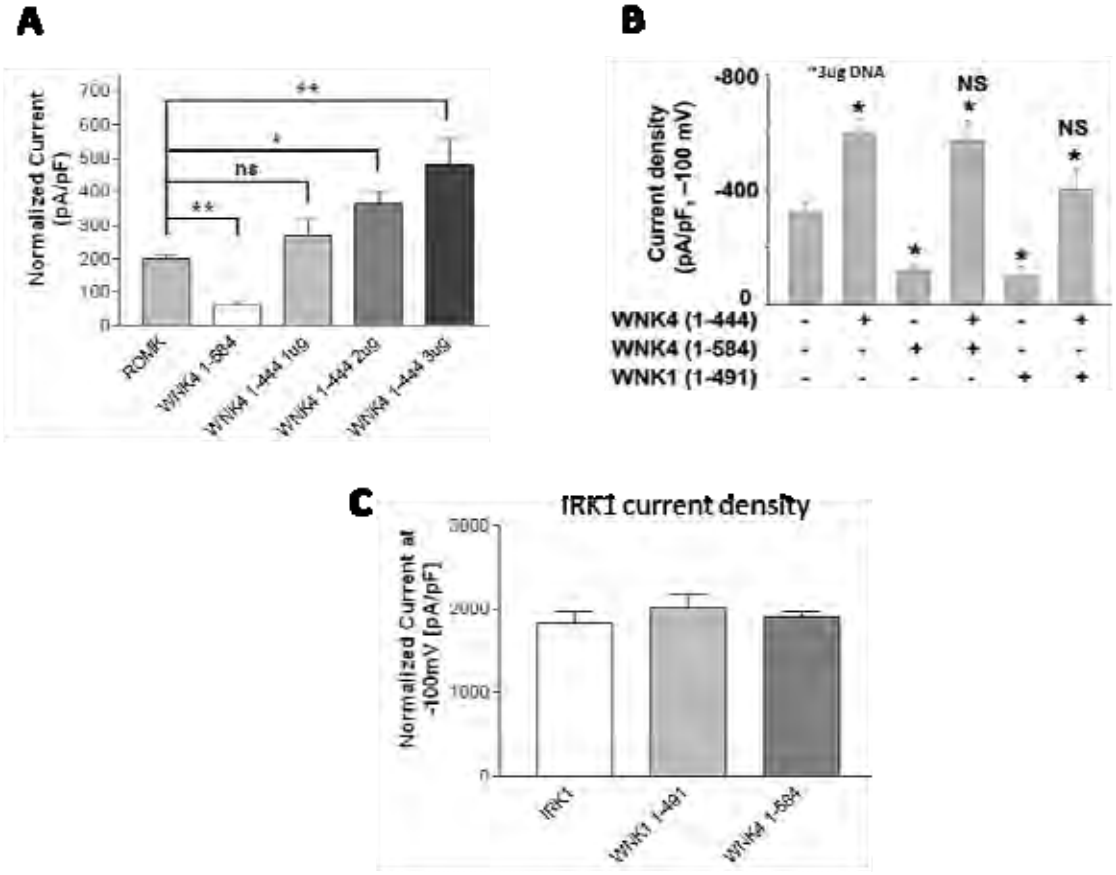

Figure 3-6. The function of WNK4 kinase domain

A. The dose response of WNK4 kinase domain (1-444) exhibited dominant negative effect on ROMK. When over-expressed in large amount (~3ug), WNK4 (1-444) significantly increases ROMK basal current. All the groups were balanced with vector DNA.

B. Effects of various combinations of WNK1 and WNK4 constructs on ROMK currents. Over-expression of WNK1 (1-444) (~3ug) antagonized the inhibitory effects of both WNK1 and WNK4. †P < 0.01 versus ROMK alone. NS, not significantly different versus ROMK plus WNK4 1–444. Modified statistics adjusted for multiple comparisons were used (see Methods).

C. WNK1 and WNK4 had no effects on IRK1 current density. It is reported by He G *et. al.* that WNK41–584 interacts with the C terminus of ROMK but not with the C terminus of IRK1. H6-tagged ROMK and IRK1 C termini were used to pull down myc-tagged WNK11–584 from transfected cell lysates. Western blot analysis of proteins was performed using anti-H6 and anti-myc antibody. WNK4 1–584.

WNK1 and WNK4 inhibition of ROMK requires N-WASP but not cdc42

Recently, the contribution of actin polymerization to endocytosis has received more recognition [Kaksonen M, 2005, 2006]. N-WASP is a major protein to promote actin polymerization. Recent studies confirmed that N-WASP contributes to clathrin-mediated endocytosis by polymerizing actin cytoskeleton to generate the driving force necessary for the completion of endocytosis fission [Kaksonen M, 2005].

I found that WNK1 and WNK4 inhibition of ROMK also depends on N-WASP. As shown in Figure 3-8 A/B, when co-expressed with N-WASP siRNA (WASL), both WNK1 and WNK4 lost inhibition of ROMK. WNK1 and WNK4 co-expressed with or without control siRNA oligo can still inhibit ROMK, suggesting that the expression of WNK1 and WNK4 was not affected by siRNA co-transfection. Furthermore, I also tested the effects of WNK1 and WNK4 on ROMK in 7R cells, a N-WASP deficient cell line (N-WASP-/-) immortalized from N-WASP knockout mouse [Gift from Yin HL]. Both WNK1 and WNK4 failed to inhibit ROMK in R1 cell lines, though they can still inhibit ROMK in the control cell line (N-WASP +/-, 1R). [Figure 3-8 C-D].

Usually, N-WASP is kept in an autoinhibited state through an intramolecular association of GBD domain with its C-terminus, which prevents the C-terminal VCA from interacting with the Arp2/3 complex to stimulate actin polymerization. Cdc42, a GTPase, together with PIP2 were thought to be the major regulators of N-WASP. Cdc42 binds with GBD domain and dissociate it from VCA domain, which is facilitated by PIP2 binding to certain basic residues in the N-WASP [Rohatgi R, 1999]. Upon activation, the VCA domain of N-WASP recruits the Arp2/3 complex to mediate actin polymerization.

The DH domain of the long form intersectin is found to be a specific Guanine nucleotide Exchange Factor (GEF) for cdc42 [Hussain NK, 2001]. Although long form intersectin has been

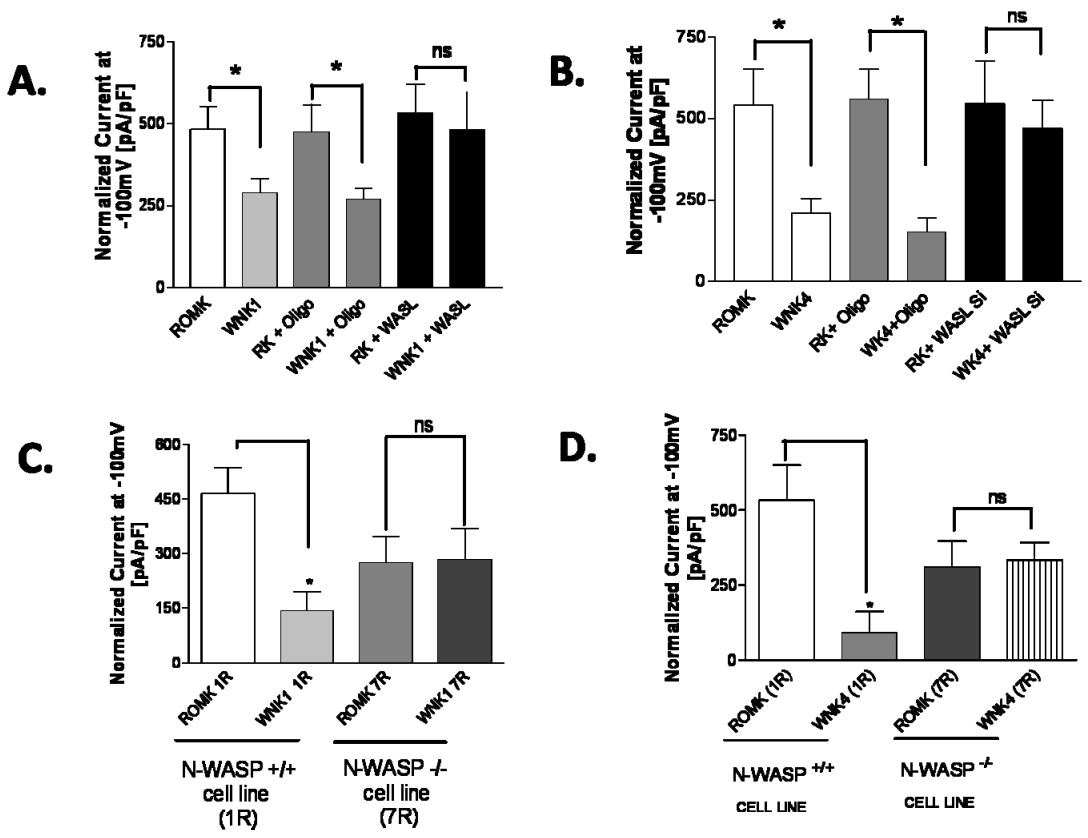

Figure 3-7. N-WASP is required for WNK1 and WNK4-mediated stimulation of endocytosis of ROMK.

- **A.** siRNA knockdown of N-WASP gene (WASL) prevented the inhibition of ROMK by WNK1, whereas WNK1 still inhibit ROMK in groups transfected without siRNA or transfected with scramble oligo RNA.
- **B.** siRNA knockdown of N-WASP gene (WASL) abolished WNK4 inhibition of ROMK as well.
- **C.** WNK1 failed to inhibit ROMK in N-WASP deficient cell line: 7R cells, but not in control cell line: 1R cells.
- **D.** WNK4 failed to inhibit ROMK in 7R cells (N-WASP -/-) , but not in 1R cells: 1R cells (N-WASP+/+).

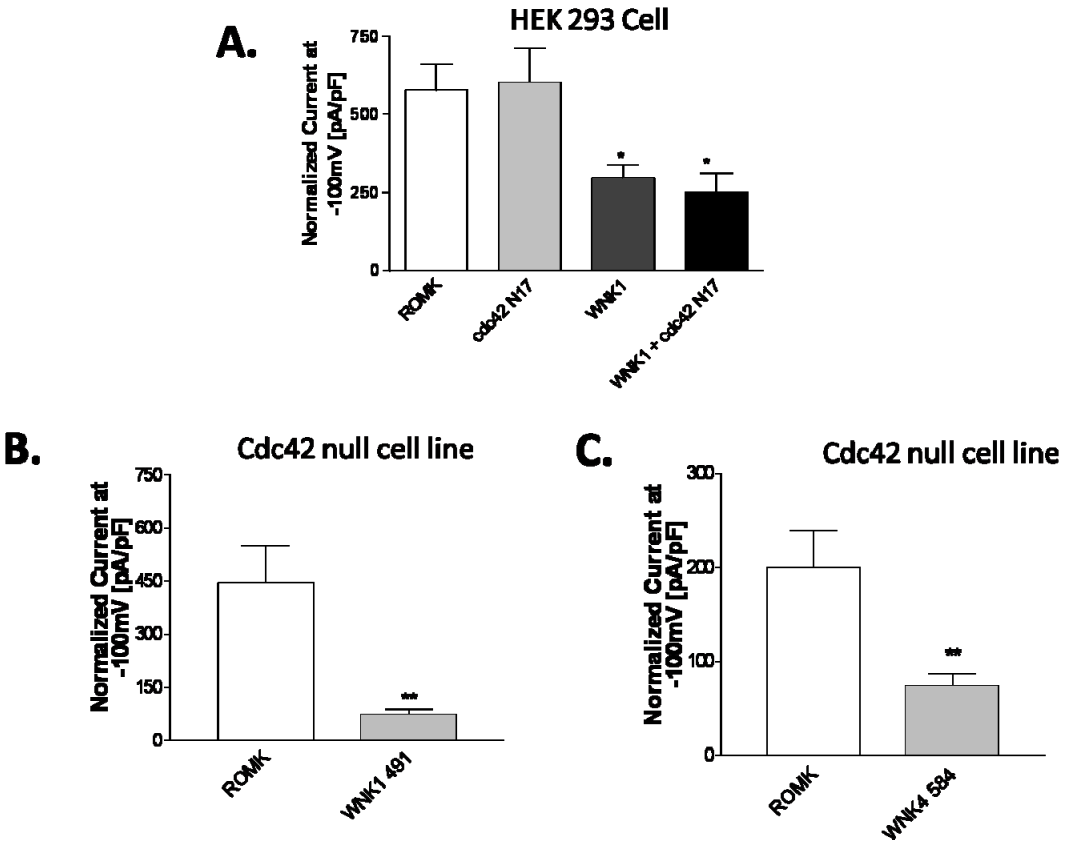

Figure 3-8. Cdc42 is not required for WNK1 and WNK4-mediated stimulation of endocytosis of ROMK.

- **A.** Dominant negative form of cdc42 N17 did not prevent WNK1 inhibition of ROMK.
- **B.** WNK1 (1-491) still inhibit ROMK in cdc42 null cells.
- **C.** WNK4 (1-584) retained inhibition of ROMK in cdc42 null cell line.

reported to be expressed exclusively in the neuronal cell [Hussain NK, 2001], our RT-PCR result suggested that the long form intersectin may be expressed in HEK cell. It is appealing to hypothesize that WNK kinase may recruit intersectin long form which activates cdc42 and subsequently activates N-WASP to facilitate endocytosis of ROMK. We then test to see if cdc42-mediated N-WASP activation is important for WNK-mediated endocytosis of ROMK. Cdc42 N17 is a dominant-negative form of cdc42. Co-expressing cdc42 N17 with WNK1 did not prevent WNK1 inhibition of ROMK, suggesting that cdc42 may not be involved in WNK-mediated endocytosis of ROMK [Figure 3-8 A]. In line with this result, WNK1 and WNK4 are still able to inhibit ROMK in a cdc42 null cell line [Figure 3-8 B-C]. Thus, WNK1 and WNK4 kinase can inhibit ROMK without cdc42. How could N-WASP get activated if it is required for WNK regulation of endocytosis of ROMK?

Intersectin short form activates N-WASP and actin polymerization

Recently, SH3 containing proteins were also reported to activate N-WASP independent of cdc42 and PIP2 [Carlier MF, 2000; Kessels MM, 2006]. Multiple SH3 domains, linked together at a proper distance, is proposed to be a mechanism for activating N-WASP. Each molecule of intersectin binds 3 molecules of N-WASP, and intersectin also forms dimer through the central coiled coil domain, which makes it a good candidate to activate N-WASP [Hussain NK, 2001]; however, a role for intersectin SH3 domains in activating N-WASP has not been reported. Once being activated, N-WASP will recruit Arp2/3 complex to promote actin polymerization, which produces actin filaments (F-actin) [Hussain NK, 2001]. We utilized Alexar 564 labeled phalloidin, a toxin that binds specifically to F-actin, to stain NIH 3T3 cells transfected with GFP, GFP+ intersectin short form, GFP+ intersectin SH3 domain A-E, and GFP tagged intersectin DH- PH domain [Figure 3-9].

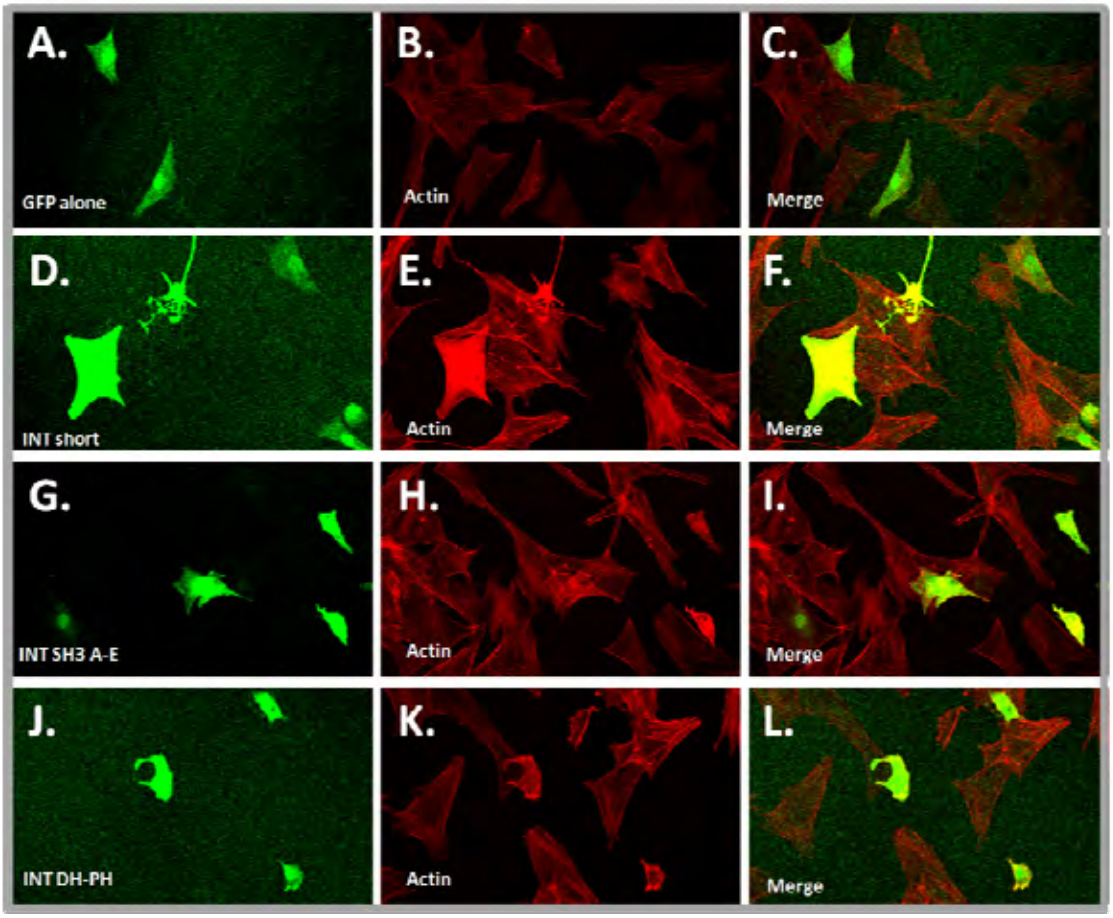

Figure 3-9. Intersectin enhances actin polymerization in NIH 3T3 cells.

A.B.C. GFP transfected cell

D.E.F. Intersectin 1 short form transfected cells

G.H.I. Intersectin SH3 A-E domain transfected cells

J.K.L. Intersectin DH-PH domain transfected cells

The figures are representative images for each group. Left panel (green) are GFP channel representing transfected cells. Middle panel (red) are F-actin stained by Alexar 564 labeled phalloidin. Right panel are the merged images.

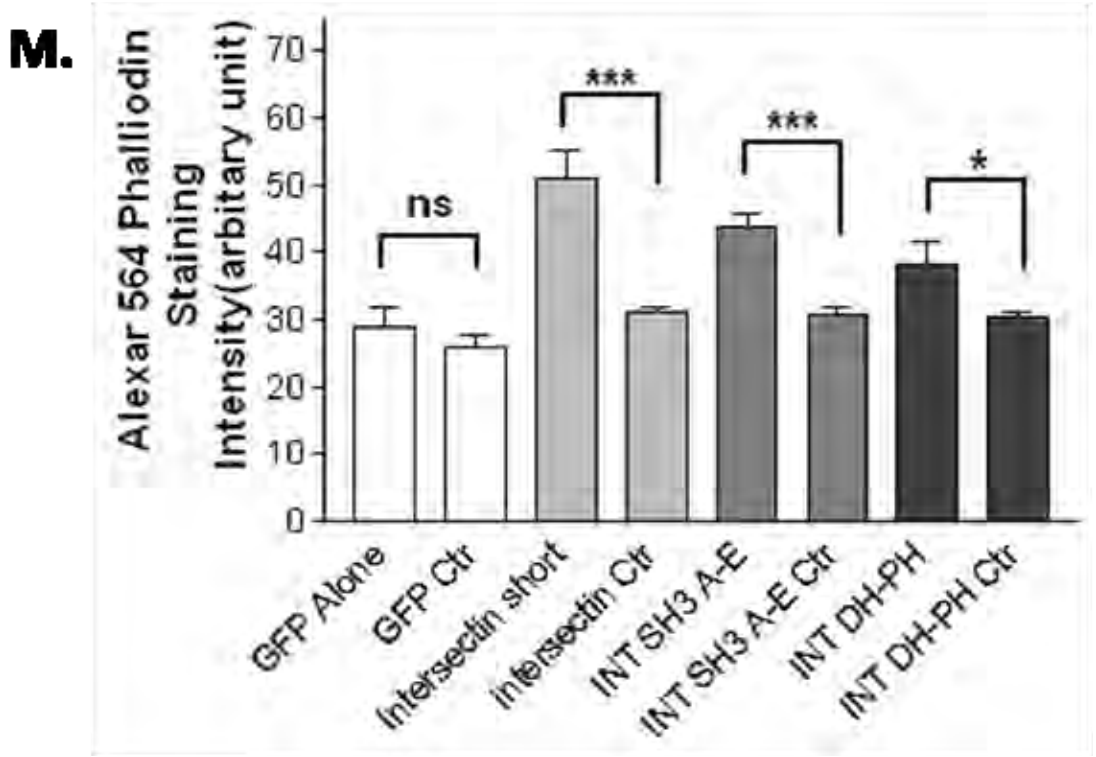

Figure 3-9. Intersectin enhances actin polymerization in NIH 3T3 cells.

M. Quantitation of the F-actin signals in transfected cells.

GFP transfection did not affect F-actin intensity. Overexpression of intersectin 1 short form increased F-actin intensity most dramatically. Intersectin SH3 A-E domains also increased F-actin intensity but with a less effect compared with control. Intersectin DH-PH domain is used as positive control, which activates cdc42 activity and indirectly activate N-WASP-mediated actin polymerization.

We then compare the signal intensity of visualized F-actin in transfected cell and non-transfected cell to quantitate the induced actin polymerization [similar methods used by Kessels MM, 2006]. Figure 3-9 are representative confocal image of cells in each groups. Figure A. D. G. J represent the GFP signal of transfected cells; figure 3-9 B., E., H., K. are image of the actin skeleton stained by Alexar-564 labeled phalloidin; figure 3-9 C.,F., I., L. are the merged images of the previous two images. Figure 3-9M is the quantitation of the actin signal intensity in each groups compared with the adjacent non-transfected cells. From the representative figures and the quantitated signals, intersectin short form and SH3 domains significantly increased the F-actin production, whereas GFP transfection itself does not affect F-actin amount. Intersectin DH-PH domain, which functions as positive control, increased the F-actin production, presumably by activating cdc42 [Hussain NK, 2001]. Surprisingly, in comparison with the effect of DH-PH domain, intersectin SH3 A-E domains seems to function even better to activate N-WASP. The central coiled-coil domain may potentiate the effect of SH3 domains, since full length intersectin 1s performs best in activating actin polymerization. The stimulation of actin polymerization by intersectin depend on N-WASP, since intersectin 1s and SH3 domains or DH-PH domain does not stimulate actin polymerization in N-WASP null cell lines (data not shown).

Intersectin SH3 domains activate dynamin 1 and dynamin 2 GTPase activity in vitro

Dynamin is a GTPase protein [De Camilli P 1995]. Under normal conditions, dynamin is in its inactive state. It has been recognized that the major GTPase Activator Protein or GAP for dynamin is dynamin itself, although recently, the phox domain of PLD turned out to be another GAP for dynamin. SH3 domain containing proteins like Grb2 forms a dimer, and Grb SH3 domain binding with dynamin brings multiple dynamin molecules close enough to activate each other. PIP2 binding to dynamin also synergistically activate dynamin GTPase activity [Barylko B, 1998].

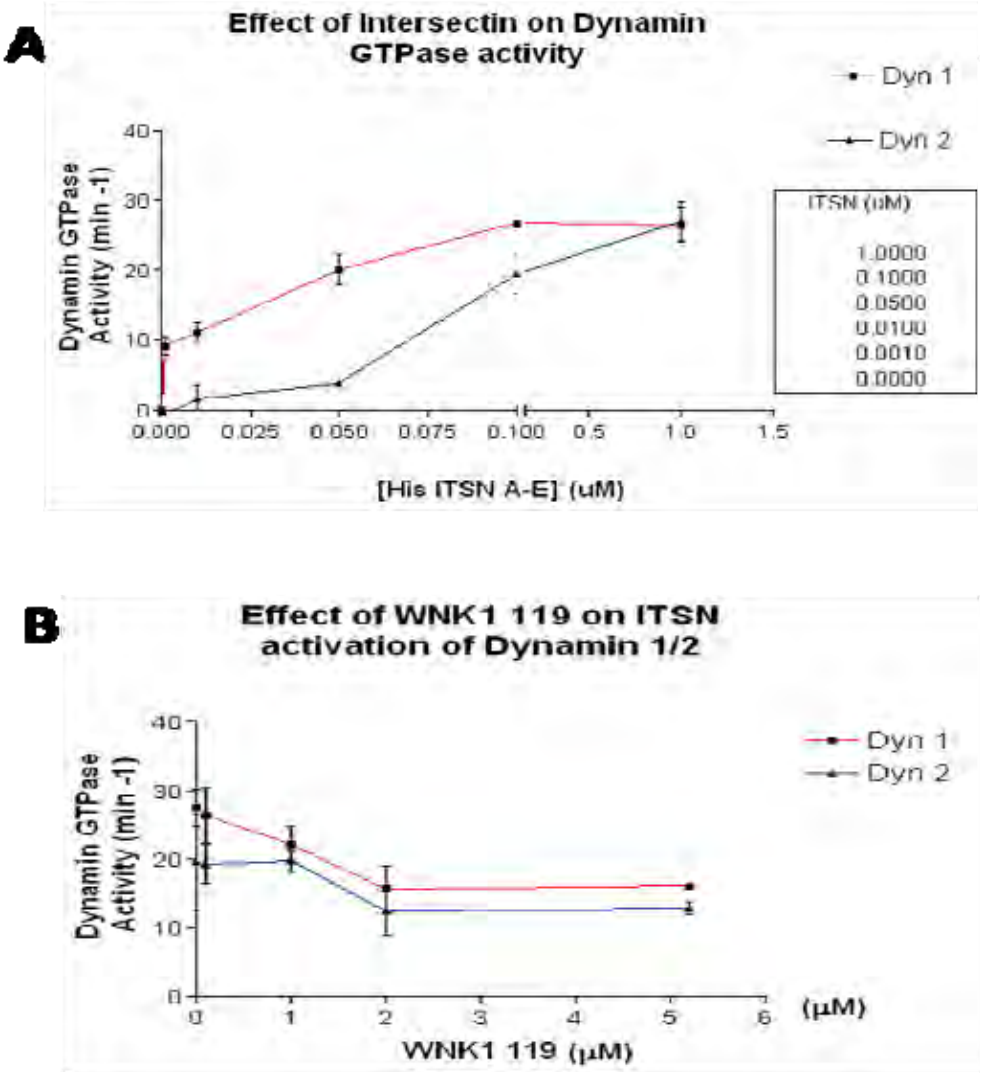

Figure 3-10. GTPase activity of Dynamin 1 and 2 are activated by purified intersectin SH3 A-E domains.

A. The activation curve of dynamin GTPase activity by His-tagged intersectin. 0.1µM dynamin 1 and dynamin 2 were used. 0, 0.001, 0.01, 0.05, 0.1 and 1.0 µM of His-intersectin SH3 A-E protein were used to activate dynamin. At concentration as low as 0.001 µM, intersectin significantly activated dynamin 1. With 0.01 µM intersectin, dynamin 2 was significantly activated.

B. Addition of purified WNK1 PRD protein as much as 5 µM did not greatly interfere with the activation of GTPase activity of dynamin 1 and dynamin 2 by 0.1 µM of intersectin SH3 A-E protein.

Activated dynamin hydrolyses GTP and oligomerizes into a collar like structure, which can squeeze the neck of endocytic vesicles and promote the fission of endocytic vesicles [Ramachandran R, 2008].

Intersectin contains five SH3 domains. ITSN SH3 A, C and E bind dynamin [Koh TW, 2004], however, the function of ITSN SH3 domains and dynamin activation has not been reported. I have constructed and purified His 6-tagged intersectin SH3 A-E domains (His tag will not oligmerize as GST tag and eliminated any artifact caused by tag oligomerization).

As shown in Figure 3-10A, purified His-intersectin SH3 A-E protein activated GTPase activity of both dynamin 1 and 2 at concentration as low as 10nM, and reached plateau at 50nM-1uM range, respectively. The activation of dynamin by intersectin were comparable with that of GST-Grb2 [Barylko B, 1998], a well-established SH3 domain for activating dynamin. Consider that intersectin will dimerize by its coiled-coil domain and further being concentrated by WNK recruitment, the recruitment and activation of dynamin may be even more dramatic with full length proteins *in vivo.*

Furthermore, dynamin activation by intersectin was not blocked by WNK1 binding to intersectin, even in the presence of concentration of WNK1 (1-119) protein as high as 5uM [Figure 3-10 B]. It suggests that dynamin binds more tightly with intersectin SH3 domains than WNK1 does, and that the recruitment of intersectin by WNK1 may not interfere with further recruitment and activation of dynamin by intersectin.

Localization of ITSN1 to the distal nephron

ROMK channels are present in the distal nephron, including the distal convoluted tubule (DCT), the connecting tubule (CNT), and the cortical collecting duct (CCD)[Huang CL, 2007]. We examined the distribution of ITSN1 in rat kidney by immunofluorescent staining. The staining for ITSN1 by a specific anti-ITSN1 antibody was strong in the distal nephron, including DCT and CCD [Figure 3-11A, arrowhead and arrow, respectively], but was not apparent in the proximal tubule or glomerulus.

No staining was observed without the anti-ITSN1 antibody [Figure 3-11B]. Sodium-calcium exchanger isoform 1 (NCX1) is present in the basolateral membrane of DCT and CNT [Loffing J, 2003]. Aquaporin-2 (AQP2) is present in the apical membrane of CNT and CCD [Nielsen S, 2002]. Staining for ITSN was present in NCX1-positive tubules [Figure 3-11C] and in AQP2-positive tubules [Figure 3-11D]. The patterns of cellular and subcellular staining observed for ITSN1, NCX1, and AQP2 are consistent with the idea that ITSN1 is predominantly distributed intracellularly and near the apical membrane of DCT, CNT, and CCD. WNK1 and WNK4 are also present in the distal nephron [Wilson FH, 2001]. These results support the hypothesis that ITSN1 in important for endocytosis of ROMK by WNKs.

In addition, I found that intersectin is also highly expressed in the basolateral side of the medullary collecting duct (MCT) as marked by Dolichos Biflorus Agglutinin(DBA) [Figure 3-11E] [Murata F, 1983]. It is possible that intersectin may regulate the trafficking of ion channel or transporters in the basolateral side of inner medullary collecting duct, however, it is unknown whether WNK kinase is involved or not.

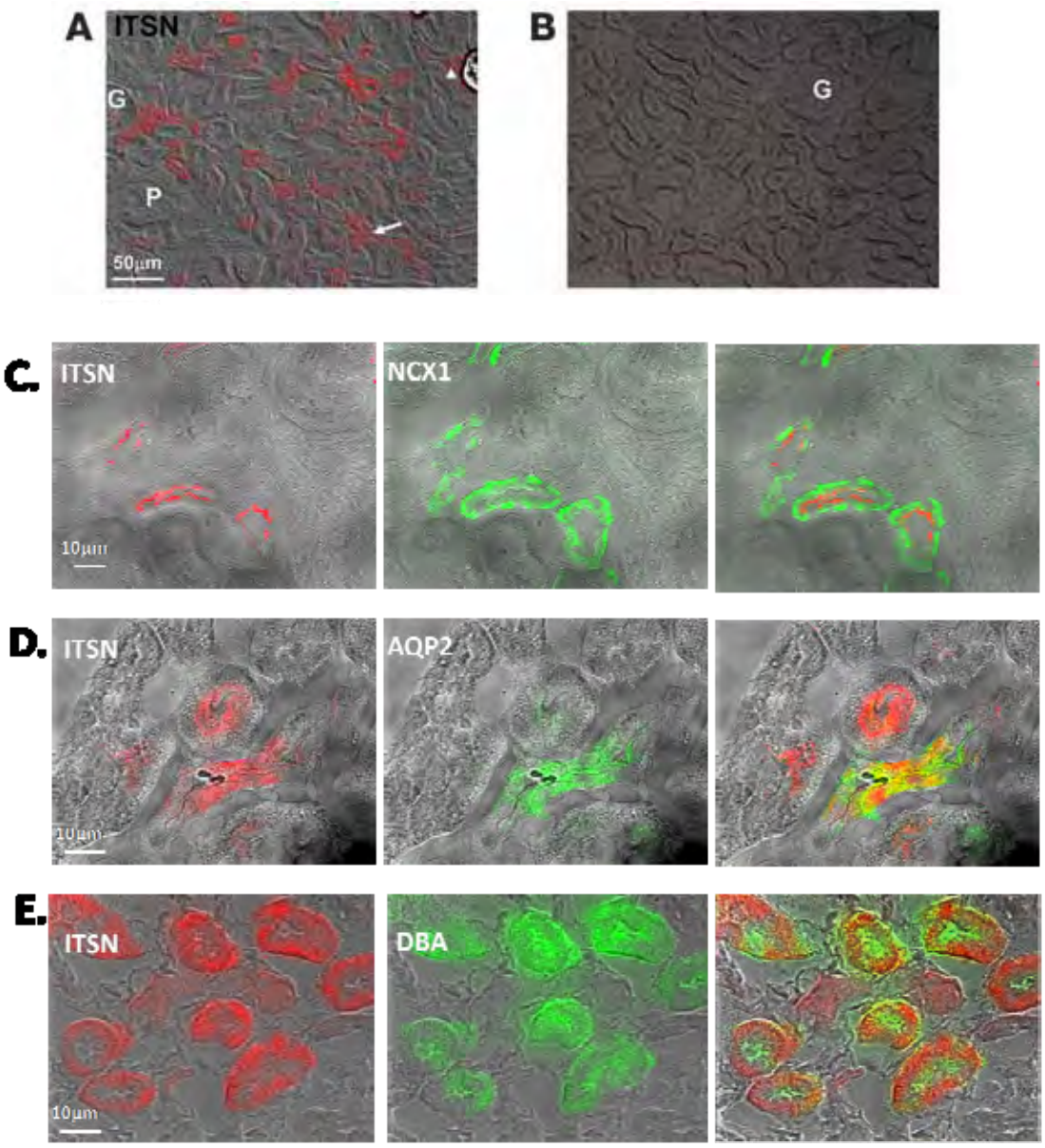

Figure 3-11. Localization of ITSN1 in rat kidney.

- **A.** A .Cortical section stained by anti-ITSN antibody. Fluorescent image was merged with differential interferencecontrast image. White arrow and arrowhead indicate a CCT and a DCT, respectively. G, glomerulus; P, proximal tubule. Scale bar is shown.
- **B.** Cortical section stained as in panel A, but without the anti-ITSN antibody.
- **C.** Cortical section double-stained by antibodies against ITSN (red) and NCX1 (green).
- **D.** Cortical section doublestained by antibodies against ITSN (red) and AQP2 (green).
- **E.** Inner medullar section double-stained by antibody against ITSN (red) and DBA (green) Scale bars: 50 µm (A and B); 10 µm (C , D and E).

Discussion

Intersectin and WNK: Previously, we identified that specific PRD in WNK1 and WNK4 mediates the inhibition of ROMK. How the WNK1 and WNK4 PRD stimulate clathrin-mediated endocytosis of ROMK is unknown. In this chapter, we found that intersectin links WNK kinase with endocytosis of ROMK; the recruitment of intersectin further activated dynamin and N-WASP [as shown in the working model, Figure 3-12].

PRD of both WNK1 and WNK4 interacted specifically with the SH3 domains of ITSN1, and the interaction is critical for inhibiting ROMK by WNK. Interestingly, the critical PXXP motifs for interaction with ITSN in WNK4 were located right before the conserved acidic region, where PHA2-causing mutations are clustered. Disruption of those PXXP motifs in WNK4 abolished the enhanced interaction with ITSN and the enhanced inhibition of ROMK elicited by disease-causing mutations of WNK4. WNK4 PHAII mutations also enhanced the interaction between WNK4 and intersectin (data not shown, by He G). Amino acids 559–562 of the acidic region in WNK4 are also conserved in WNK1, however, the 3 conserved PXXP motifs located in front of the conserved EADQ amino acids 559–562 of WNK4 are not present in WNK1. To date, there are no reports of missense mutations in this region of WNK1 that will cause PHA2, which is consistent with our finding that the integrity of PXXP motifs is important for PHA II mutations to cause the disease. In WNK1, the PXXP motifs in the N terminus are critical for interaction with ITSN and the inhibition of ROMK however, the context specificity of WNK sequences for binding with different SH3 domains of intersectin remains elusive.

SH3 domains interact with different PXXP motifs at different steps of CCV formation [Simpson F, 1999]. For example, ITSN SH3A, SH3C, and SH3E interact stronger with dynamin and N-WASP than with SH3B and SH3D [Sengar AS, 1999; Hussain NK 2001]. ITSN SH3A is required for early

events leading to formation of constricted pits, whereas other SH3 domains are preferentially involved in the late events leading to CCV fission [Sengar AS, 1999]. We found that WNK1 interacted with ITSN SH3C, whereas WNK4 interacted with SH3A, SH3B, and SH3C, raising the possibility that WNKs may be important in coordinating the recruitment of endocytic proteins by ITSN at different stages of CCV formation. The differential interactions with ITSN may also underlie the synergism between WNK1 and WNK4 in stimulating the endocytosis of ROMK [Lazrak A, 2006]. WNK1 is ubiquitous, and WNK4 also has broad tissue distribution in epithelia cells [Kahle AT 2003, 2004]. A recent siRNA-based kinome-wide screen using virus entry as a measure of clathrin-mediated endocytosis identifies WNK4 as a target [Pelkmans L, 2005]. Knockdown of WNK1 also appears to decrease the endocytosis [Pelkmans L, 2005]. Thus, WNKs may regulate clathrin-mediated endocytosis in general through recruitment of ITSN.

WNK4 kinase domain and ROMK interaction: WNK4 interacts with ROMK [Kahle KT, 2003]. We found that the interaction with ROMK, though not sufficient by itself, is essential for WNK4 stimulation of endocytosis of ROMK. ROMK is targeted to coated pits via a C-terminal NPXY motif [Zeng WZ, 2002]. Interaction with WNK4 may also help targeting ROMK to coated pits. Most WNK4 mutations in PHA2 are missense mutations of amino acids localized immediately distal to the first coiled-coil domain [Kahle KT, 2003]. We found that missense mutations of WNK4 enhanced the ability of WNK4 to interact with ITSN1 as well as with ROMK, which increased the endocytosis of ROMK caused by PHA2 mutations of WNK4.

Dynamin: ITSN was originally named as Dynamin Associating Protein (160kD) or DAP160 in Drosophila and was thought to be stabilizing dynamin rather than activating it. Knockout of intersectin in *Drosophila* greatly reduced the levels of dynamin and caused severe defects in vesicle endocytosis. For the first time, I found that the SH3 domains of ITSN could also directly

activated dynamin GTPase activity *in vitro*, presumably by concentrating dynamin and promote its self-activation via dynamin GED domain.

The physiological significance of this finding was previously suggested by a detailed in vivo study of lamprey reticulospinal axons [Evergren E, 2007]. By using electron microscopy, they found that intersectin binds large quantity of dynamin in the synaptic vesicle cluster during the resting state, supporting the idea that ITSN stabilizes dynamin. Upon neuronal stimulation, dynamin dissociated from intersectin and bound to the clathrin-coated vesicles (CCV), possibly with the help other SH3 proteins. Subsequently, intersectin also traveled separately to the CCV [Evergreen E, 2007]. In the same paper, antibody against intersectin SH3 domains was injected into the axon, which competes with dynamin for binding with SH3 domains. Upon ITSN antibody injection, free dynamin in the cytosol and dynamin localized in the neck region of clathrin-coated vesicles were both increased [Evergren E, 2007]. However, even with the increased concentration of dynamin in CCV, the dynamin-mediated vesicle fission was blocked. In other words, without binding with ITSN, dynamin cannot be activated at the neck region of CCV, which indirectly suggests ITSN may activate dynamin *in vivo*. My *in vitro* data provided the first direct evidence that ITSN could activate dynamin by concentrating dynamin via SH3 domains.

How could intersectin be both a positive and a negative regulator of dynamin? Is it possible that the recruitment by WNK kinase could switch the functions of intersectin from being a "warehouse" to being a "spur"? These are interesting questions that deserve future studies.

N-WASP: Another downstream player of intersectin is N-WASP. Different from the previous view that only the DH domain of neuronal expressed long form of Intersectin activates N-WASP,

I found for the first time that ubiquitous short form of intersectin could directly activate N-WASP via SH3 domains.

N-WASP is an important regulator of actin polymerization which is normally kept in an inactive form. In conventional pathways, cdc42 and PIP2 binding to N-WASP synergistically activate the actin polymerization activity of N-WASP. DH domain of Intersectin long form could activate cdc42 and induce N-WASP-mediated actin polymerization. In this study, I first found that WNK-mediated endocytosis of ROMK depends on endogenous N-WASP in HEK cells, since both WNK1 and WNK4 cannot inhibit ROMK in N-WASP null cell line; however, intersectin 1 short form, being the predominant endogenous form of intersectin in HEK cell as shown in the western blotting [He G and Wang HR, 2007], lacks the DH domain of the long form intersectin to activate cdc42. In addition, I found that cdc42 is not required for WNK-mediated ROMK endocytosis. These data suggested that N-WASP may be activated independent of cdc42.

It has been reported that SH3 domain proteins like Grb2, Syndapin activate N-WASP independent of cdc42 and PIP2. Whether intersectin SH3 domain activates N-WASP or not is not known. To test it, I overexpressed full length ITSN 1s, ITSN SH3 domains and ITSN DH domain in NIH 3T3 cells and examined their influence on actin polymerization. I found that over expression of ITSN SH3 A-E, by themselves, dramatically increased the F-actin content *in vivo*, suggesting that SH3 domains of ITSN can activate N-WASP-mediated actin polymerization independent of cdc42 and PIP2. The activation of actin polymerization is even stronger in cells transfected with full length ITSN 1s, possibly because the central coiled-coil of ITSN further concentrated N-WASP protein and enhanced activation. It has been reported that the Dbl homology (DH) domain of intersectin long form (neuronal) stimulates the formation of filapodia through activation of

cdc42 and N-WASP [Hussain NK, 2001]. Notably, the DH-PH domain of ITSN 1 long form only weakly induced actin polymerization in my experiments, suggesting that it may only play a minor role in mediating actin polymerization compared with the function of SH3 domains.

The function of Intersectin to the downstream players: a role of scaffold v.s. activator

In contrast with the old view that endocytic scaffolding proteins like intersectin simply provide the docking sites for other endocytic proteins. I found that the SH3 domains of intersectin may also participate in activating GTPase activity of dynamin and stimulating actin polymerization activity, possibly via N-WASP. These results revealed a more dynamic life of intersectin beyond simply being a "scaffold". It not only suggests a mechanism for WNK1 and WNK4 mediated endocytosis of ROMK, but also provides a more general "Recruit and Activate" mechanism for intersectin to facilitate endocytosis.

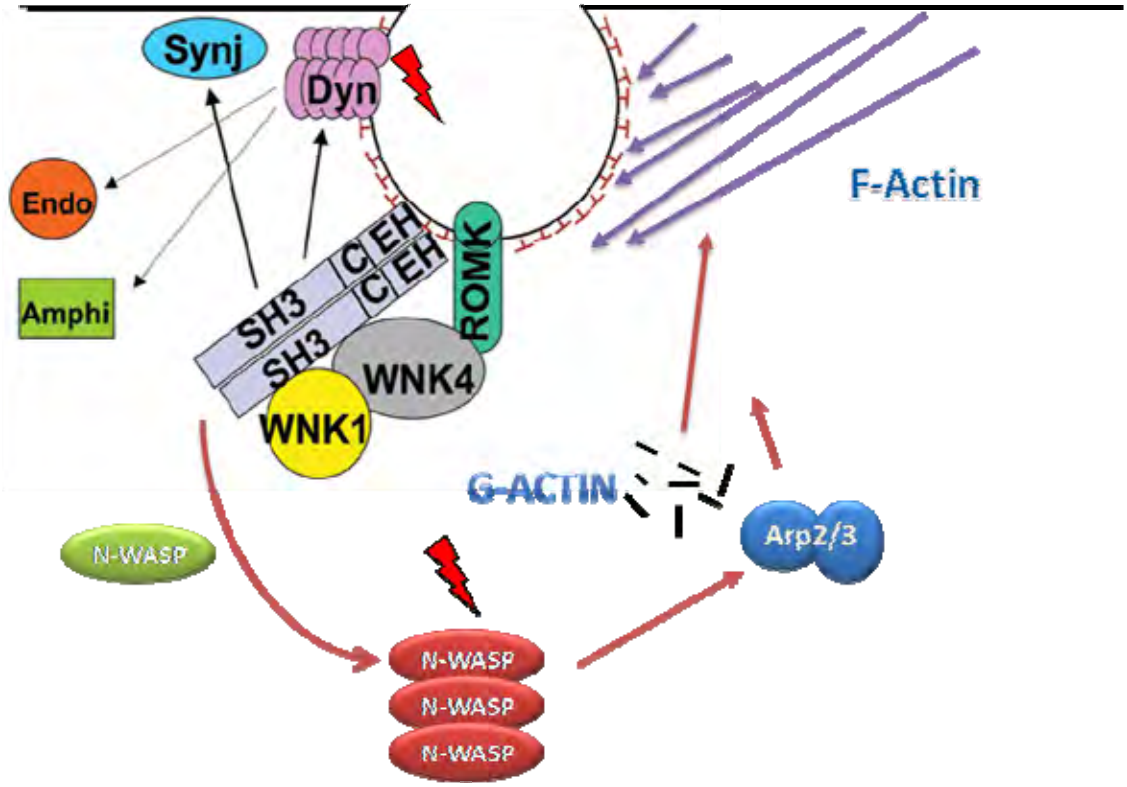

Figure 3-12.Model for endocytosis of ROMK regulated by WNK kinases and intersectin.

WNK4 kinase domain specifically binds with C-terminus of ROMK. WNK1 binds WNK4. WNK1 and WNK4 stimulate endocytosis of ROMK by binding to the SH3 domains of intersectin. Intersectin then recruit dynamin, N-WASP, and synaptojanin etc.. Intersectin SH3 activates the GTPase activity of dynamin and the actin-polymerization activity of N-WASP, thus promote endocytosis of ROMK. Dynamin could also recruit endophilin and amphiphysin to help endocytosis of ROMK.

CHAPTER FOUR: Domains of WNK1 kinase in the regulation of ROMK

ABSTRACT

WNK1 inhibits renal potassium channel ROMK by enhancing its endocytosis, likely contributing to hyperkalemia in affected patients. The domains of WNK1 involved in inhibition of ROMK have not been completely elucidated. Here, we reported that an N-terminal proline-rich domain (N-PRD; amino acids 1-119) is necessary and sufficient for WNK1 inhibition of ROMK. Inhibition of ROMK by WNK1 (1-119), but not WNK1 (1-491), may depend on endogenous WNK1, possibly for targeting. A region named "NL" for N-linker (amino acids 120-220) located between N-PRD and the kinase domain of WNK1 (amino acids 220-491) antagonized the inhibition of ROMK caused by N-PRD. The WNK1 kinase domain reversed the antagonism of NL on N-PRD. Mutagenesis studies revealed that charge-charge interactions between two conserved catalytic residues (Lys-233 and Asp-368) within the kinase domain (not the kinase activity) is critical for kinase domain to reverse the antagonism of NL domain. The WNK1 auto-inhibitory domain (AID; amino acids 491-555) also affected ROMK, presumably by modulating the kinase domain conformation. Mutations of two conserved phenylalanine abolished the ability of AID to modulate ROMK. Finally, the first coiled-coil domain (CC1; amino acids 555-640) of WNK1 alleviated the effect of AID domain towards kinase domain. In sum, multiple intra- and/or intermolecular interactions of WNK1 domains are at play for the regulation of ROMK by WNK1.

INTRODUCTION

WNK1 consists of about 2,100 amino acids and a 270 amino acid kinase domain located near the amino terminus [Xu BE, 2000]. The human WNK1 gene consists of 28 exons [Wilson FH, 2001]. The full-length WNK1 transcript encoded by all 28 exons is present in every tissue examined. WNK1 is highly expressed in the DCT and CCD, where ROMK functions to secret K+ [Wilson FH, 2001; Choate KA, 2003]. In the previous chapters, we found out that PRD of WNK1 stimulate endocytosis of ROMK channel via recruiting intersectin [He G and Wang HR, 2007]. However, the regulatory domains of PRD domain in WNK have not been identified, and the mechanism for the regulation is yet to be understood. In this study, I designed different WNK1 constructs containing different WNK1 domains to study their function in regulating PRD domain of WNK1.

As shown in chapter 2, WNK1 (1-119) is required and sufficient to inhibit ROMK. However, it has been reported that kinase dead WNK1 and WNK1 (1-220) (does not contain kinase domain) failed to regulate ROMK. These results appear to be contradicted, since the proline-rich domain for recruiting intersectin is still intact in kinase dead WNK1. To solve the conundrum, we hypothesize that PRD of WNK1 is functionally coupled to its kinase domain. WNK1 AID inhibits WNK1 kinase activity by binding to the kinase domain. WNK1 1-555 does not exhibit kinase activity [Xu BE, 2002]. We also found that WNK1 1-555 failed to regulate ROMK. Thus, it is also of interest to study the effect of AID to WNK1 (1-491)-mediated inhibition of ROMK. Furthermore, though mutation of the catalytic lysine abolished the inhibition of ROMK by WNK1, whether kinase activity is important for regulating ROMK is not known, and it will be studied in detail in this chapter.

Another question is how a small WNK1 peptide of amino acids 1-119 could induce the endocytosis of ROMK, potently and specifically, without being able to bind with ROMK. I hypothesize that the endogenous WNK1 may contribute to WNK1 (1-119)-mediated endocytosis of ROMK. To support this, multiple domains in WNK1 is reported to interact with each other, and full length WNK1 protein exists as a tetramer. Lenertz LY et al. reported that WNK1 (1-220), including the PRD, interacts strongly with the C-terminal sequences of WNK1, which may provide docking site for PRD to regulate ROMK. These interactions may represent an intra- and/or inter-molecular regulation mechanism by which WNK1 PRD is tightly modulated to control the endocytosis of ROMK.

MATERIAL AND METHODS

Molecular biology. GFP-ROMK was described previously [He G and Wang HR, 2007]. Various cDNAs encoding different domains of rat WNK1 (gifts of M. Cobb and B. Xu, University of Texas Southwestern Medical Center) were cloned in pCMV5-myc vector [Wang HR, 2008]. Point mutations were generated by site-directed mutagenesis (QuickChange kit; Stratagene) and confirmed by sequencing. Sense and antisense oligonucleotide for WNK1 siRNA were 5′-UGU CUA ACG AUG GCC GCU U dT dT and 5′-AAG CGG CCA UCG UUA GAC A dT dT, respectively.

Cell culture, transfection and patch-clamp recording as described in Chapter 2.

Statistics. Statistical comparisons between 2 groups of data were made using a 2-tailed unpaired Student's *t* test. Multiple comparisons were made using 1-way ANOVA followed by 2-tailed Student's *t* test adjusted for multiple comparisons. *P* values less than 0.05 and 0.01 were considered significant for single and multiple comparisons, respectively.

RESULTS

Both WNK1 kinase domain and proline-rich domain are important in the regulation of ROMK

WNK1 inhibits ROMK channel by increasing its endocytosis [Lazrak A, 2006]. We have shown in previous chapters that the N-terminal proline-rich domain (N-PRD) of WNK1 is important for the inhibition of ROMK [Figure 2-5]. Here, we further examined the role of WNK1 kinase domain and N-PRD using full-length WNK1 as the backbone for mutagenesis of the kinase domain and N-PRD.

Figure 4-1A shows the domain structure of WNK1 referenced in the present study and a working model for WNK1 inhibition of ROMK by recruitment of intersectin to enhance the endocytosis of the channel. As shown in Figure 4-1B, full-length WNK1(F-WNK1), a WNK1 fragment containing amino acid 1-1200 ($WNK1_{1-1200}$) or amino acids 1-491 ($WNK1_{1-491}$) each inhibited ROMK current density. Consistent with previous reports that WNK1 kinase domain is important for regulation of ROMK, mutation of the conserved catalytic lysine-233 to methionine (K233M) in Full length WNK1, WNK1(1-1200) or WNK1(1-491) abolished their inhibition of ROMK [Figure 4-1B].

N-PRD of WNK1 (amino acid 1-119) is sufficient for the inhibition of ROMK [Figure 2-5B]. Mutations of proline-94, -103, and -114 within N-PRD of WNK1 (1-491) disrupt its interaction with intersectin and its inhibition on ROMK [Figure 2-5C]. Full length WNK1, however, contains more than 20 proline-rich domains [Xu BE, 2000]. It is not known whether proline-94, -103, and -114 are the only proline residues of full length WNK1 essential for inhibition of ROMK. Here, we found that triple mutations of proline-94, -103, and -114 to alanine ("$FWNK1/PA_3$") abolished the inhibition of ROMK by full length WNK1 [Figure 4-1C, 4^{th} and 5^{th} bar from left]. Western blot analysis of WNK1 revealed that the loss of inhibition by triple-proline mutant of full length WNK1 was not due to reduced or lack of expression of the protein. Similar as Figure 2-5B, triple mutations of proline-94, -103, and -114 to alanine ("$FWNK1/PA_3$") abolished the inhibition of

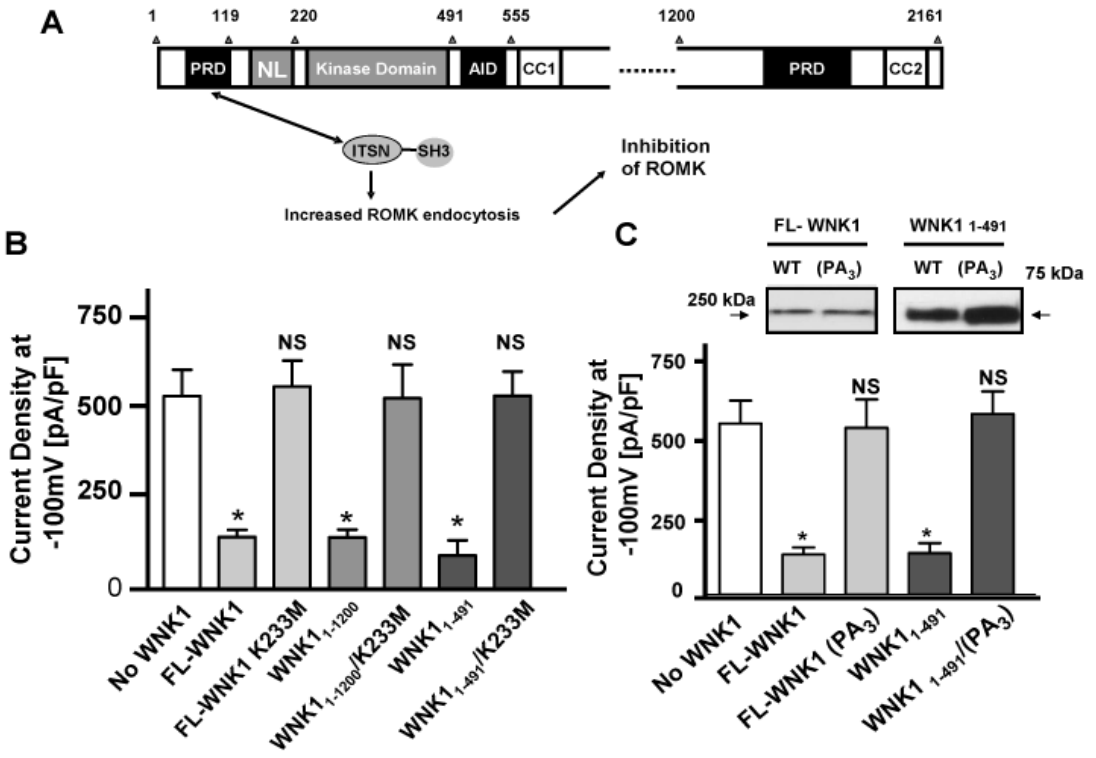

Figure 4-1. Role of kinase domain and proline-rich domain in the regulation of ROMK.

A. Schematic diagram of rat full-length WNK1. N-PRD, proline-rich domain; NL, N-linker domain; CC1 and 2, coiled-coil domain 1 and 2 (predicted using the COILs program [14]); AID, autoinhibitory domain; ITSN, intersectin; SH3, Src-homology domain-3. As shown, N-PRD of WNK1 binds ITSN via SH3 domain. This recruitment of ITSN stimulates endocytosis of ROMK.

B. Effect of lysine-233 to methionine (K233M) mutation of full length WNK1 (FWNK1), amino acids 1-1200 (WNK11-1200) or amino acids 1-491 of WNK1 (WNK11-491) on the regulation of ROMK. HEK cells were co-transfected by ROMK and indicated WNK1 construct or an empty vector ("No WNK1"). About 36-48 hrs after transfection, whole-cell ROMK current density (pA/pF at -100 mV) was measured. *, $p < 0.01$ vs control ("No WNK1"). NS, statistically not significant vs control. Of note, HEK cells express endogenous FWNK1, which exerts inhibition of ROMK in the absence of exogenous WNK1.

C. Effect of triple mutations of proline-94, -103, -114 (to alanine; labeled as PA3) on WNK1 regulation of ROMK. Expression of myc-tagged wild type (WT) and PA3 mutant in transfected HEK cells was examined using anti-myc antibody. *, $p < 0.01$ vs control ("No WNK1"). NS, statistically not significant vs control.

ROMK by WNK1(1-491) [Figure 4-1C, 2nd and 3rd bar from left]. Thus, N-PRD is not only sufficient but also essential for full-length WNK1 to inhibit ROMK.

Endogenous WNK1 is required for WNKI PRD to inhibit ROMK

It has been puzzling as to why a small piece of protein likes WNK1 (1-119), probably lacking the necessary sequences to target clathrin-coated vesicle, could still stimulate a complicate process like endocytosis, and how WNK1 (1-119) could have achieved the specificity to inhibit ROMK?

Since WNK1 PRD domain binds with C-terminal sequence, and there is abundant full length WNK1 existing in the HEK cells. I hypothesize that WNK1 (1-119) may bind with endogenous full length WNK1 and utilize it as scaffold to inhibit ROMK specifically. To prove it, we need to knockdown endogenous WNK1 when over-expressing exogenous WNK1. I take advantage of the siRNA targeting for human WNK1 kinase domain. Our exogenous expressed WNK1 are rat proteins with mis-match target sequence of siRNA [Figure 4-2D]. WNK1 (1-119) also does not contain the target sequence of WNK1 siRNA; thus, co-expression with siRNA will not affect the expression of WNK1 (1-119) or WNK1 (1-491). As shown in Figure 4-3A, siRNA knockdown of endogenous WNK1 abolished the effect of WNK1 (1-119), whereas WNK1 (1-491) inhibition was not affected by WNK1 knockdown [Figure 4-3B]. It suggests that the proline rich domain of WNK1 still needs endogenous WNK1 to inhibit ROMK, which possibly dock WNK1 (1-119) to the proximity of ROMK endocytic machinery. WNK1 (1-491) does not require endogenous WNK1, suggesting the kinase domain of WNK1 may also function to provide the targeting of WNK1.

To support this, WNK1 kinase domain (220-491), antagonized the function of WNK1 (1-119) [Figure 4-3C]. The over-expressed kinase domain 220-491 may compete with endogenous WNK1 to bind with WNK1 119, and thus antagonizing WNK1 (1-119) in a dominant negative fashion.

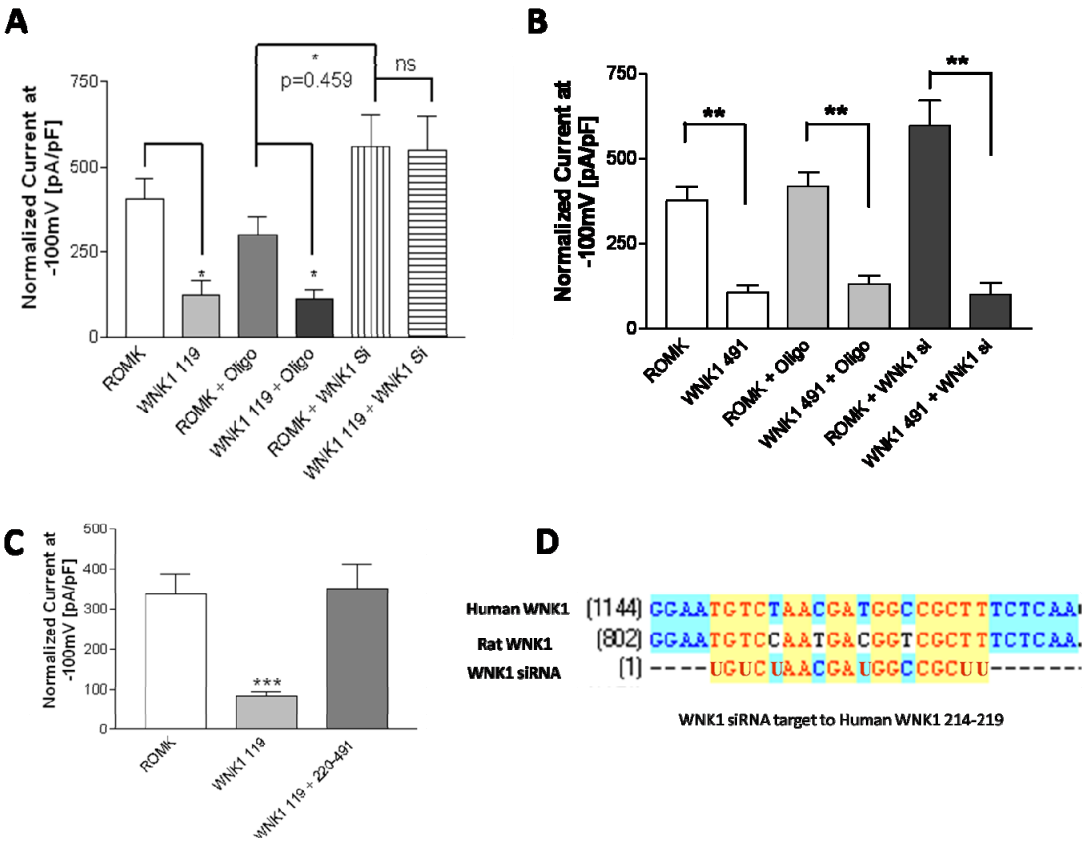

Figure 4-2.The role of endogenous WNK1 in the regulation of ROMK by exogenously expressed WNK1

- **A.** siRNA knockdown of endogenous WNK1 abolished WNK1 1-119 inhibition, whereas scramble siRNA had no effect. WNK1 siRNA increased the basal level of ROMK compared with ROMK oligo group and ROMK group, suggesting that WNK1 siRNA did knock down endogenous WNK1.
- **B.** WNK1 (1-491) effect is not affected by knockdown of endogenous WNK1. WNK1 siRNA also increased ROMK basal level in the 5th bar compared with control group.
- **C.** WNK1 (220-491) (the kinase domain) prevented the effect of WNK1 (1-119).
- **D.** The target sequence of human-WNK1 siRNA is different with rat WNK1 sequence.

The role of amino acids between N-terminal PRD and the kinase domain

The above results, however, create an apparent contradiction: why K233M mutants of FWNK1, WNK1 (1-1200) and WNK1 (1-491) fail to inhibit ROMK while they contain the N-PRD domain sufficient for inhibition of the channel? To resolve this conundrum, we hypothesized that the region located between N-PRD and the kinase domain WNK1 (including amino acids 120-220; named N-linker [NL]; see Figure. 4-1A) plays an important role in modulating WNK1(1-491) regulation of ROMK. In this hypothesis, NL region antagonizes the inhibition of ROMK by N-PRD and WNK1 kinase domain further reverses the antagonism of NL to N-PRD.

In support of the hypothesis that NL domain plays a role, we compared the effects of WNK1(120-220) and WNK1(120-491) on WNK1(1-119)-mediated inhibition of ROMK. WNK1(120-491) differs from WNK1(120-220) by having a kinase domain in addition to a NL domain. WNK1(120-220) reversed WNK1(1-119)-mediated inhibition of ROMK [2^{nd} and 3^{rd} bar, Figure 4-3B]. In contrast, WNK1(120-491) did not reverse the inhibition caused by WNK1(1-119) [5^{th} bar, Figure 4-3B], supporting that WNK1 kinase domain neutralizes the function of WNK1(120-220) on WNK1(1-119)-mediated inhibition of ROMK. Western blot analysis confirmed that all three domains could express as independent peptides in HEK 293 cells (data not shown). Together, these results support the hypothesis that N-PRD inhibits ROMK, NL domain reverses N-PRD-mediated inhibition of the channel, and that kinase domain neutralizes the function of NL domain.

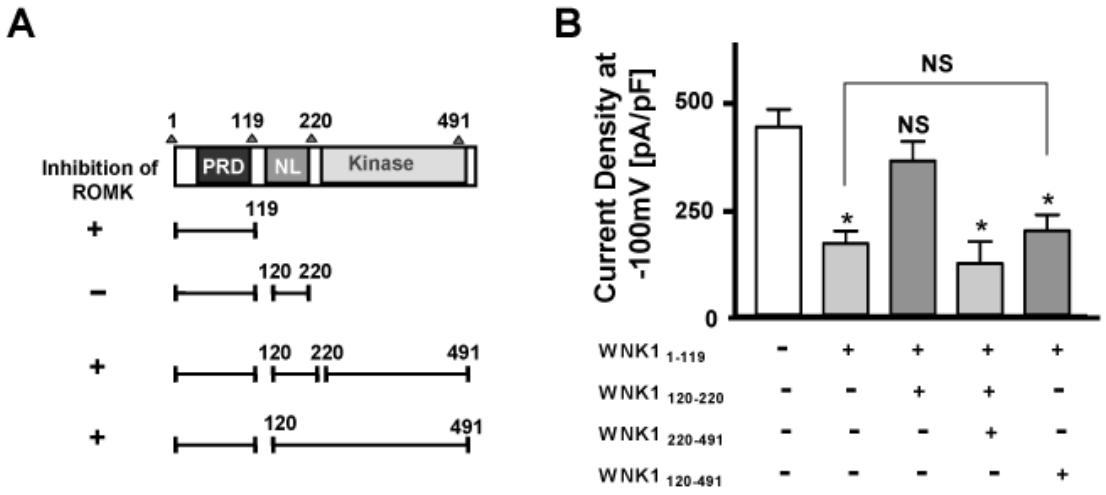

Figure 4-3. Role of amino acids between N-terminal proline-rich domain (PRD) and kinase domain of WNK1 in the regulation of ROMK.

A. Amino acids (1-491) of WNK1 include N-terminal PRD (amino acids 1-119), N-linker domain (NL, amino acids 120-220), and kinase domain (amino acids 221-491). Effects of these domains on ROMK("inhibition of ROMK") from a previous study [5] and the present study shown inpanel B are summarized. "+" and "-" indicate inhibition and no inhibition of ROMK,respectively.
B. Role of NL domain in the regulation of ROMK. HEK cells were co-transfected with ROMK plus various combinations of WNK1 constructs as indicated. *, $p < 0.01$ vs control (no WNK1; white bar). NS, statistically not significant vs control or between indicated groups. Fig. 3. Electrostatic interaction between lysine-233 and aspartate-368 of kinase

Conformation of WNK1 kinase domain rather than kinase activity is critical for inhibition of ROMK.

Autophosphorylation of Ser-382 in the activation loop of WNK1 is critical for its kinase activity [Xu BE, 2002]. Mutation of the serine residue abolishes kinase activity [Xu BE, 2002]. We used Ser-382 to alanine mutant of WNK1(1-491) to further test the role of the kinase activity in the regulation of ROMK. We found that WNK1(1-491)/S382A inhibited ROMK as well as wild type WNK1(1-491) [2nd and 4th bar, Figure 4-4A]. For comparison, WNK1(1-491)/K233M did not inhibit ROMK [3rd bar, Figure 4-4A]. These results provide further support for the idea that WNK1 kinase activity is not necessary for the regulation of ROMK.

The crystal structure of the kinase domain of WNK1 reveals that Lys-233 and Asp-368 are in close proximity and may form a salt-bridge [Min X, 2004] [Figure 4-5A]. We hypothesized that an electrostatic interaction between Lys-233 and Asp-368 is critical for maintaining a structure of the kinase domain to inhibit ROMK. In support of this hypothesis, we have shown that a double mutant of WNK1(1-491) carrying charge-reversal double mutations of Lys-233 (K233) and Asp-368 (D368) (namely, double mutations of K233 to Asp and of Asp-368 to Lys; K233D/D368K) remains capable of inhibiting ROMK [3rd bar, Figure 4-5E], while possessing no kinase activity (by Shao-Kui Huang, [He G and Wang HR, 2007]). WNK1(1-491)/K233R, which carries a charge conservation mutation of Lys-233 to arginine, inhibited ROMK similarly as the wild type [4th bar, Figure 4-5E]. The functional data above correlated well with their binding with ITSN SH3-C as shown in Figure 4-5F, where WNK1 (1-491) wild-type and KDDK double mutant bind with ITSN SH3-C, while WNK1 (1-491) KD or DK single mutant does not (by Shao-Kuei Huang, [He G and Wang HR, 2007]).

The results of Figure 4-3 suggest that WNK1(120-220) antagonized WNK1(1-119)-mediated inhibition of ROMK while WNK1(120-491) did not antagonize the inhibition. To know if the

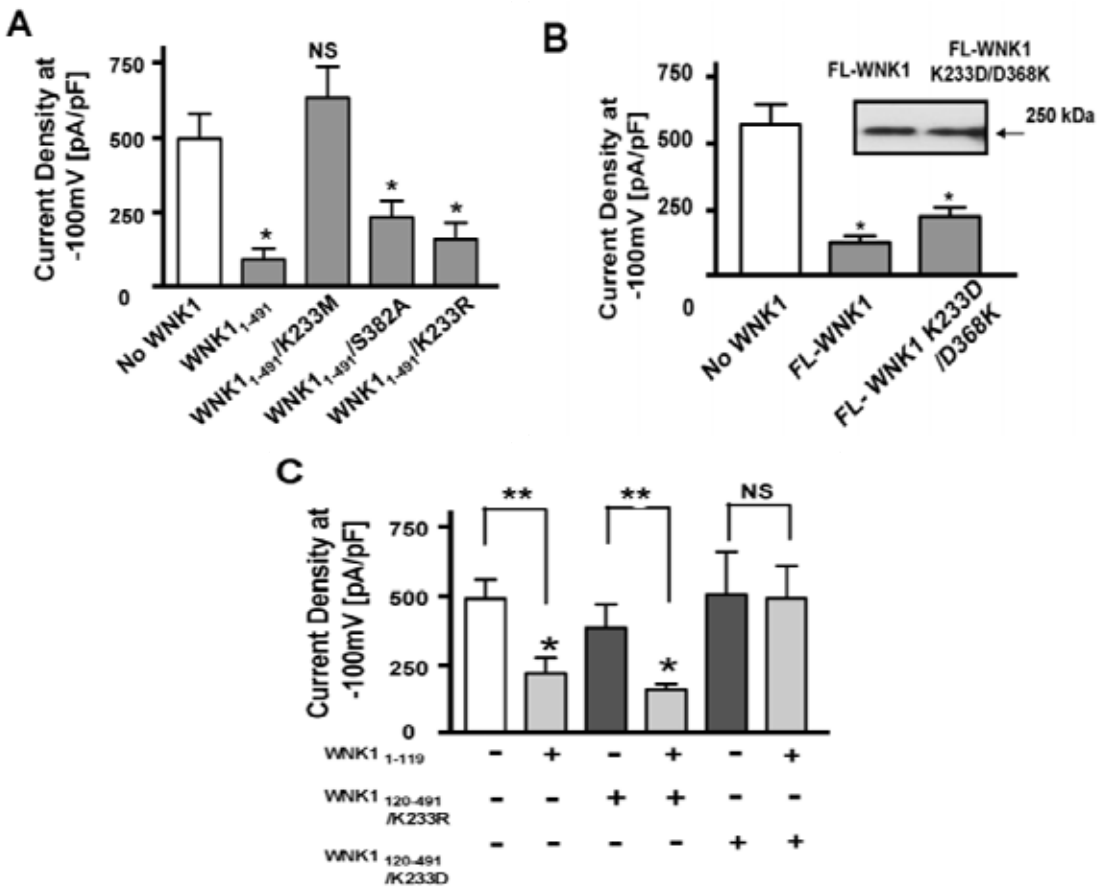

Figure 4-4. Electrostatic interaction between lysine-233 and aspartate-368 of kinase domain of WNK1 is critical for inhibition of ROMK.

A. Effect of serine-382 to alanine(S382A), lysine-233 to methionine (K233M) or lysine-233 to arginine (K233R) mutation. *, $p < 0.01$ vs control ("No WNK1"). NS, statistically not significant vs control.
B. Effect of charge-conservation (K233R) and charge-disruption mutation (lysine-233 to aspartate; K233D). *, $p < 0.01$ vs control (white bar). **, $p < 0.001$ between indicated groups. NS, statistically not significant between indicated groups.
C. Effect of charge-reversal mutation between K233 and D368 (i.e., double mutations of lysine-233 [K233] to aspartate and of aspartate-368 [D368] to lysine; K233D/D368K) of full-length WNK1 (FWNK1). *, $p < 0.01$ vs control ("No WNK1"). Expression of myc-tagged wild type and mutant WNK1 in transfected HEK cells was examined using anti-myc antibody.

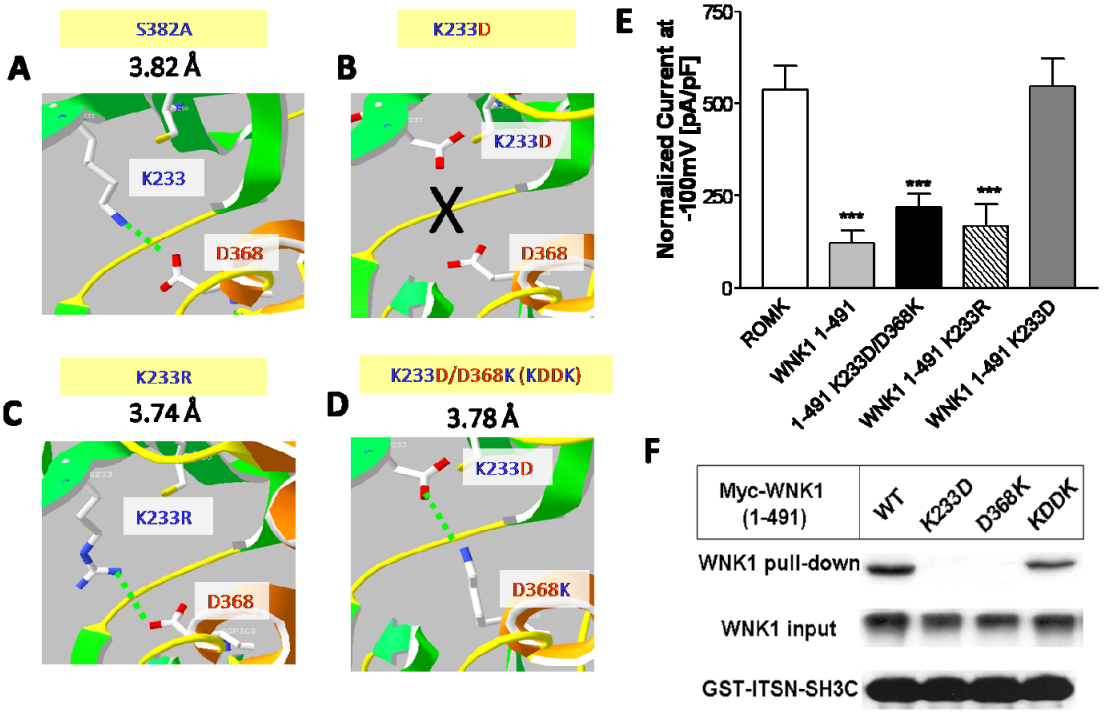

Figure 4-5. The role of WNK1 kinase domain conformation in regulating endocytosis of ROMK.

- **A.** The conformation of catalytic lysine and aspartic acid residues in crystal structure of WNK1 kinase domain. The distance between K233 and D368 is 3.82A, which is close enough to form salt bridge.
- **B.** In silico simulation of the conformation of K233D mutation. Due to the disruption of charge, the salt bridge cannot be maintained in WNK1 K233D mutant.
- **C.** Mutation of lysine to arginine at site K233 maintained both the distance and the charge requirement for maintaining the salt bridge.
- **D.** In WNK1 KDDK mutant, the charge switch of K233 and D368 also keeps the salt bridge necessary for keeping the kinase domain conformation.
- E. Regulation of ROMK by WNK1 mutants. WNK1 K233D/D368K and WNK1 K233R mutants both inhibited ROMK as well as wild-type WNK1, however, WNK1 K233D mutant failed to regulate ROMK, probably due to its disrupted kinase domain conformation.
- **F.** GST-ITSN-SH3C was used to pull down wild-type WNK11–491 or mutants. WNK1 WT and KDDK mutant can recruit ITSN SH3C, but not WNK1 K233D and D368K mutants. It suggests WNK1 kinase domain conformation regulates recruitment of intersectin to WNK1 proline-rich domain. (by Shao-Kuei Huang)

electrostatic interaction between Lys-233 and Asp-368 on the regulation of ROMK is related to these interactions, we examined the effects of mutation of Lys-233 of WNK1(120-491). We found that WNK1(120-491) carrying the mutation of Lys-233 to aspartate WNK1 (120-491)/K233D antagonized WNK1(1-119)-mediated inhibition of ROMK [6th bar Figure 4-4C] in contrast to its wild type counterpart [5th bar Figure 4-4C]. By itself, WNK1(120-491)/K233D had no effect on ROMK. These results indicate that the mutant kinase domain no longer neutralizes the function of NL domain toward N-PRD. For comparison, a charge-conservation mutation of WNK1(120-491)that preserves the electrostatic interaction (K233 to arginine; WNK1(120-491)/K233R) did not antagonize N-PRD-mediated inhibition of ROMK [4th bar Figure 4-4C]. By itself, WNK1(120-491)/K233R had no effect on ROMK [3th bar Figure 4-4C]. Thus, the ability of the kinase domain to antagonize NL domain depends on the charge interaction between K233 and D368 in the kinase domain. Next we examined the role of the electrostatic interaction of the kinase domain for full-length WNK1. Full-length WNK1 bearing K233D/D368K double mutations inhibited ROMK [Figure 4-4B]. Thus, the electrostatic interaction between Lys-233 and Asp-368 is important for the inhibition of ROMK by full-length WNK1.

The role of the autoinhibitory domain

The auto-inhibitory domain (AID; amino acids 491-555; Figure 4-6A) of WNK1 negatively regulates its kinase activity (Xu AID). A WNK1 fragment containing the AID domain in addition to the kinase domain (WNK1 (1-555), consisting of amino acids 1-555) does not exhibit kinase activity [Xu BE, 2002]. We examined the role of WNK1 AID domain in the regulation of ROMK. We found that WNK1(1-555) had no effect on ROMK [5th bar Figure 4-6B]. Two phenylalanine residues (Phe-524 and -526; Figure 4-6A) within the AID domain of WNK1 are critical for the inhibition of the kinase activity; Mutations of both residues abrogate the function of AID domain

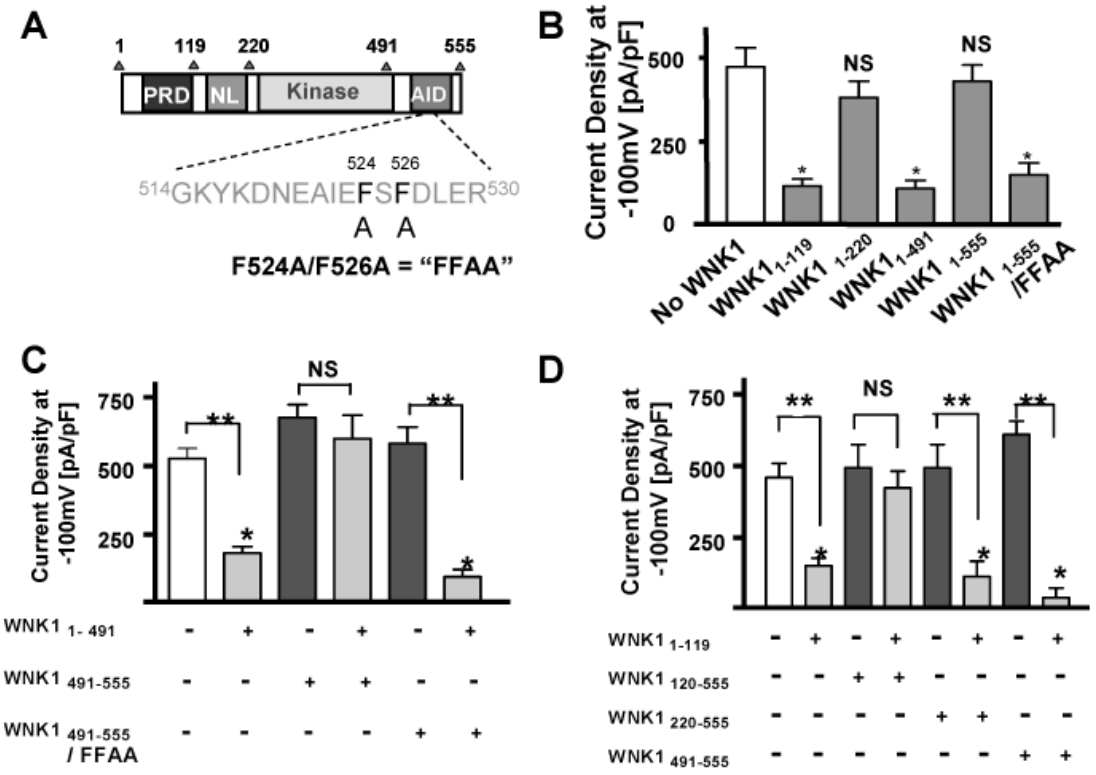

Figure 4-6. Role of autoinhibitory (AID) domain of WNK1 in the regulation of ROMK.

A. Diagram of WNK1 domains in the study. Amino acids of AID surrounding phenyalanine-524 and -526 critical for the function of AID domain are shown. Doublevmutations of phenylalanine to alanine is abbreviated as "FFAA".
B. HEK cells were co-transfected with ROMK plus indicated construct or no WNK1 (control). *, $p < 0.01$ vs control ("No WNK1"). NS, statistically not significant vs control.
C. Cells were co-transfected with ROMK plus indicated construct. *, $p < 0.01$ vs control (white bar). **, $p < 0.001$ between indicated groups. NS, statistically not significant between indicated groups.
D. Cells were co-transfected with ROMK plus indicated construct. *,$p < 0.01$ vs control (white bar). **, $p < 0.001$ between indicated groups. NS,statistically not significant between indicated groups.

to inhibit kinase activity. WNK1 (1-555) carrying double mutations of phenylalanine to alanine ($WNK1_{1-555}$/FFAA) inhibited ROMK [6th bar, Figure. 4-6B], confirming that a functional AID domain is necessary for the antagonism toward WNK1(1-491). When expressed as a separate peptide from WNK1(1-491), AID domain WNK1(491-555) still prevented inhibition of ROMK by WNK1(1-491) [4th bar, Figure. 4-6C]. In contrast, WNK1(491-555)/FFAA did not prevent inhibition of ROMK by WNK1(1-491) [6th bar, Figure. 4-6C]. Neither WNK1(491-555) nor WNK1(491-555)/FFAA affected ROMK in the absence of WNK1(1-491) [3rd and 5th bar, Figure 4-6C].

In our model of multiple domain interactions for WNK1 regulation of ROMK, the effect of the NL domain to reverse N-PRD-mediated inhibition of ROMK is an essential step. We next examined the role of NL domain (amino acids 120-220) in the regulation of ROMK by the AID domain. In these experiments, we made WNK1 constructs containing amino acids 120-555 WNK1(120-555) and amino acids 220-555 WNK1(220-555) and compared their effects on WNK1(1-119)-mediated inhibition of ROMK.

As expected from the results by using a continuous peptide WNK1(1-555), we found that WNK1(120-555) reversed WNK1(1-119)-mediated inhibition of ROMK [4th bar Figure 4-6D]. WNK1(120-555) had no effect on ROMK in the absence of WNK1(1-119) [3rd bar Figure 4-6D]. In contrast, WNK1(220-550) did not reverse WNK1(1-119)-mediated inhibition of ROMK [2nd bar Figure 4-6D], indicating that amino acids 120-220 are necessary for the effect of AID domain through the kinase domain. WNK1(220-555) had no effect on ROMK in the absence of WNK1(1-119) [5th bar Figure 4-6D]. As additional controls, we found that the AID domain (WNK1 491-555) by itself did not cause inhibition of ROMK nor did it affect WNK1 (1-119)-mediated inhibition of ROMK [7th and 8thF bar, Figure 4-6D].

Together, these results suggest that WNK1 AID domain regulates the functional coupling between the kinase domain and the NL domain, thus influencing the functionality of the N-PRD domain to cause endocytosis of ROMK. The AID domain itself cannot directly affect the inhibition of N-PRD on ROMK.

The role of amino acids C-terminal to AID domain

One of the two coiled-coil domains of WNK1 (labeled CC1 for 1st coiled-coil domain; located within amino acids 555-640) is localized adjacent to the AID domain [Figure 4-7A]. In a kinase activity assay, the CC1 domain antagonizes the inhibitory effect of AID domain on the kinase domain [16]. We hypothesized that CC1 domain may also be important in modulating the effect of AID domain on the kinase domain with respect to the regulation of ROMK. The hypothesis may explain why full-length WNK1 and WNK1$_{1\text{-}1200}$ each inhibited ROMK [Figure 4-1B], while WNK1(1-555) did not [Figure 4-6B]. We tested this by adding CC1 to AID domain and examined ability of the construct to reverse inhibition of ROMK by WNK1(1-491). We found that WNK1(491-640), which contains both AID and CC1 domains, did not reverse WNK1(1-491)-mediated inhibition of ROMK [4th bar Figure 4-7B]. In contrast, WNK1(491-550) (which contains AID alone) was capable of reversing the inhibition of ROMK by WNK1(1-491) [3rd bar Figure 4-7B]. As a control, WNK1(491-640) carrying double phenylalanine mutation (WNK1$_{491\text{-}640}$/FFAA) had no effect on the inhibition of ROMK by WNK1(1-491) [5th bar Figure 4-7B], suggesting that (without an intact AID domain) CC1 domain did not exert a nonspecific effect on ROMK and/or WNK1(1-491).

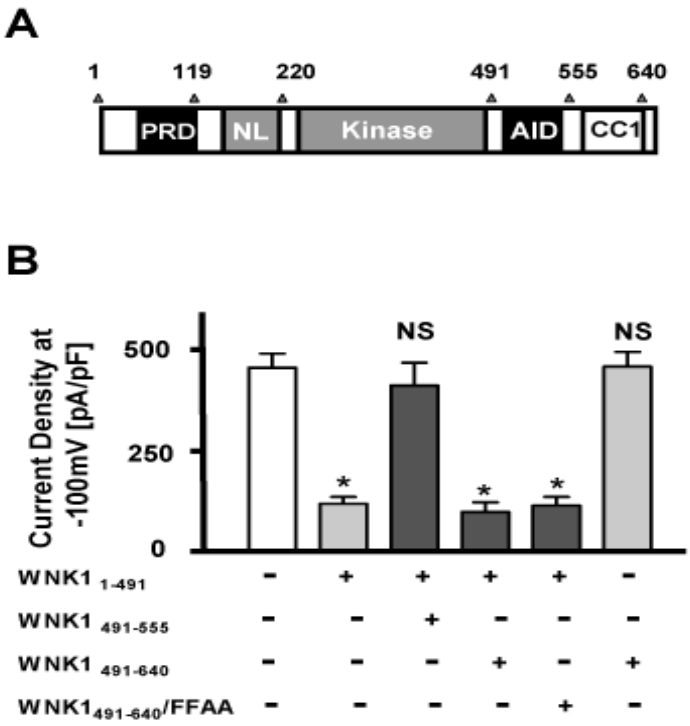

Figure 4-7. Role of the first coiled-coil (CC1) domain of WNK1 in the regulation of ROMK.

A. Diagram of WNK1 domains in the study.
B. HEK cells were co-transfected with ROMK plus various combinations of WNK1 constructs as indicated. *, $p < 0.01$ vs control (no WNK1 constructs; white bar). NS, statistically not significant vs control.

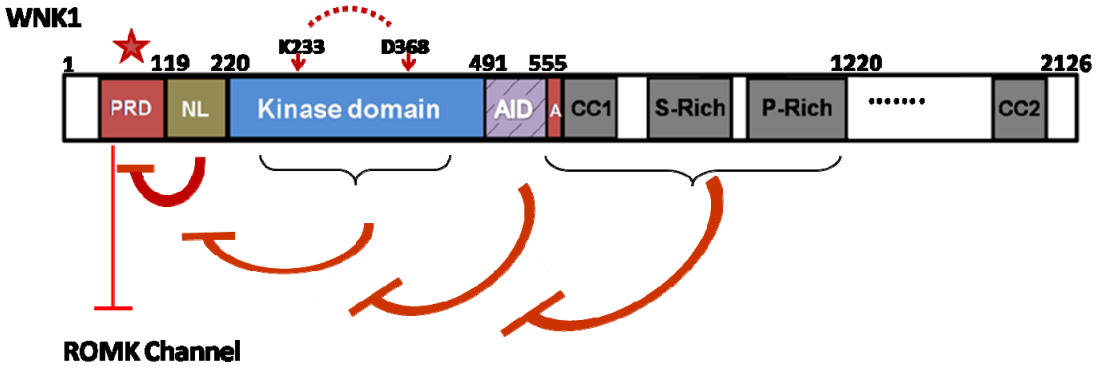

Figure 4-8.A model of role of different domains of WNK1 in regulating ROMK. (See text for detail)

DISCUSSION

In previous chapters, I showed that specific proline-rich motifs in the N-terminal proline-rich domain of WNK1 (N-PRD) are important for the inhibition of ROMK. N-PRD binds and recruits the endocytic scaffold protein intersectin to enhance the endocytosis of the channel.

In the present study, I further examined the role of the entire domain structure of WNK1 in the regulation of ROMK as shown in the working model [Figure 4-8]. Though we cannot exclude the possibility that some of the expressed short peptide domains may act differently from the full-length protein, the totality of our results in this and previous studies are consistent and allow us to propose the following working model for WNK1 regulation of ROMK [Figure 4-8].

As shown in the working model [Figure 4-8], The N-terminal PRD domain inhibits ROMK (through recruitment of intersectin, not shown in Figure 4-8). The N-linker (NL) domain inhibits the function of N-PRD domain. WNK1 kinase domain antagonizes the function of NL domain towards N-PRD domain. The AID domain further modulates the coupling between WNK1 kinase domain and N-Linker domain, indirectly regulates the function of N-PRD domain. Finally, the first C-terminal coiled-coil domain (CC1) prevents the AID from inhibiting the kinase domain. The role of WNK1 kinase domain deserves further discussion. Previously, we and others reported that kinase-dead WNK1 caused by charge-neutralization mutations of the catalytic lysine or a conserved aspartate fails to regulate ROMK [Kahle KT, 2005; Lazrak A, 2006]. In a more recent study, we suggested that the role of WNK1 kinase domain in regulating ROMK requires a properly folded kinase domain maintained by charge-charge interactions between the catalytic lysine (L233) and the conserved aspartate (D368). Thus, disruption of the charge-interaction

within the kinase domain abolished WNK1 regulation of ROMK, presumably by preventing the PRD from binding with ITSN.

How does the kinase domain affect the function of the N-PRD? We identified an N-Linker domain between N-PRD and kinase domain. The NL domain inhibits the function of N-PRD domain and the inhibition can be reversed by a properly folded kinase domain. The AID domain of WNK1 inhibits catalytic activity of the kinase domain, presumably by direct binding to the kinase domain [Lenertz LY, 2005]. In our model, AID domain also affects the function of kinase domain in the regulation of ROMK. The first coiled-coil domain (CC1) following AID domain, in turn, interferes with the inhibitory effect of AID domain towards kinase domain. This effect of CC1 on AID domain may underscore that full-length WNK1 or constructs that contain the region of amino acids 1 through 640 of WNK1 [such as WNK1(1-1200) and WNK1(1-555) plus WNK1 (555-640)] inhibit ROMK.

In sum, our data provide a scenario where these different domains of WNK1 are coupled through physical interactions to regulate clathrin-mediated endocytosis of target proteins. Since WNK1 forms multimers, the multiple domain interactions may occur between subunits as well as within subunits. Moreover, it may occur between different WNK kinases [Lenertz LY, 2005; Yang CL, 2003, 2007].

CHAPTER FIVE: Kidney Specific WNK1 in the regulation of ROMK channel

ABSTRACT

WNK1 has multiple alternatively spliced isoforms including a ubiquitously expressed long form (WNK1) and a kidney-specific form (KS-WNK1) predominantly expressed in the kidney. As previously reported, KS-WNK1 does not inhibit ROMK, but antagonizes the inhibition of ROMK by WNK1. Here, we identified that two regions of KS- WNK1 are involved in the antagonism of WNK1: One region is a 4a domain encoded by the alternatively spliced initiating exon4A of KS-WNK1; the other region is equivalent to the autoinhibitory domain (AID) of WNK1. KS-WNK1 from forms SDS/DTT resistant oligomer (most likely 16mer) that could bind tightly with the membrane, which is possibly facilitated by palmitoylation. Mutations of 4 cysteines in the 4a domain abolished the antagonism of 4a domain of KS-WNK1 to WNK1. Inhibition of palmitoylation by 2-BP prevent 4a domain from antagonizing WNK1, and decreased its binding with WNK1, but the function of AID domain was not affected by 2-BP. In biochemical and imaging experiments, deletion or mutation of the 4a domain disrupted the membrane targeting of KS-WNK1. The inhibition of palmitoylation subtly affects the location of KS-WNK1 in the lipid raft. Hydrogen peroxide, which is produced during K deficiency, abolished the function of the 4a domain, possibly by oxidizing the cysteines residues important for palmitoylation. Finally, KS-WNK1 4a domain binding with WNK1 decreased the interaction between WNK1 and Intersectin SH3 C domain.

INTRODUCTION

A WNK1 transcript produced from all 28 exons (encodes a peptide referred to herein as WNK1, WNK1) is ubiquitously expressed [Xu BE, 2000]. A shorter WNK1 transcript produced by an alternative 5'exon (exon4A) and exon5-28 is expressed exclusively in the kidneys, encoding the so called kidney-specific WNK1 (KS-WNK1) [Xu Q, 2002; Verissimo F, 2001].

KS-WNK1 is about 1,700 amino acids in length and lacks amino acids 1-437 of the WNK1 that are encoded by exon1-4. The first 30 amino acids of KS-WNK1 are encoded by exon4A [Delaloy C , 2003]and is unique to KS-WNK1. In the kidney, KS-WNK1 is predominantly expressed in the distal convoluted tubule, the connecting tubule, and the cortical collecting duct, suggesting a role in these tubules. KS-WNK1 transcription is induced by aldosterone and enhances ENaC-mediated Na+ transport [Náray-Fejes-Tóth A, 2004]. KS-WNK1 transcript in the kidneys is more abundant than that for WNK1, accounting for 90% of the transcript of WNK1 gene as reported previously [Delaloy C , 2003]. Yet, the relative protein abundance of KS-WNK1 v.s. WNK1 has not been determined. Large deletions within the first intron of *WNK1* increase the abundance of *WNK1* transcript and cause PHAII. One recent report suggests that the deletion of the first intron causes ectopic expression of KS-WNK1 outside of kidney [Delaloy C, 2008]. However, the effects of KS-WNK1 ectopically expressed in other tissues have not been studied.

Kidney specific WNK1 does not inhibit ROMK but antagonizes the inhibition of ROMK caused by WNK1 [Lazrak A, 2006]. K+ secretion by the kidneys is critical for controlling serum K+ levels and overall K+ homeostasis. As an important pathway for K+ secretion in kidney, the abundance of ROMK on the apical membrane of the distal nephron is regulated by dietary K+ intake [Wang

WH, 2006]. We found that dietary K+ restriction in rats increases the expression of WNK1 and decreases that of KS-WNK1 [Zeng WZ, 2002]. The increase in WNK1 to KS-WNK1 ratio would cause inhibition of ROMK. These results suggest that KS-WNK1 is an important physiological antagonist of WNK1 and the ratio of WNK1 to KS-WNK1 regulates surface abundance of ROMK and renal K+ secretion during changes in dietary K+ intake.

In the present study, we further examined the mechanism by which KS-WNK1 antagonizes WNK1 regulation of ROMK. We identified two regions within amino acids 1-253 of KS-WNK1 that are involved in binding to and antagonizing WNK1. One of them is the cysteine-rich domain known as the 4a domain (encoded by exon 4A), the other is the AID. Similar to the function of AID, KS-WNK1 4a domain has been reported to inhibit WNK1 kinase activity. The antagonism may be related to an alteration of the kinase domain conformation by binding.

K+ deficiency induced oxidative stress in the kidneys. Application of H_2O_2 quickly (within 10 min) inhibited ROMK activity in isolated CCD cells [Wei Y, 2007]. The inhibition of ROMK by H_2O_2 was not due to direct inhibition of ROMK activity, but was caused by clathrin-mediated endocytosis. It was proposed that tyrosine kinase c-SRC and MAPK is responsible for stimulating endocytosis of ROMK; however, application of the tyrosine kinase c-SRC and MAPK inhibitors individually did not significantly reverse inhibition of ROMK by H_2O_2. The author reported that an addition of 3 inhibitors for c-SRC, p38 and ERK altogether blocked the inhibition of ROMK by H_2O_2. However, the addition of 3 potent inhibitors of important kinases together may result in unexpected and complicated results [Wei Y, 2007]. It has been reported that the kinome (including all the kinase) plays critical roles in endocytosis [Pelkmans L, 2005], the effects of multiple kinase inhibitors may not be specific to H_2O_2-mediated endocytosis of ROMK but rather inhibited endocytosis in

general. Thus, the H_2O_2 -mediated endocytosis of ROMK in CCD cells has not been satisfactorily explained.

KS-WNK1 is abundant in distal nephrons [Delaloy C, 2003]. 4a domain is unique throughout the proteome in that it clustered as many as 6 cysteine residues within a sequence of only 30 amino acids long. Cysteine is the most reductive residues among all amino acids. We hypothesize that the cysteine-rich 4a domain functions as a sensor for oxidative stress during K+ deficiency. Oxidation of the cysteine residues by H_2O_2 could have affected the antagonism of KS-WNK1 to WNK1-mediated endocytosis of ROMK, thus increased endocytosis of ROMK by WNK1.

The clustered cysteines residues in the 4a domain are predicted to be protein palmitoylation sites by CSS-Palm2.0 [http://csspalm.biocuckoo.org/]. The residues preceding the cysteine cluster are hydrophobic, and oligomerization may further enhance the hydrophobicity of the 4a domain. These features favor a palmitoylation process, since palmitoyl-transferases reside in the lipid membrane.

Protein palmitoylation or protein S-acylation is the thioesterification of fatty acyl moieties, typically the 16 carbon palmitoyl moiety, to certain protein cysteines. Palmitoylation could orchestrate a variety of cellular processes, including protein interaction with membrane/ lipid raft or other proteins, protein function, sorting and stability, so on and so forth. For example, many G proteins like H- and N-Ras, or some Rho proteins, rely on palmitoylation for proper membrane-localization in order to function [Baekkeskov S, 2009].

Another unique feature of protein palmitoylation is the reversibility. The regulated addition and removal of the palmitoyl moiety from protein by palmitoyl-transferases and acyl protein

thioesterases respectively, provide an attractive mechanism for controlling membrane and/or raft association or protein interaction [Baekkeskov S, 2009]. It is also interesting to note that palmitoylation is sensitive to the cellular redox state, for example, the palmitoylation and membrane trafficking of newly synthesized caveolin can greatly be blocked by oxidative stress [Parat MO, 2002].

Palmitoylation requires the un-oxidized cysteine residue to accept the palmitoyl moiety, while oxidative stress may oxidize the thiol group in cysteine, thus prevented palmitoylation. Due to the fact that cysteine is the most sensitive residue towards oxidation because of the thio group, by accepting the palmitoyl moiety, palmitoylation seems like an attractive mechanism in sensing the redox state and regulating the function of KS-WNK1 *in vivo*.

Material and Methods

Molecular Biology: pEGFP-ROMK, pCMV-Myc-WNK1(1-491), pIRES-Flag-KS-WNK1(1-253) were described previously [Liu Z, 2009]. WNK1 fragments were amplified by PCR using rat WNK1 cDNA as the template and subcloned into a pCMV5-Myc vector. Fragments of rat KS-WNK1were amplified by PCR and subcloned into a vector with C-terminal Flag (pIRES-hrGFP-1a)(Stratagene). Point mutations were generated by site-directed mutagenesis (Quickchange kit, Stratagene) and confirmed by sequencing.

Patch clamp assay and cell culture: as described in the chapter 2.

Membrane preparation: Preparation of the cytosol and membranes Cells were washed with PBS and scraped in a solution containing 0.25 M sucrose, 20 mM Tris/HCl (pH 7.5), 0.1 M NaCl, 1 mM EDTA, 0.2 mMPMSF, and protease inhibitor cocktail. Cells were lysed by two freeze–thaw cycles and passed through a 27.5 gauge needle. Lysates were then centrifuged at 1000 g for 5 min to obtain PNS (post-nuclear supernatants). The PNS were then centrifuged at 200000 g for 15 min to separate the cytosol from membranes. The resulting membrane pellets were homogenized in one of the following solutions: (i) To extract peripheral proteins: 0.1 M Na2CO3 (pH 11) . (ii) To extract integral proteins : 1% (v/v) Triton X-100, 20 mM Tris/HCl (pH 7.5) and 1 mM EDTA. After homogenization, samples were again centrifuged at 200000 g for 15 min at 4◦C to remove insoluble material. Next, proteins are solved in loading buffer and western blotted. In some cases, the membrane fractions can be prepared sequentially. In these cases, pellets were first homogenized in 0.1 M Na2CO3 (pH 11) to extract peripheral proteins and then homogenized in solutions containing 1%Triton X-100 to extract integral proteins [Barylko B, 2009].

Co-immunoprecipitation and GST pull down, GST pull down is done as described in the chapter 3. For coimmunoprecipitation, proteins were immunoprecipitated from cell lysates by using monoclonal anti-Flag antibody (1:100 dilution; Sigma) followed by protein A sepharose beads. Precipitates were washed three times with 50 mM Tris-HCl (PH 7.4), 150 mM NaCl, 0.5% Triton X-100. For western blot analysis, total lysates, immunoprecipitates or kidney homogenate were resolved by SDS-PAGE gel electrophoresis, and proteins were transferred onto nitrocellulose membranes. Membranes were incubated with the indicated antibodies and developed by using enhanced chemiluminescence.

Immunostaining: the procedure for immunostaining is as described in Chapter 3. Mouse or rabbit anti-flag antibody were used to stain Flag tagged KS-WNK1 constructs. Mouse antibody against Caveolin 1 were used to stain caveolar structure. Alexar 564 labeled goat-anti-rabbit secondary antibody and Cy5 labeled goat-anti-mouse (sigma) were used for visualization.

Quantitation of bands in western blotting: image of western blotting were imported to Adobe photoshop, and the cooresponding bands were circled. The pixel and signal intensity median of the circled band were then multiplied to represent the protein amount. The band of interest was then normalized to the corresponding control band. The ratio was plotted in Prizm 3.0.

RESULT

4a domain and AID domain in KS-WNK1 are both involved in the antagonism of WNK1

Our lab has reported that both full length and KS-WNK1 (1-253) antagonize inhibition of ROMK by WNK1 full length and WNK1(1-491). The inhibition is mediated by KS-WNK1(1-253) binding to (1-491) of WNK1 [Lazrak A, 2006]. To further define the domains of KS-WNK1 involved in antagonism of WNK1 regulation of ROMK, several small fragments of KS-WNK1(1-196), (1-137), (1-77), and (31-253) respectively, shown in Figure 5-1A, were generated (by Dr. Liu Z).

As shown in Figure 5-1B, WNK1(1-491) inhibits ROMK and that KS-WNK1(1-253) reverses WNK1(1-491)-mediated inhibition of ROMK as we previously reported [Lazrak A, 2006]. Expression of KS-WNK1(1-253) alone slightly increased ROMK basal current [Lazrak A, 2006], possibly due to the antagonism of endogenous WNK1. Surprisingly, we found that basically every smaller piece of the KS-WNK1 we constructed reversed the inhibition of ROMK caused by WNK1(1-491) [4^{th}-6^{th} bar, Figure 5-1B].

WNK1 contains a conserved autoinhibitory domain (AID) adjacent to the kinase domain. The AID binds with and suppresses the activity of the kinase domain [Xu BE, 2002]. We have recently shown that AID domain of WNK1 can antagonize WNK1(1-491)-mediated inhibition of ROMK [Wang HR, 2008]. As KS-WNK1(1-253), KS-WNK1(1-196), KS-WNK1(1-137), and KS-WNK1(31-253) each contains the AID domain, it is not surprising that they each can reverse the effect of WNK1(1-491). However, the ability of KS-WNK1(1-77) to reverse the inhibition of ROMK by WNK1(1-491) is unexpected and suggests that additional region in KS-WNK1 is involved in interacting with and reversing the effect of WNK1(1-491). KS-WNK1 (1-77) contains a unique 30

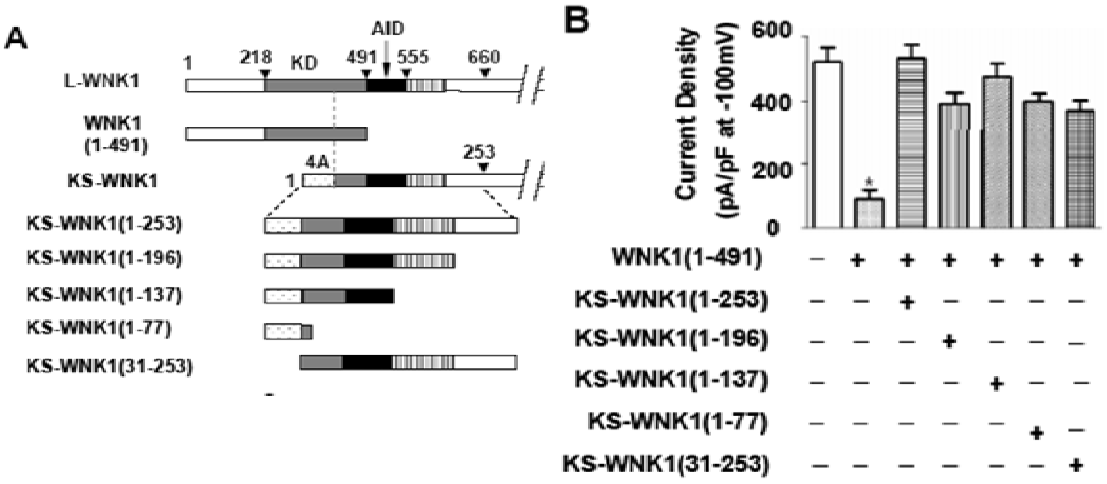

Figure 5-1. Effects of KS-WNK1 fragments on regulation of ROMK by WNK1.

A. Domain structure of WNK1 and KS-WNK1 (not drawn in scale). KS-WNK1 lacks the first 437 amino acids of WNK1 (encoded by exon1-4) but contains unique 30 amino acids coded by an alternatively spliced exon4A (the first exon of KS-WNK1). The vertical dotted line defines position of amino acid in WNK1 equivalent to amino acid 31 of KS-WNK1. Amino acids of WNK1 (438-2126) and KS-WNK1 (31-1719) distal to the dotted line (encoded by exon5-28) are identical. Amino acid 660 of WNK1 is equal to amino acid 253 of KS-WNK1. "AID", "KD", and "4A" indicate autoinhibitory domain, kinase domain,and region of KS-WNK1 encoded by exon4A, respectively. Fragments of KS-WNK1 used in the present study are shown.

B. Effects of various KS-WNK1 constructs on WNK1(1-491) inhibition of ROMK. HEK cells were transfected with ROMK alone or cotransfected with ROMK plus WNK1(1-491) and with KS-WNK1(1-253), KS-WNK1(1-196), KS-WNK1(1-137), KS-WNK1(1-77) or KS-WNK1(31-253) as indicated. Inward ROMK current density (pA/pF, at -100 mV) was shown. Results are mean ± sem; n = 5-10 for each group. * denotes $p < 0.05$ vs ROMK alone (white bar, 1st bar from left) by unpaired student's t-test. Results of experiments shown in 3rd-7th bar are not statistically significantly different from ROMK alone (white bar).

(Interaction between WNK(1-491) and various KS-WNK1 fragments. Lysates from cell cotransfected with Myc-tagged WNK1(1-491), Flag-tagged KS-WNK1(1-253), KSWNK1(1-77), KS-WNK1(31-253) or an unrelated Flag-tagged protein Pod1 were immunoprecipi -tated by anti-Flag antibody and probed for western blot analysis by anti-Myc and by anti-Flag antibody as indicated.)

amino acids domain encoded by exon4A. We hypothesized that this domain encoded by exon4A, or so called 4a domain is responsible for the antagonism of WNK1(1-491)-mediated inhibition of ROMK by KS-WNK1(1-77). The AID and 4a domain within KS-WNK1(1-253) may interact with WNK1(1-491) independently to reverse the effect of WNK1(1-491).

To support this hypothesis, we have reported that KS-WNK1(1-253), KS-WNK1(1-77) or KS-WNK1(31-253) all interact with (1-491) by co-immunoprecipitation. Consistently, the interaction between WNK1 (1-491) and KS-WNK1(1-253) is stronger than KS-WNK1(1-77) and with KS-WNK1(31-253) (Done by Zhen Liu, data not shown), suggesting that 4a and AID work together to antagonize WNK1 effect on ROMK in full length KS-WNK1.

The role of WNK1 kinase domain in regulation by KS-WNK1

In previous chapters, I found that several proline-rich motifs within WNK1(1-119) are necessary and sufficient for the inhibition of ROMK in HEK cells. Other regions of WNK1, including kinase domain, AID domain, etc contribute to the regulation of ROMK by direct or indirect modulation of the effect of WNK1(1-119). These findings raise the question of whether KS-WNK1 can antagonize WNK1(1-119) or the antagonism requires amino acids of WNK1 beyond 1-119. To answer this question, I co-transfected ROMK and WNK1 (1-119) with either KS-WNK1(1-253) [4th bar Figure 5-2B), KS-WNK1(1-77) (5^{th} bar) or KS-WNK1(31-253) (6^{th} bar). In addition, I examined the effect of WNK1(491-555) (3^{rd} bar), which is the AID of WNK1 and is identical to the AID of KS-WNK1. As shown, we found that none of the above constructs reversed the inhibition of ROMK caused by WNK1(1-119) [Figure 5-2B]. These results are in contrast to results in Figure 5-1B, which show that KS-WNK1 (1-253), KS-WNK1(1-77) and KS-WNK1(31-253) each reversed the inhibition of ROMK by WNK1(1-491). Thus, amino acids 120-491 of WNK1 are required for

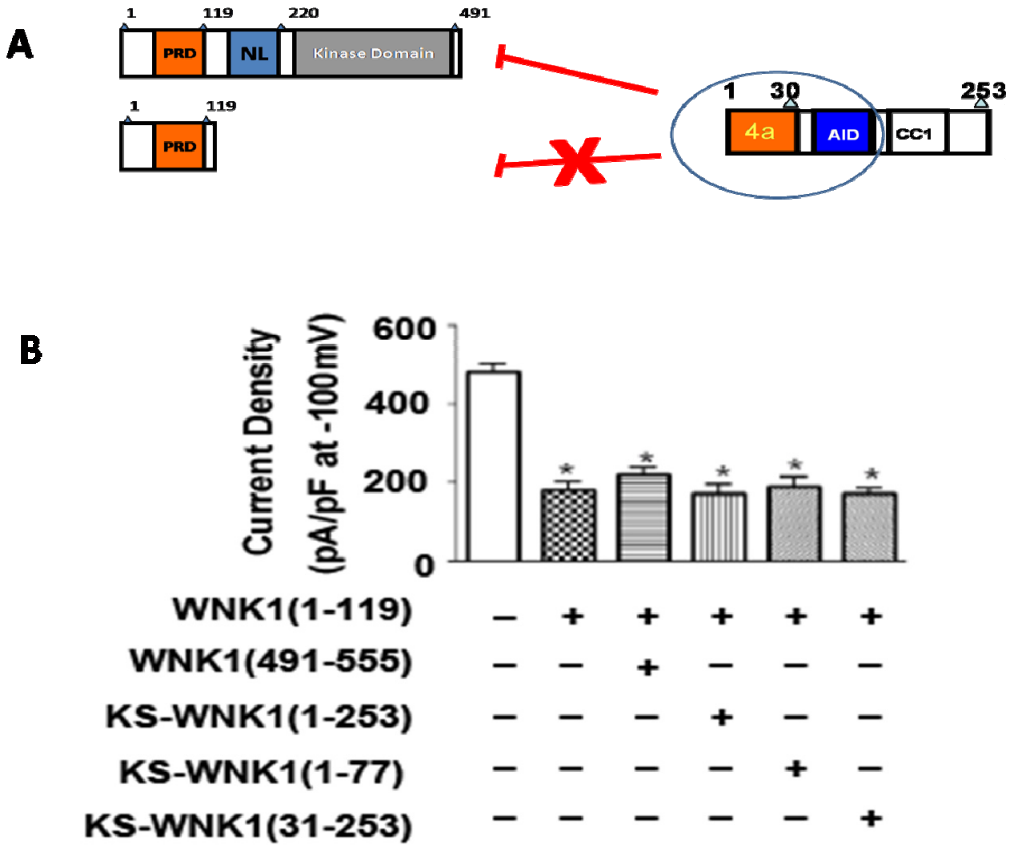

Figure 5-2. Role of WNK1 kinase domain for regulation by KS-WNK1.

A. Partial sequences of LWNK1 (amino acids 491-555) and KS-WNK1 (amino acids 84-149) surrounding AID domain. Amino acids phenylalanine-524 and -526 of WNK1 (= phenylalanine-117 and -119 of KS-WNK1) critical for the function of AID domain are shown. Double mutations of phenylalanine to alanine is abbreviated as "FFAA".

B. Effects of double phenylalanine mutations on KS-WNK1 antagonism of WNK1(1-491) inhibition of ROMK. Cells were co-transfected with ROMKand indicated constructs and recorded for ROMK current density using whole-cell recording. "FFAA"indicates double mutations of phenylalanine-117 and -119 of KS-WNK1 to alanine. Results are mean ±sem; n = 5-10 for each group. * denotes $p < 0.05$ between two indicated groups. NS, indicates statistically not significant. *D*, expression of KS-WNK1(1-253)/FFAA, KS-WNK1(31-253), and KSWNK1(31-253)/FFAA confirmed by western blot analysis using an anti-Flag antibody.

(Phenylalanine residues in AID domain are critical for interaction with WNK1(1-491). Lysates from cell cotransfected with Myc-tagged WNK1(1-491) and with Flag-WNK1(491-555) or Flag-WNK1(491-555)/FFAA were immunoprecipitated by anti-Flag antibody and probed in western blot analysis by anti-Myc or anti-Flag antibody as indicated.)

the antagonism by KS-WNK1 (1-253). To support the physiological data, our published data by Dr. Liu in our lab also suggested that both 4a and AID domain of KS-WNK1 does not bind with WNK1 (1-119). Together, these results support the hypothesis that amino acids 120-491 of WNK1 (which include the kinase domain [amino acids 220-491] and N-linker region [amino acids 120-220]; see ref. #21 for more details) are required for 4a and AID domain of KS-WNK1(1-253) to antagonize WNK1 inhibition of ROMK channel.

Role of the AID domain of KS-WNK1 in the regulation of ROMK

In chapter 4, I found that AID reverses WNK1 (1-491) inhibition of ROMK. Mutations of two phenylalanine residues within the AID of WNK1 abolished its binding and regulation of the kinase activity [Xu BE, 2002]. Mutations of these two conserved phenylalanine abolished the ability of AID to reverse WNK1(1-491)-mediated inhibition of ROMK [Xu BE, 2002]. I found that mutation of AID in KS-WNK1(31-253) ("KS-WNK1(31-253)/FFAA") prevented it from reversing WNK1(1-491)-mediated inhibition of ROMK [Figure 5-3C,compare 5th and 6th bar from left].

These results support that the AID within KS-WNK1(31-253) is responsible for reversing WNK1(1-491) inhibition of ROMK. In contrast, double phenylalanine mutations of the AID of KS-WNK1(1-253) did not significantly affect its ability to reverse WNK1(1-491) inhibition of ROMK [Figure 5-3C, compare 3rd and 4th bar from left]. These results support the hypothesis that the 4a domain within KS-WNK1(1-253) by itself is sufficient for interacting and reversing the effect of WNK1(1-491). All the KS-WNK1 constructs used in patch clamping are expressed at the similar level as revealed by western blotting (by Zhen Liu).

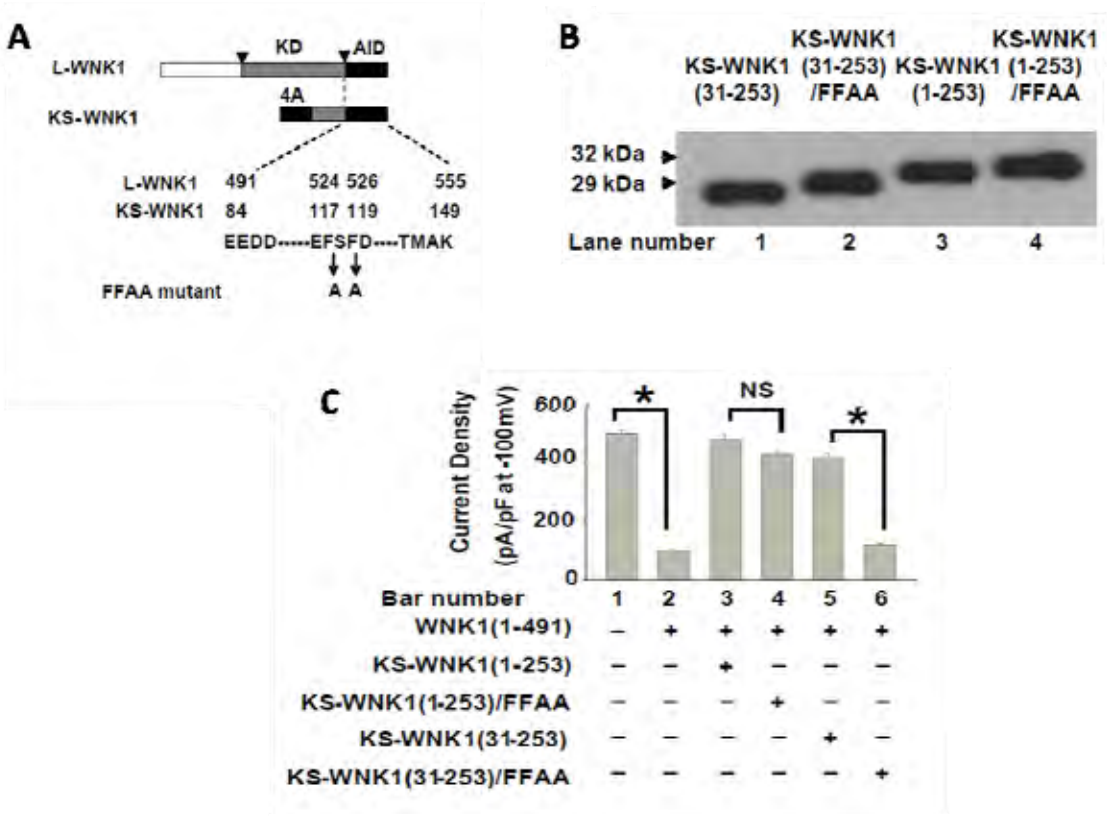

Figure 5-3. Role of KS-WNK1 autoinhibitory domain in regulation of ROMK.

A. Partial sequences of LWNK1 (amino acids 491-555) and KS-WNK1 (amino acids 84-149) surrounding AID domain. Amino acids phenylalanine-524 and -526 of WNK1 (phenylalanine-117 and -119 of KS-WNK1) critical for the function of AID domain are shown. Double mutations of phenylalanine to alanine is abbreviated as"FFAA".

B. Effects of double phenylalanine mutations on KS-WNK1 antagonism of WNK1(1-491) inhibition of ROMK. Cells were co-transfected with ROMK and indicated constructs and recorded for ROMK current density using whole-cell recording. "FFAA" indicates double mutations of phenylalanine-117 and -119 of KS-WNK1 to alanine. Results are mean ±sem; n = 5-10 for each group. * denotes $p < 0.05$ between two indicated groups. NS, indicates statistically not significant.

C. Expression of KS-WNK1(1-253)/FFAA, KS-WNK1(31-253), and KSWNK1(31-253)/FFAA confirmed by western blot analysis using an anti-Flag antibody.

Cysteine residues in the region encoded by exon4A is crucial for the KS-WNK1(1-77) oligomerizing and antagonism of WNK1

We found that KS-WNK1(1-77) can antagonize WNK1(1-491) and associate to form oligomers[Figure 5-4C]. We thus asked whether oligomerization is important for KS-WNK1(1-77) antagonism of WNK1(1-491). Within amino acids 1-77 of KS-WNK1, the region of unique NH2-terminal 30 amino acids encoded by exon4A is rich in cysteine residues [Figure 5-4A]. We examined the role of 4 successive cysteine residues at positions 19 through 22 in oligomerization and the role of oligomerization in the binding of KS-WNK1(1-77) and the antagonism of WNK1(1-491) [Figure 5-4C]. We first examined the role of these cysteine residues in the reversal of inhibition of ROMK by WNK1(1-491). As shown, a mutant KS-WNK1(1-77) carrying quadruple mutations of cysteine-19, -20,-21, and -22 ("KS-NK1(1-77)/CCCCSSSS") didn't reverse WNK (1-491)'s effect on ROMK[Figure 5-4*B*, compare 3rd and 4th bar from left]. For comparison, wild type KS-WNK1(1-77) [3rd bar Figure 5-4B] and a mutant carrying double mutations of cysteine-20 and -21 to serine ("KS-WNK1(1-77)/CCSS", 5th bar) remain capable of antagonizing WNK1(1-491) inhibition of ROMK. These results suggest that all four cysteine residues at position 19 through 22 are essential for the reversal effect of KS-WNK1(1-77) on WNK1(1-491).

The region encoded by exon4A contains a stretch of two phenylalanine residues (at position8 and 12) spaced by 3 amino acids [Figure 5-4A]. We asked whether this stretch of amino acids may function similarly to the FXF motif of the AID with respect to the reversal of WNK1(1-491). To test this possibility, Dr Liu Z in the lab made double mutations of phenylalanine ("KS-WNK1(1-77)/FFAA"), and I found that the mutant didn't affect the ability of KS-WNK1(1-77) to reverse WNK1(1-491)-mediated inhibition of ROMK [6th bar Figure 5-4B]. I further examined the ability of double cysteine, quadruple cysteine, and double phenylalanine mutants to oligomerize and to

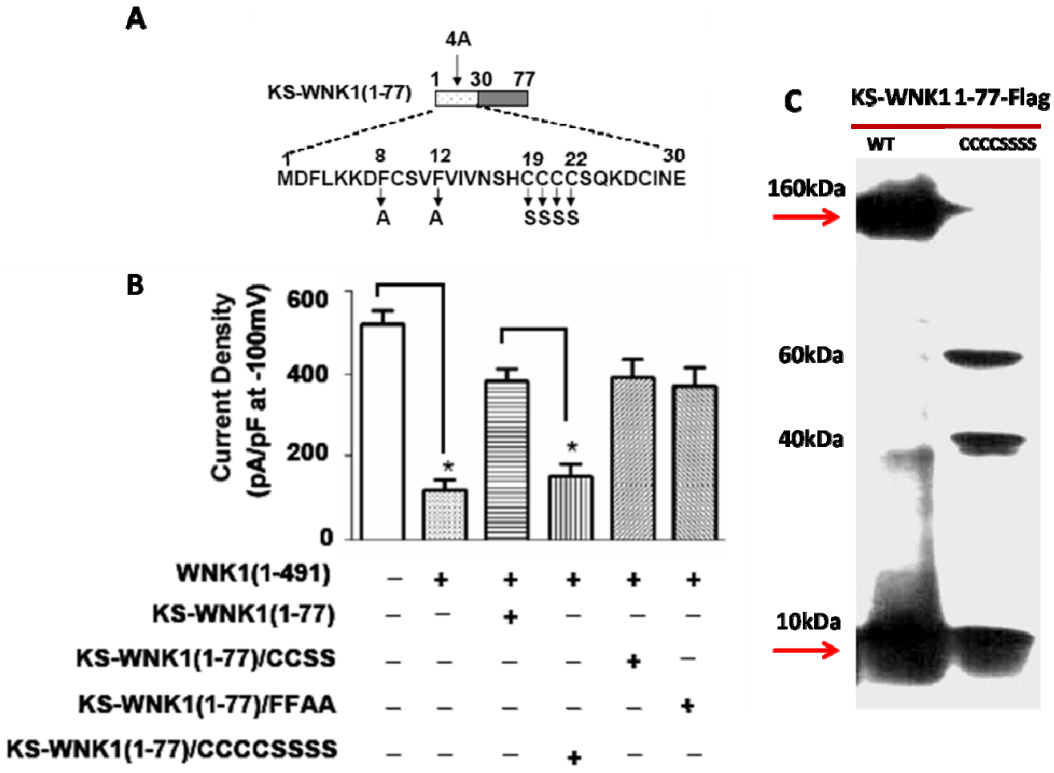

Figure 5-4. Role of cysteine residues within 4A domain in antagonism of WNK1 inhibition of ROMK.

A. Amino acid sequence of the region of KS-WNK1 encoded by exon4A showing multiple cysteine residues.

B. Effects of cysteine and phenylalanine mutations on antagonism of WNK1(1-491) inhibition of ROMK by KS-WNK1(1-77). Experimental paradigm is the same as in Fig.1*B*. "CCCCSSSS" and "CCSS" indicates quadruple and double cysteine to serine mutations, respectively. "FFAA" indicates double mutations of phenylalanine-8 and -12 to alanine. Results are mean ± sem; n = 5-8 for each group. * denotes $p < 0.05$ between indicated groups by unpaired student's t-test.

C. Western blotting of KS-WNK1(1-77) and KS-WNK1(1-77) 4CS mutant.

interact with WNK1 (1-491). Wild type KS-WNK1 (1-77) migrates in monomeric as well as oligomeric forms in SDS-PAGE gel electrophoresis [Figure 5-4C]. The quadruple cysteine mutant exists mostly as a monomer and did not interact with WNK1 (1-491) in the co-immunoprecipitation assay. In contrast, the double cysteine mutant and double phenylalanine mutant retain the ability to oligomerize (160 kDa band) and to interact with WNK1 (1-491) (done by Zhen liu, data not shown). The ~20, 40, 60 kDa molecular size band (indicated by arrows) probably represent tetramers and 6mers [Figure 5-4C]. These results support the hypothesis that the four cysteine residues at positions 19 through 22 are critical for KS-WNK1(1-77) to oligomerize, and that oligomerization is important for its antagonism of WNK1(1-491).

Palmitoylation may be important for the function of KS-WNK1 4a domain

After blasting the four cysteine residues of 4a domain [Figure 5-5A] in the genome, I found that this type of structure is very rare. The appearance of such rare structure usually indicates its important functional/regulatory role. Among them, modification of the cysteines by palmitoylation poised as an attractive hypothesis.

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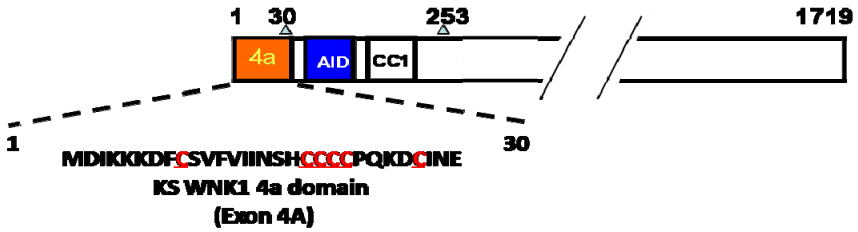
CSS-Palm 2.0 is a popular software for predicting protein palmitoylation sites based on sequences of palmitoylated proteins that are experimentally identified [Ren J, 2008]. As shown in Figure 5-5B: CSS-Palm 2.0 predicts that multiple cysteines in KS-WNK1 4a domain may be palmitoylated, with the 4 clustered cysteines scoring the highest. The most definitive way to identify protein palmitoylation is through 3H palmitate acid labeling of interested proteins, which usually takes weeks even months for exposure. Due to technical difficulties, till now, I haven't got any positive result from the labeling experiments. However, there are other experiments suggesting that palmitoylation of KS-WNK1 may contribute to the functionality of

A

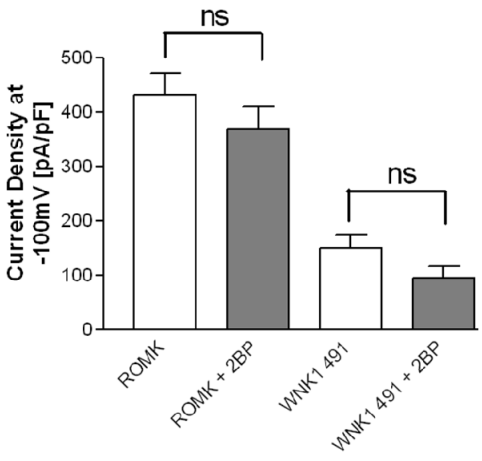


B

Palmitoylation site prediction of KS WNK1 4a domain by CSS-Palm 2.0

Position	Peptide	Score	Cutoff	Palmitoylation Type
9	KDFCSVF	1.209	0.8	TypeIII: Others
19	NSHCCCC	1.905	0.8	TypeII: -CXXC-
20	SHCCCCS	4.062	0.7	TypeI: -CC-
21	HCCCCSQ	3.781	0.7	TypeI: -CC-
22	CCCCSQK	1.429	0.8	TypeII: -CXXC-

C



D

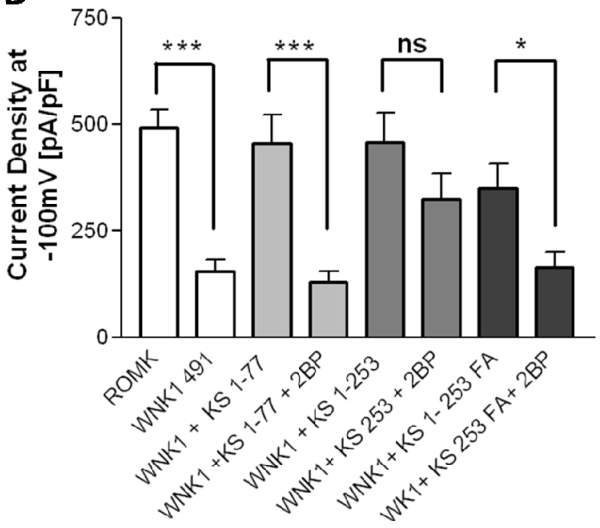


Figure 5-5. Palmitoylation inhibitor 2-BP abolishes antagonism of WNK1 (1-491) by the 4a domain of KS-WNK1

- **A.** Diagram of KS-WNK1 with the sequence of 4a domain in the N-terminus.
- **B.** Palmitoylation site prediction of KS-WNK1 4a domain by CSS-Palm 2.0. the 4 clustered cysteines scores highest for potential palmitoylation
- **C.** 2-BP did not affect the current density of ROMK nor the function of WNK1 (1-491).
- **D.** The effect of 2-BP on KS-WNK1 constructs. 2-BP abolished the inhibition of WNK1 491 by KS-WNK1 4a domain; 2-BP does not affect the function of AID domain as KS-WNK1 1-253 can still antagonize WNK1; disruption of AID domain make KS-WNK1 1-253 FFAA completely sensitive to 2-BP inhibition as WNK1 1-77 does.

KS-WNK1. 2-bromopalmitate (2-BP, Sigma) is a widely used inhibitor of palmitoylation. As shown in Figure 5-5D, when treat the cell was treated with 100uM 2-BP over-night, the KS-WNK1 4a domain (1-77) lost the ability to antagonize WNK1 inhibition of ROMK. ROMK current density in ROMK alone and WNK1 491 group were not affected by 2-BP treatment, suggesting 2-BP does not affect ROMK activity or WNK1 inhibition of ROMK by itself [Figure 5-5C]. The effect of 2-BP seems to be specific to the 4a domain. KS-WNK1 1-253, which also harbors an AID besides 4a domain, still partially reversed WNK1 effect upon 2-BP treatment. However, when we disrupted the AID by mutating the two conserved phenylalanine residues, KS-WNK1(1-253)FFAA antagonism of WNK1 became completely 2-BP sensitive. These results suggest that palmitoylation of KS-WNK1 may be required to antagonize WNK1.

The binding of KS-WNK1 to WNK1 is important for antagonizing WNK1. We then ask the question of whether blockage of palmitoylation by 2-BP treatment affects the interaction between WNK1 and KS-WNK1. We expressed myc-WNK1 1-491 with different KS-WNK1-flag constructs (KS-WNK1 1-77, KS-WNK1 1-253 and KS-WNK1 (33-253) lacking 4a domain) in HEK cells treated with or without 100μM 2-BP for 24hrs. Mouse Flag antibody and Agarose A beads were then used to pull down KS-WNK1 complex. As shown in Figure 5-6A upper panel (western blotting of the immunoprecipitated myc-WNK1 $_{1\text{-}491}$), agarose A beads does not pull down WNK1 491, (7^{th} lane from the left), while all the KS-WNK1 constructs co-precipitate with myc-WNK1 491 with different affinity in the control groups. More importantly, 2-BP treatment decreased the binding affinity of both KS-WNK1 (1-77) and KS-WNK1 (1-253) [2nd and 4th lane Figure 5-6A], which contains the 4a domain. In contrast, WNK1 (1-491) binding with KS-WNK1 (33-253), which lacks the 4a domain, were not affected by 2-BP suggesting that the AID binding with WNK1 may not be affected by 2-BP [5th and 6th lane Figure 5-6A]. These results correlate well with the

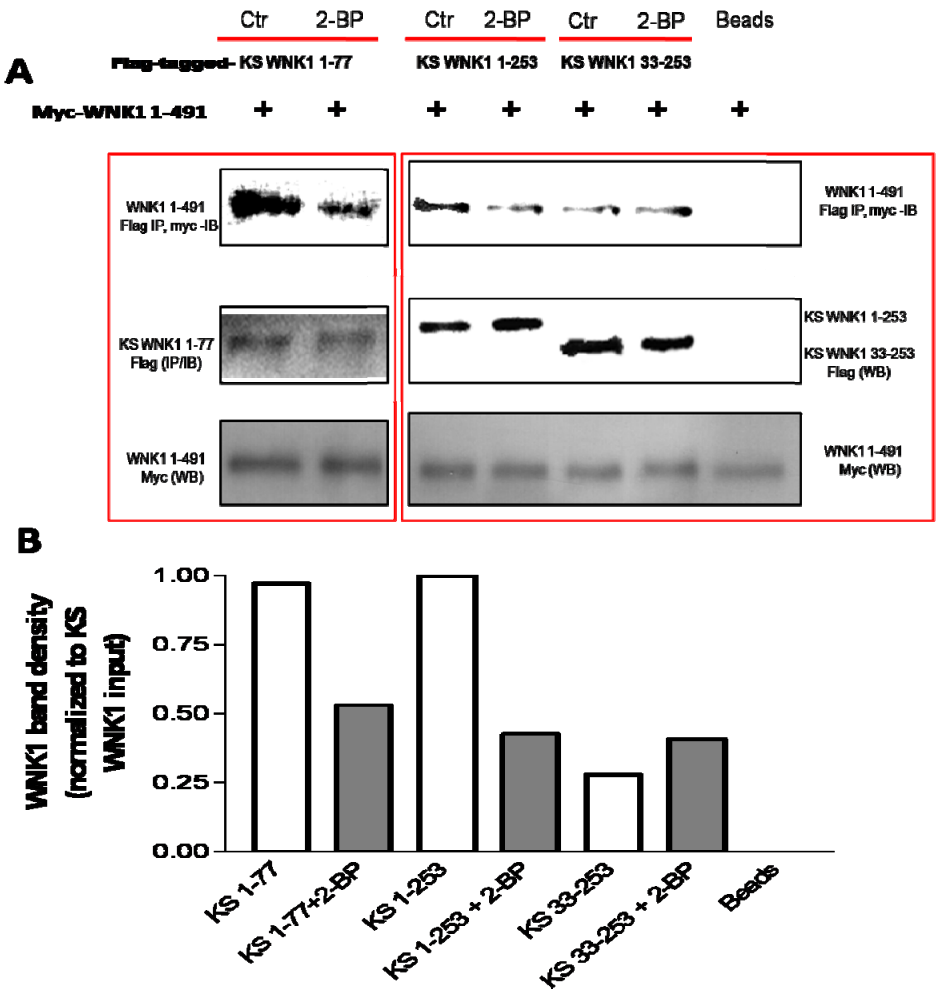


Figure 5-6. Physical interaction between KS-WNK1 4a domain and WNK1 491 was impaired by palmitoylation inhibitor 2-BP.

- **A.** The effect of 2-BP on KS-WNK1 constructs interaction with WNK1 491. In the left panel, 2-BP impaired the interaction of WNK1 491 by KS-WNK1 1-77 compared with control, given similar KS-WNK1 1-77 and WNK1 expression in control and 2-BP group; In the right panel, 2-BP does not affect the binding of KS-WNK1 33-253 to WNK1; In the middle panel, WNK1 1-253 binds more WNK1 than KS-WNK1 33-253 even at lower abundance as shown in the WB of KS-WNK1 constructs, however, 2-BP treatment decreased the binding affinity of KS-WNK1 1-253 with WNK1 to level comparable with that of KS-WNK1 33-253 AID domain. WNK1 (1-491) are expressed in similar amount in all comparable groups.
- **B.** Quantitation of the WNK1 491 co-immunoprecipited with KS-WNK1 constructs. The bands intensity was calculated by size × intensitity, each WNK1 bands were normalized to the corresponding precipitated KS-WNK1 bands.

physiological data, in which KS-WNK1 (1-77) antagonism of WNK1 were completely abolished by 2-BP, while WNK1 1-253 were less affected due to its AID. Figure 5-6B shows the immunoprecipitated WNK1(1-491) bands normalized to the corresponding immunoprecipitated KS-WNK1 constructs in each lane shown above.

The sub-cellular distribution of KS-WNK1

The localization of protein is important for its function. Protein palmitoylation regulate the trafficking of protein to the membrane. A protein must also first bind to membrane structures to be palmitoylated since most of the palmitoyl-transferase resides in the plasma membrane. Localization in plasma membrane may help a protein to regulate endocytosis. It has been know that WNK (1-491) binds to plasma membrane; however, there is no report of the localization of KS-WNK1, which is important for the function of KS-WNK1. In the following work, I utilized biochemical methods and imaging technique to study the distribution of KS-WNK1.

Firstly, we over-expressed KS-WNK1 (1-77) and KS-WNK1 (1-77) 4CS into HEK cells, and used ultra-centrifuge to extract the cytosol fraction (in TNE buffer, Tris-HCL 50mM, NaCl 140mM, 1mM EDTA pH 7.4), peripheral membrane (in Na2CO3, pH 11), and integral membrane fraction (in TNE buffer + 1% Triton). Western blotting of each fraction showed that KS-WNK1 (1-77) wt and KS-WNK1 (1-77) 4CS has distinctive distribution pattern. As shown in Figure 5-7C, KS-WNK1 (1-77) exists primarily in 16mer and monomer in the cytosol fraction, oligomerized form of KS-WNK1 has high affinity with the plasma membrane since it lies in the integral membrane fraction, but the monomer binds only loosely with the plasma membrane as suggested by its presence in the peripheral membrane fraction. Mutation of 4 cysteine residues in KS-WNK1 abolished both the formation of 16mer and the binding affinity with any membrane fraction, however, KS-WNK1 with mutations in 2 cysteine residues in still able to form 16mer and binds to

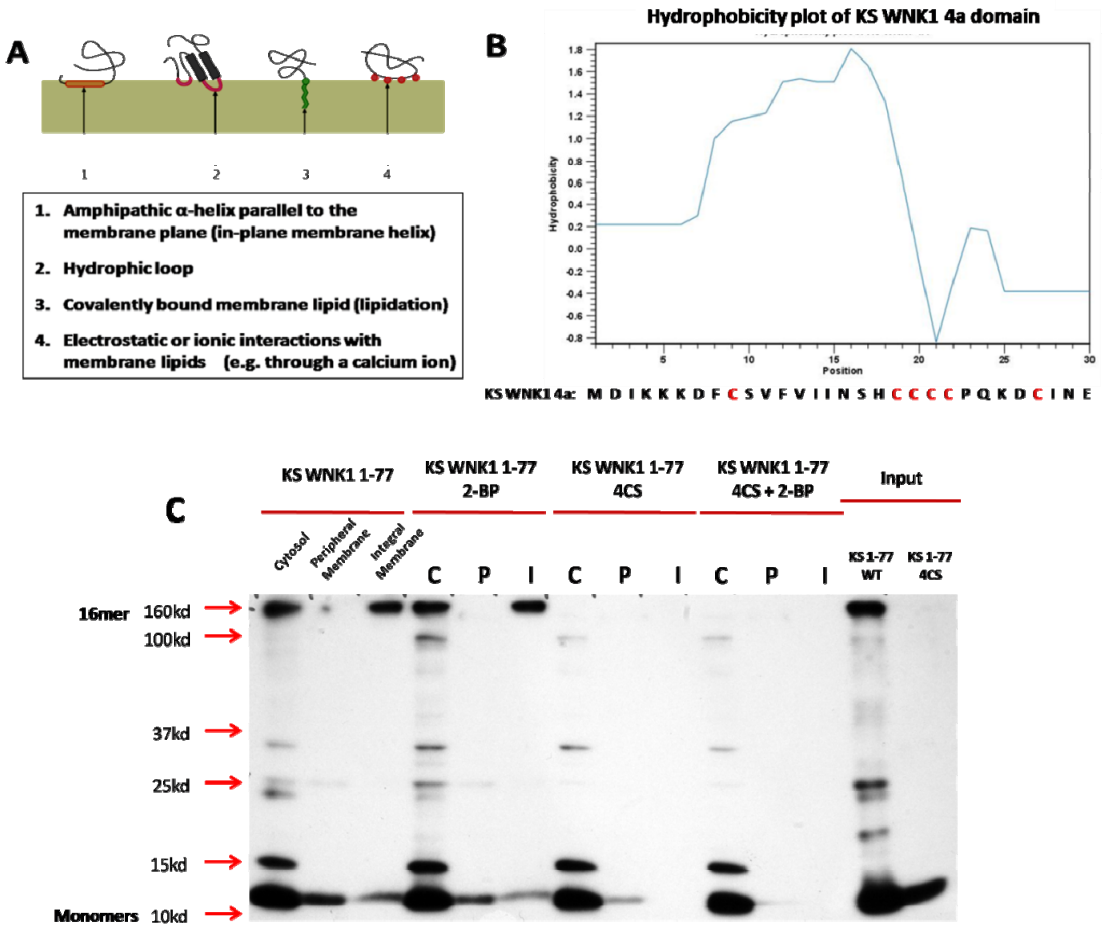


Figure 5-7. The distribution of KS-WNK1 4a domain

A. The mechanism for intracellular proteins to bind with lipid membrane. Peripheral membrane proteins usually are formed by 1.amphipathic α-helix parallel to the membrane plane; 2. hydrophic loop or 4.electrostatic or ionic interactions with membrane lipids. Mechanism 3: covalently bound membrane lipid, including palmitoylation, usually forms integral membrane proteins rather than peripheral membrane proteins.

B. KS-WNK1 4a domain is highly hydrophobic, which enable it to bind with lipid membrane as a peripheral membrane protein. Membrane binding is required for palmitoylation by enzymes. Oligomerization may further increase the hydrophobicity of KS-WNK1 4a.

C. KS-WNK1 1-77 exist in all fractions. The monomer can bind to peripheral membrane, while oligomer exist as integral membrane protein. 2-BP does not affect the distribution or oligomerization of KS-WNK1 greatly. However, mutation of cysteines in 4a domain abashed the oligomerization, and KS-WNK1 1-77 4CS mainly exists in the cytosol.

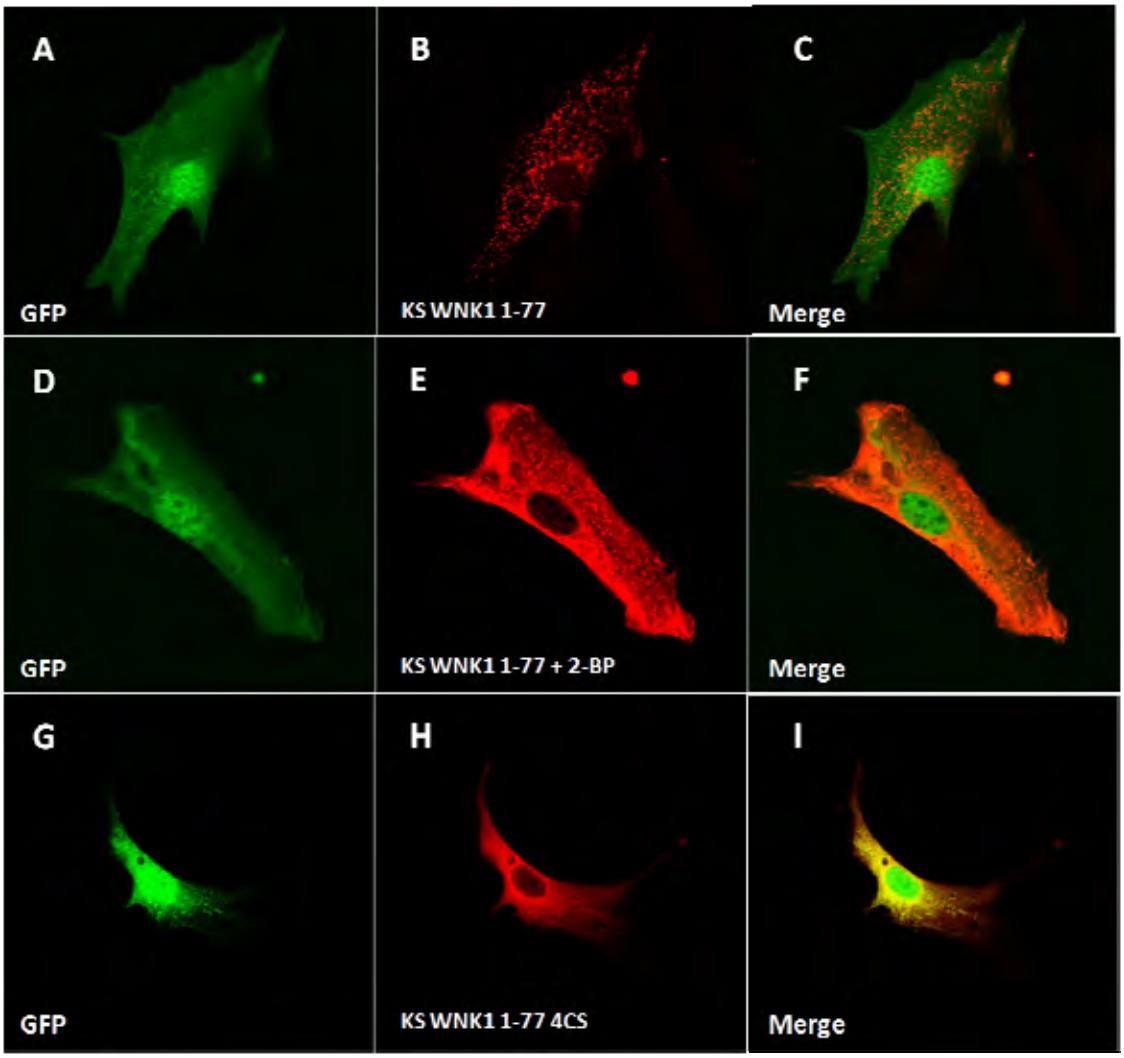


Figure 5-8. KS-WNK1 (1-77) WT and 4CS mutant has distinct subcellular distribution

- **A.** Panel **A.D.G.** are GFP signals of tranfected NIH 3T3 cells.
- **B.** Panel **B.E.H.** are flag-tagged KS-WNK1 1-77, KS-WNK1 1-77+ 2-BP and KS-WNK1 1-77 4CS respectively. Rabbit anti-Flag antibody and goat anti-rabbit secondary antibody labeled with alexar-564 were used for immunostaining.
- **C.** Panel **C.F.I** are merged imagine of GFP and flag-staining.

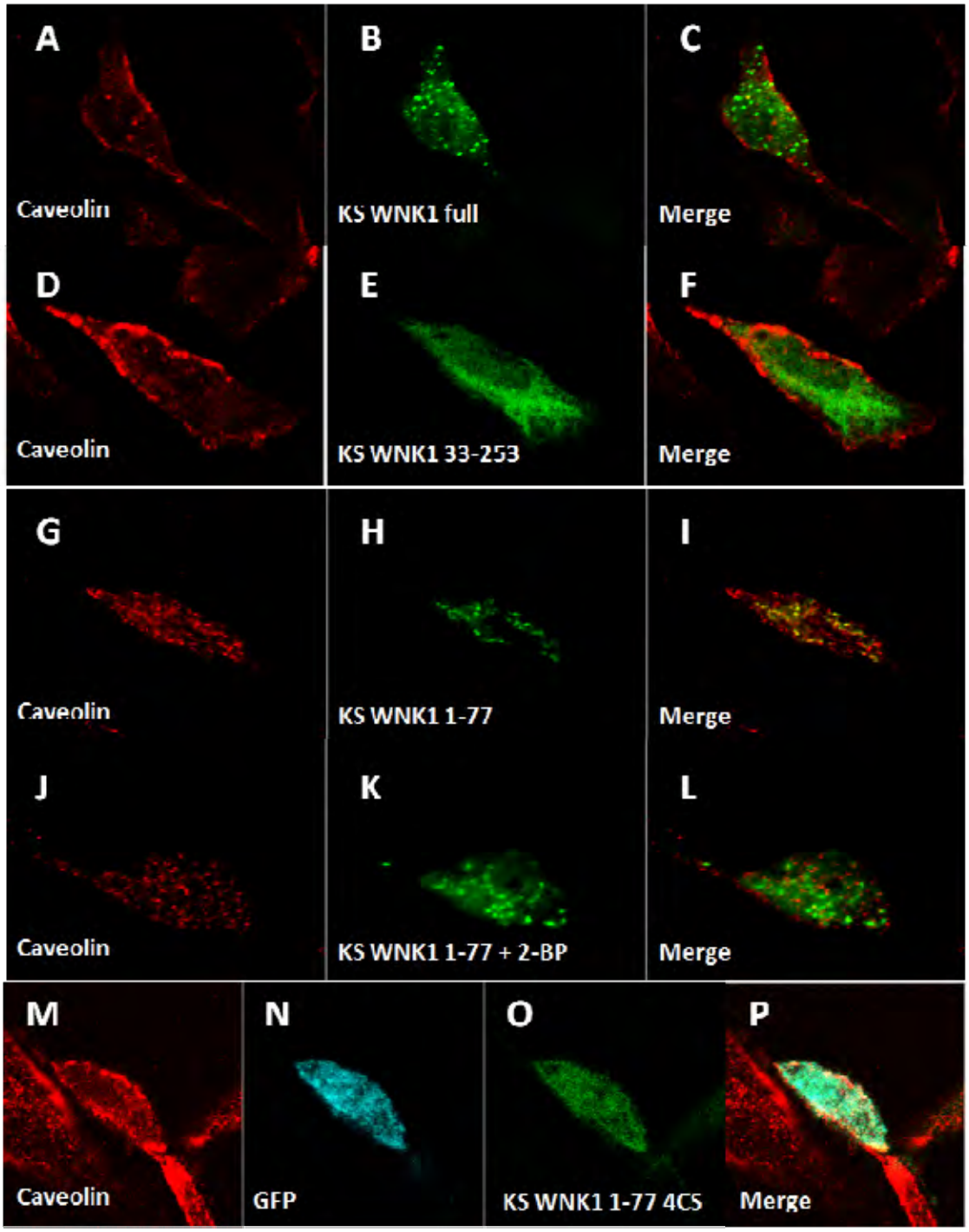


Figure 5-9.The distribution of different KS-WNK1 constructs in comparison to caveolin.

Red channel represents caveolin staining; green channel represents various KS-WNK1 constructs stained by mouse anti-flag antibody. Right panel are merged images of previous channels. GFP signal are in blue in KS-WNK1 1-77 4CS group. (See text for detail.)

the membrane (data not shown). Thus, the ability to form oligomer determines the membrane affinity of KS-WNK1, which may also help its function in antagonizing WNK1 effect on ROMK by targeting KS-WNK1 close to endocytosis machinery. However, the subcellular distribution of KS-WNK1 (1-77) in different cell fractionation is not significantly changed upon 2-BP treatment.

Furthermore, immunostaining of KS WNK1 in NIH 3T3 cells was done to study the distribution of KS-WNK1 constructs. KS-WNK1 (1-77) and KS-WNK1 (1-77) 4CS was transfected into NIH 3T3 cells growing on coverslips. Rabbit anti-flag antibody and Alexar 564 labeled goat-anti-rabbit secondary antibody were used to stain KS-WNK1 proteins. As shown in Figure 5-8, in transfected cells as shown in green, KS-WNK1 (1-77) wild-type exhibited *puncta* staining pattern. However, KS-WNK1 (1-77) 4CS exhibited a diffused pattern in the cell, co-localizing perfectly with GFP throughout the cytosol. This is supporting our biochemical evidence that KS-WNK1 (1-77) 4CS only exist in the cytosol. 2-BP treatment (24hr, 100μM) has a subtle effect on the distribution of KS-WNK1 (1-77), which seems to be more diffused rather than clustered in 2-BP group compared with control group.

Palmitoylated proteins often reside in the lipid-raft structures containing caveolin. We then asked whether KS-WNK1 presents in the lipid-raft. As in Figure 5-9, we double-stained cells expressing KS-WNK1 full length, KS-WNK1 33-253, KS-WNK1 (1-77)(ctr. and 2-BP treated cells) and KS-WNK1 (1-77) 4CS with rabbit anti-flag antibody and mouse anti-caveolin 1 antibody, followed by staining of secondary antibodies of GAR Alexar 564 and GAM Cy5. As shown in Figure 5-9 A-C, KS-WNK1 full length also exhibited *puncta* staining similar as KS-WNK1 (1-77) [Figure 5-9 G-I], it also co-localized partially with caveolin 1 staining. However, KS-WNK1 33-253 lacking the 4a domain exhibited a diffused pattern similar to WNK1 (1-77) 4CS and does not co-localized with caveolin structures [Figure 5-9 D-F]. KS-WNK1 (1-77) co-localized well with

caveolin as shown in [Figure 5-9 G-I]. The co-localization seems to be affected by in 2-BP treated cells [Figure 5-9 J-L]. Finally, KS-WNK1 (1-77) 4CS diffused throughout the cytosol. The mutant colocalized extensively with GFP signal but not with caveolin staining [Figure 5-9 M-P].

KS-WNK1 oligomerization and palmitoylation

As shown in Figure 5-10A, KS-WNK1 oligomer is highly resistant to SDS/ β-ME. In non-reducing gels, KS-WNK1 exclusively existed in an oligomerized form, elevating of β-ME to 10% increased the monomer fraction, but still did not completely abolished the oligomer. Mutation of the cysteines completely abolished the formation of 16mer.

Palmitoylation helps protein to form stable SDS/ β-ME resistant high molecular weight oligomer, which are often critical for protein function as the case of synaptotagmin, CD95 and some other proteins[Feig C, 2007; Kang R, 2004]. The formation of 16mer in KS-WNK1 may be facilitated by palmitoylation. Unlike KS-WNK1 (1-77) expressed in HEK cells, bacteria expressed His-KS-WNK1 (1-77) does not form high order of oligomer as shown in the first lane of Figure 5-10B, possibly due to the lack of palmitoyl-transferase in prokaryote. The supplementation of HEK cell lysate to the purified His-KS-WNK1 (1-77) protein enable KS-WNK1 (1-77) to form 16mer *in vitro* [2nd lane Figure 5-10B], suggesting that some components in HEK cells help KS-WNK1 to form 16mer. The oligomerization process also depends on the oxidization of the cysteines groups since addition of H_2O_2 further enhance the formation of oligomer [3rd lane Figure 5-10B].

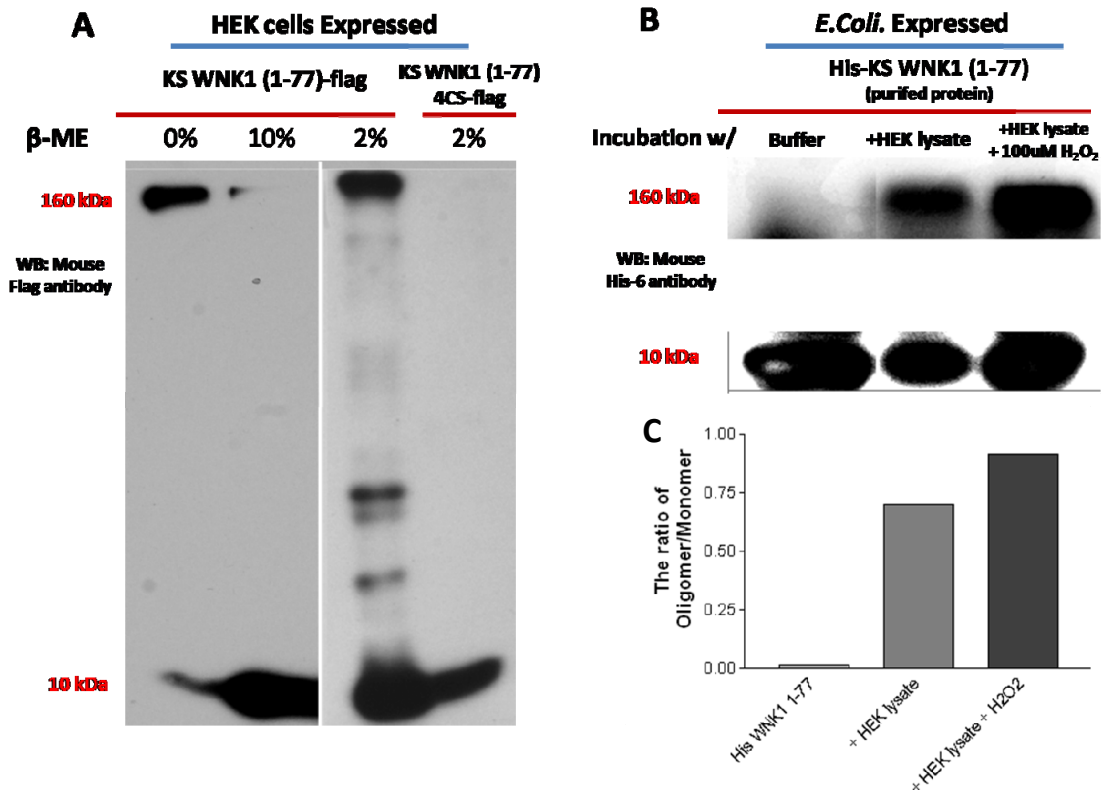


Figure 5-10. Biochemical analysis of KS-WNK1 oligomerization

A. As shown in the left panel, without reducing agent β-ME, KS-WNK1 (1-77) exists solely in oligomerized form in SDS loading buffer. Addition of 10% β-ME depolymerized KS-WNK1 1-77. At 2% β-ME, KS-WNK1 (1-77) exists in both monomer and various types of oligomer, whereas mutation of the 4 cysteine groups completely abolished the oligomerization.

B. Upper panel, bacteria expressed His-KS-WNK1 (1-77) does not form stable 16mer in SDS loading buffer, possibly due to the lack of a certain key enzyme, such as palmitoyl-transferases which only express in eukaryote cells. Palmitoylation helps to stabilize oligomer as reported by several groups. Without palmitoylation, any formed oligomer will dissociate in loading buffer containing 2% β-ME. Supplement of HEK lysate to the purified His-KS-WNK1 helps the bacteria expressed protein to form 16mer. The oligomerization is facilitated by addition of H_2O_2 suggesting that the nature of KS-WNK1 oligomerization indeed involve oxidizing.

C. Quantitation of the oligomerized His-WNK1 (1-77)v.s. the monomer. HEK cell lysate greatly increased the formation of oligomerization which is also facilitated by H_2O_2.

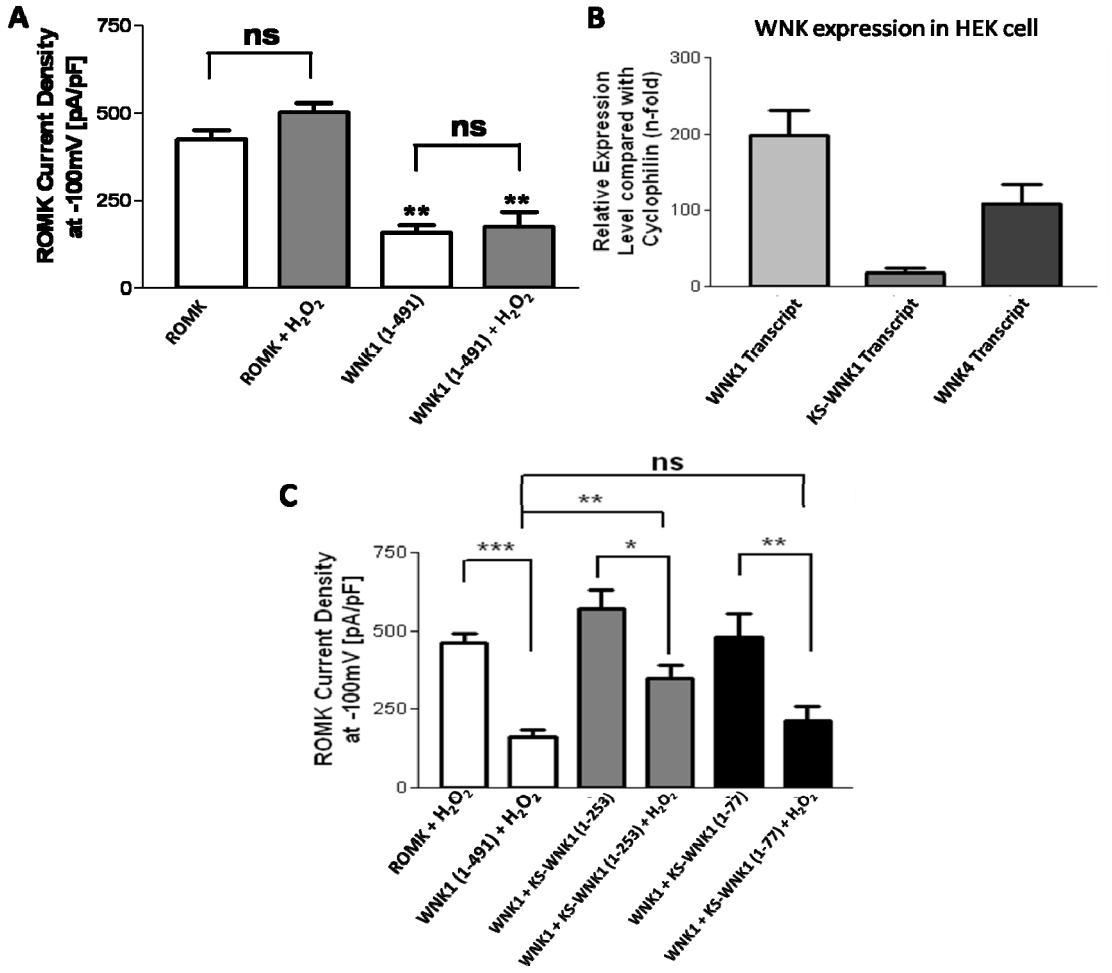


Figure 5-11. H_2O_2 abolishes the function of KS-WNK1 4a domain.

- **A.** H_2O_2 (50μM overnight incubation) did not significantly affect current density in ROMK and WNK1 (1-491) groups compared with corresponding control groups. WNK1 (1-491) inhibits ROMK in both control and H_2O_2 group as shown. This result is different from results obtained in native CCD cells, where H_2O_2 is shown to inhibit ROMK current. It suggests that HEK and CCD cells may have different protein expression profile.
- **B.** In contrast the reported high expression of KS-WNK1 in kidney, HEK cells does not express significant amount of endogenous KS-WNK1. As positive control, WNK1 expression is the highest in HEK cells, and WNK4 is also expressed abundantly.
- **C.** H_2O_2 greatly affected the function of KS-WNK1 (1-77). KS-WNK1 (1-253) can still partially prevent WNK1 inhibition, suggesting AID domain is not affected by H_2O_2.

H_2O_2 did not significantly affect current density in ROMK and WNK1 control groups, probably because there is no endogenous KS-WNK1 for H_2O_2 to inhibit. In CCD cells, H_2O_2 may antagonize endogenous KS-WNK1 and increased the WNK1-mediated endocytosis of ROMK. It has been reported that H_2O_2 does not inhibit ROMK directly.

KS-WNK1 and oxidative stress

It has been reported that K+ deficiency will induce oxidative stress in the kidneys, which will inhibit ROMK by stimulating its endocytosis [Wei Y, 2007]. However, the mechanism is not completely understood. Since the 4a domain of KS-WNK1 contains multiple cysteine residues which is sensitive to oxidative stress. I tested the effect of H_2O_2 on the function of KS-WNK1. Intriguingly, H_2O_2 completely abolished the function of exogenous expressed KS-WNK1 (1-77). Again, the effect of H_2O_2 to KS-WNK1 (1-253) is less dramatic, suggesting that H_2O_2 may not affect the function of AID [Figure 5-11C]. Interestingly, the effect of H_2O_2 to full-length KS – WNK1 is more dramatic compared with KS-WNK1 (1-253) [data not shown]. It is likely that full length WNK1 relies more on the function of 4a domain, since the AID domain function is blocked by the CC2 domain [Xu BE, 2002].

Furthermore, over-night incubation of H_2O_2 at 50uM did not significantly affect ROMK current density in HEK cells [Figure 5-11A], denouncing a role for tyrosine kinase and MAPK in H_2O_2-mediated endocytosis of ROMK in HEK cells. Real time PCR result shows that the mRNA transcript of KS-WNK1 in HEK cell is very low (if any), whereas WNK1 and WNK4 is expressed abundantly in HEK cells. Considering the high expression of KS WNK1 in the kidney, hydrogen peroxide may stimulate endocytosis of ROMK in CCD cells by inhibiting the KS-WNK1 4a domain.

Effect of KS-WNK1 on the recruitment of intersectin by WNK1

We showed that the binding of KS-WNK1 to WNK1 antagonized the WNK1 effect on ROMK, but the detailed mechanism is not understood. We hypothesize that KS-WNK1 binding with WNK1 will preclude WNK1 from recruiting intersectin. WNK1 kinase domain conformation plays a central role in regulating its ability to inhibit ROMK.

KS-WNK1 AID domain binds and affects the kinase domain conformation of WNK1 and inhibited WNK1 kinase activity. It is also reported that KS-WNK1 4a domain also antagonize WNK1 kinase activity, probably by binding, as our result indicates. Using GST pull down, I found that co-expression of KS-WNK1 (1-77) decreased the binding of WNK1 to intersectin SH3 C domain [2nd and 3rd lane compared with 4th and 5th lane in Figure 5-12A]. However, the KS-WNK1 mutant of the cysteine residues in the 4a domain does not affect WNK1 interaction with intersectin SH3 C [6th lane in Figure 12A]. The experiments were duplicated in KS-WNK1+WNK1 and WNK1 alone groups with similar result. Figure 5-12B showed the quantitation of the corresponding WNK1 bands being pulled down v.s. the WNK1 input in different groups.

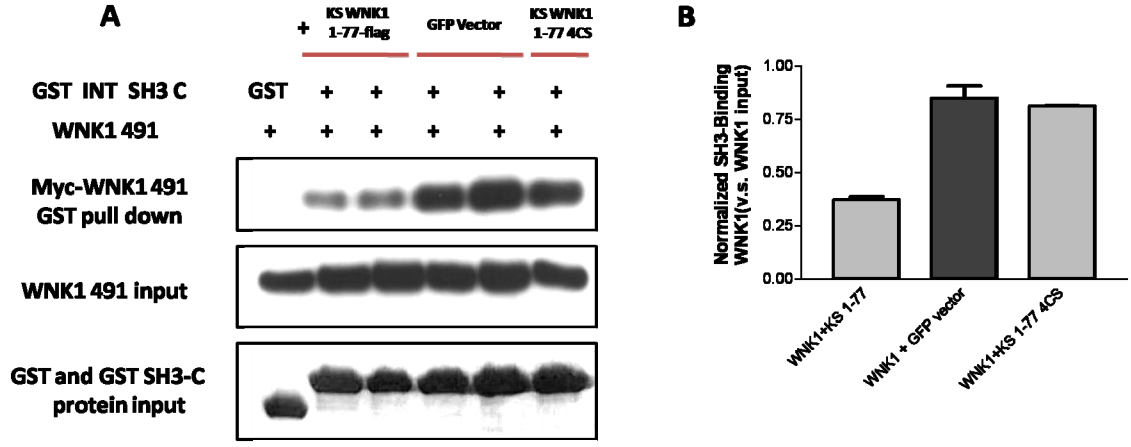


Figure 5-12. KS-WNK1 4a domain prevents intersectin from binding with WNK1.

- **A.** Co-expression of KS-WNK1 1-77, but not KS-WNK1 1-77 4CS mutant, impaired the binding affinity of WNK1 to intersectin. GST beads do not pull down WNK1 as shown. Experiments were duplicated in two lanes for KS-WNK1 1-77 and WNK1 control groups.
- **B.** Quantitation of the bands in GST pulldown experiment of myc-WNK1 (1-491). Bands intensity × bands size was normalized to the bands of WNK1 491 input.

DISCUSSION

KS-WNK1 antagonizes WNK1 by both 4a and AID domain

We have recently shown that KS-WNK1 is an antagonist of WNK1 regulation of ROMK and that amino acids 1-253 of KS-WNK1 are sufficient for the antagonism of WNK1 [Lazrak A, 2006]. In the present study, I found that two regions within amino acids (1-253) of KS-WNK1 are critical for the antagonism of WNK1 [Figure 5-13 A]. One is the region encoded by the alternative initiating exon4A. This region is unique to KS-WNK1. The other is the region equivalent to the AID of WNK1. Amino acids 84 to 148 of KS-WNK1 are identical to the AID of WNK1 (amino acids 491-556). Thus, it is not surprising that this AID-equivalent region of KS-WNK1 can antagonize WNK1 inhibition of ROMK. The function of AID domain is stated in detail in chapter 4.

The other region of KS-WNK1 (1-253) involved in the antagonism of WNK1 inhibition of ROMK is the region encoded by the alternative initiating exon4A. KS-WNK1 4a domain binds to WNK1 and antagonizes WNK1 interaction with intersectin SH3 C. The 4a domain of KS-WNK1 is rich in cysteine residues (20% of the total residues), which are important for the function of KS-WNK1 since KS-WNK1 (1-77) 4CS mutant neither binds with WNK1 nor prevent binding of intersectin to WNK1. KS-WNK1 binding with WNK1 may affect WNK1 kinase domain conformation, and prevent the recruitment of intersectin by WNK1. To support this, we previously showed that mutations that disrupted the kinase domain conformation also abolished ITSN SH3 C binding with WNK1 [by Shao-Kuei, Figure 4-5F. Consistently, KS-WNK1 (1-77) 4CS mutant, which does not bind with WNK1 [data not shown, by Zhen Liu], did not prevent WNK1 (1-491) interaction with the ITSN SH3 C [Lane 6, Figure 5-12A].

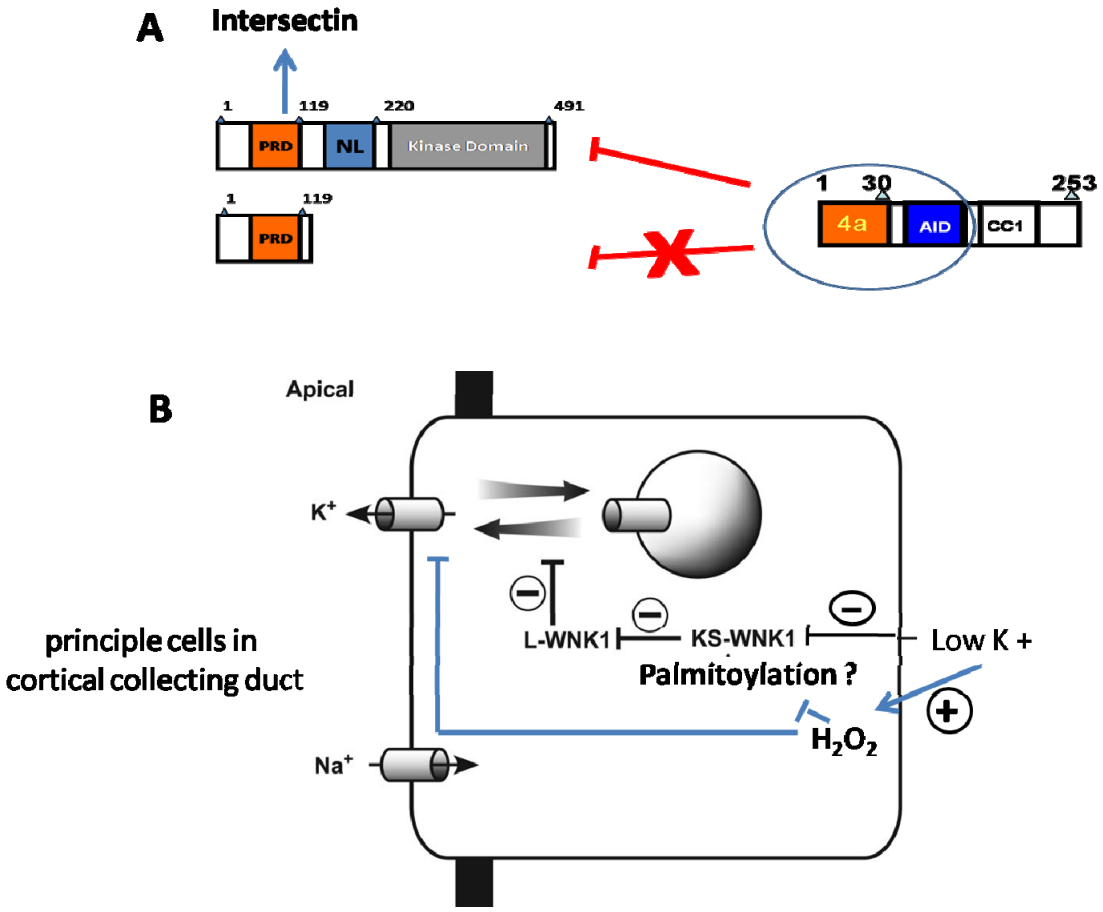


Figure 5-13. Working model for regulation of the endocytosis of ROMK by KS-WNK1

KS-WNK1 antagonizes WNK1 inhibition of ROMK. Low potassium intake suppresses KS-WNK1 expression and generates H_2O_2 as well. H_2O_2 promotes Endocytosis of ROMK in CCD cells. H_2O_2 inhibits the function of KS-WNK1 and promotes WNK1 activity to stimulate endocytosis of ROMK, which could be involved in regulation of ROMK *in vivo*.KS-WNK1 may be palmitoylated. H_2O_2 and palmitoylation inhibitor 2-BP can antagonize protein palmitoylation. Indeed, H_2O_2 and 2-BP both abolished the effect of KS-WNK1 4a domain to antagonize WNK1 in patch clamp assays. 2-BP also decreased the binding of KS-WNK1 4a domain to WNK1 (1-491).

The multiple cysteine residues are also important for oligomerization of KS-WNK1 4a domain (up to 16mer as we can see). However, the oligomer is unlikely formed solely by cysteine cross-linking since the high concentration of SDS (up to 10%) and β-ME (up to 10%) or DTT (200mM) could have disrupted the oligomer. We also observe multiple protein bands of molecular size greater than >500 kDa in gel electrophoresis of cell lysates transfected with KS-WNK1 (1-253) or with full length KS-WNK1 (not shown, by Liu Z in the lab). Further experiments are required to understand the physiological relevance of KS-WNK1 oligomerization.

In contrast to HEK cell expressed KS-WNK1, bacteria expressed His-KS-WNK1 (1-77) does not form high molecular weight oligomer [Figure 5-10B]. The supplementation of HEK cell lysate to the purified His-KS-WNK1 (1-77) protein enabled KS-WNK1 (1-77) to form 16mer *in vitro*. Addition of H_2O_2 further facilitated the formation of KS WNK1 oligomer. It suggests the oligomerization process of KS-WNK1 requires participation of eukaryote specific enzymes such as PAT, and it is also facilitated by oxidization of the cysteine groups. Oxidation plays an important role in oligomerization, but oxidation alone will not explain why KS-WNK1 16mer is stable in the presence of β-ME. Palmitoylation may stabilize the oligomer in loading buffer containing SDS/ β-ME.

Oxidative Stress and KS-WNK1 function

There is little information on physiological inhibitors of protein palmitoylation. How could the kidneys regulate and make use of palmitoylation of KS-WNK1, if there is any? I noticed that the multiple cysteines may be a good sensor towards oxidative stress. It has been known that oxidative stress occurs during dietary K+ deficiency in multiple studies [Review, Wang WH, 2006]. Oxidative stress by H_2O_2 inhibits ROMK current density in CCD cells to reduce the K+ secretion.

H_2O_2 does not inhibit ROMK current directly, but stimulated the endocytosis of ROMK. The detailed mechanism is not well-understood. It has been suggested that tyrosine kinase Src and MAPK may be the downstream of H_2O_2 mediated endocytosis of ROMK [Wei Y, 2007]. However, inhibitor of Src or MAPK alone cannot rescue the inhibition of ROMK by H_2O_2. A combination of multiple Src and MAPK inhibitors did reverse the inhibition of ROMK by H_2O_2 [Wei Y, 2007]¸ but the effect may be through inhibiting endocytosis in general rather than specifically inhibiting H_2O_2 effect on ROMK. Consistent with this notion, I further found that H_2O_2 does not increase endocytosis of ROMK in HEK cells, which expressed endogenous tyrosine kinase and MAPK similarly as CCD cells.

Protein palmitoylation can be blocked by a synthetic inhibitor like 2-BP that inhibiting palmityl-transferase, or oxidative stress that might oxidize the cysteine residues thus preventing the transfer of the palmitoyl moiety by palmity-ltransferase. There are reports showing that H_2O_2 reduces palmitoylation of caveolin and CD81 [Parat MO, 2002; Clark KL, 2004] by oxidizing the thio-group required for accepting palmitoyl group. In this study, H_2O_2 abolished the antagonism of KS-WNK1 4a domain, but not that of AID, to WNK1-mediated ROMK endocytosis------ a result similar as that of 2-BP. It suggests oxidative stress may regulate the KS-WNK1 of in vivo, esp. during K+ deficiency. The mechanism may be through reducing palmitoylation of KS-WNK1 4a domain.

All together, these results suggest that, besides being regulated at the transcription level, the functionality of KS-WNK1 may be compromised by oxidative stress during K+ deficiency. Therefore, KS-WNK1 may be the missing link connecting oxidative stress and endocytosis of ROMK during K+ deficiency [Figure 5-13 B].

KS-WNK1 localization and its function

KS-WNK1 full length, KS-WNK1 (1-253) and KS-WNK1 (1-77) exhibited *puncta* staining pattern that partially co-localized with caveolin. Deletion of the 4a domain or mutation of the cysteines residues leads to the mislocation of the KS-WNK1 within the cytosol, suggesting that KS-WNK1 4a domain helped the docking of KS-WNK1 to lipid raft. The effect of the palmitoylation inhibitor 2-BP on the localization of KS-WNK1 is subtle, and appears to decrease the co-localization of KS-WNK1 with caveolin, but it requires more immunostaining to confirm and needs a good way to quantitate the co-localization.

As shown in the biochemical fraction assay, KS-WNK1 oligomer preferentially exists in the integral membrane fraction. Inhibition of palmitoylation by 2-BP did not change the biochemical distribution of KS-WNK1 nor prevent the formation of the 16mer possibly because this assay could not differentiate lipid raft, endosome or ER membrane. Also, KS-WNK1 may be hydrophobic enough to bind to lipid membranes even without palmitoylation. As to the question why 2-BP does not affect KS-WNK1 oligomerization, it may be that there is residual palmitoylation of KS-WNK1 upon 2-BP treatment, which is sufficient to induce oligomerization but insufficient to facilitate KS-WNK1 binding with WNK1. There could be spontaneous palmitoylation that does not require enzymes as suggested in previous papers. Additionally, 2-BP may affect other proteins that require palmitoylation and affect the function of 4a domain of KS-WNK1 indirectly.

Dr Cha SK in our lab found that TRPV5 channel (a channel responsible for Ca++ reabsorption in the kidney) was inhibited by WNK1 and WNK4 via caveolin-mediated endocytosis. WNK4 PHAII mutations enhanced inhibition TRPV5, which explains the onset of hypercalciuria (high Ca++ in

the urine) in some PHAII patients. Different from WNK4 mutations, PHAII patients with WNK1 mutations do not develop hypercalciuria. The reason may be due to the antagonism of KS-WNK1, because he found that KS-WNK1 more dramatically antagonized the WNK1 inhibition of TRPV5 compared with ROMK (only 0.2μg KS-WNK1/6well is required for reversing inhibition of TRPV5 by 1μg WNK1, compared to 1μg KS-WNK1 required for ROMK). KS-WNK1 localization in lipid raft may facilitate its antagonization of WNK1 inhibition of TRPV5 by caveolin-mediated endocytosis. However, KS-WNK1 cannot antagonize WNK4 kinase effect on ROMK or TRPV5. Thus, KS-WNK1 can counteract the effect of over-expressed WNK1 on TRPV5 but not that caused by WNK4 mutations in PHAII patients. This result supports a physiological role of KS-WNK1 localization in the lipid rafts.

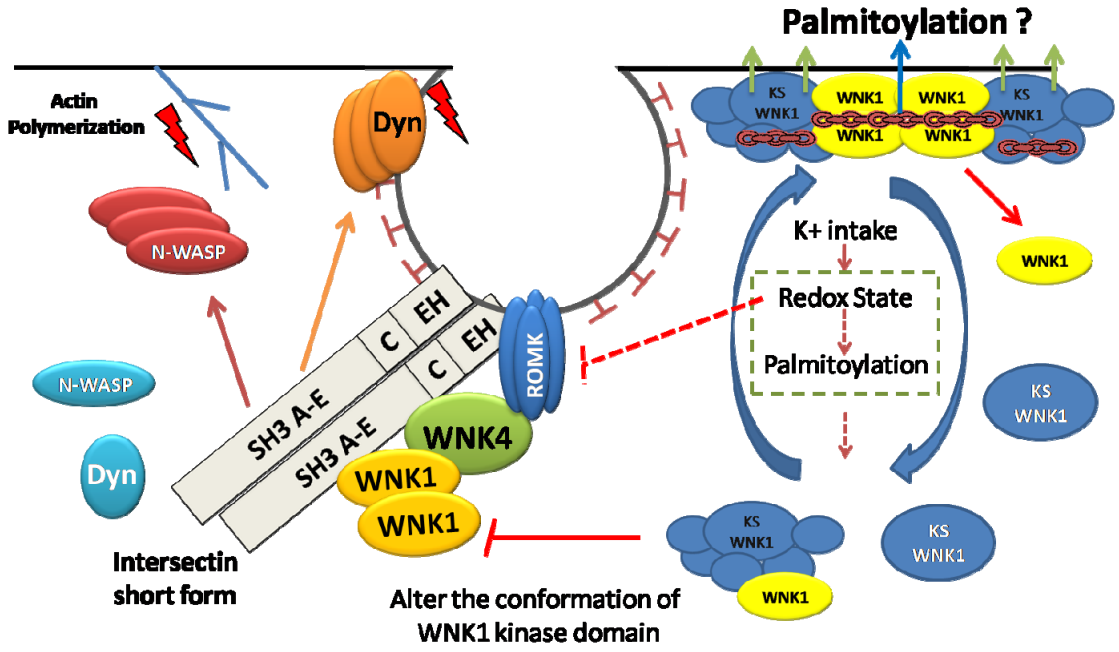


Figure 5-14. Working model for endocytosis of ROMK regulated by WNK kinase family.

WNK4 kinase domain specifically binds with the C-terminus of ROMK. WNK1 binds WNK4. WNK1 and WNK4 stimulate endocytosis of ROMK by binding to the SH3 domains of intersectin. Intersectin then recruit dynamin, N-WASP, etc.. Intersectin SH3 activates the GTPase activity of dynamin and the actin-polymerization activity of N-WASP, thus promote endocytosis of ROMK. KS-WNK1 antagonizes WNK1 effect on ROMK by binding to WNK1, which precludes the recruitment of intersectin by WNK1. KS-WNK1 forms oligomers that bind tightly with the plasma membrane. Oligomerization is mediated by the cysteine residues in 4a domain, and is possibly regulated by oxidative stress (induced by K+ deficiency) and palmitoylation. The detailed function and mechanism of redox state, palmitoylation and oligomerization of KS-WNK1 is not well understood.

Chapter six: Future Directions

Osmolarity and regulation of endocytosis of ROMK by WNK kinase

Due to fluctuations in the environment, renal cells have to withstand great changes in osmolarity and respond to them by adjusting water and electrolytes handling. WNK1 and WNK4 kinase activity can be activated by hypertonic/hypotonic stress [Lenertz LY, 2005; Zagórska A, 2007; Shaharabany M, 2008]. The activation process also involves their translocation from cytosol to membrane structures, presumably to regulate the trafficking of transporters and ion channels. ROMK in thick ascending limb(TAL) of the kidney is responsible for recycling K+ and facilitating re-absorption of NaCl by Na-K-2Cl cotransporters (NKCC2). Hyperosmolarity caused by restriction of water intake or high levels of sodium intake was reported to increase ROMK expression and abundance in the rat outer medulla, while low levels of sodium intake decrease it [Gallazzini M, 2003]. It is not known whether the endocytosis of ROMK is affected by WNK kinase activity/localization regulated by osmolarity.

Additional functional domains and binding partners for WNK kinase in the regulation of the endocytosis of ROMK

In this study, we identified intersectin as a binding partner of both WNK1 and WNK4. Since there are many other endocytic proteins containing SH3 domain, and we haven't examined all of them, it is also likely that there are SH3 proteins that bind WNK1 and WNK4. For example, I tested the SH3 domain of syndapin, it also binds to the WNK1 N-terminus, but with different PXXP motifs because the triple mutant WNK1 (1-491) also binds syndapin SH3 domain (data not shown). Triple mutant WNK1 (1-491) no longer inhibits ROMK, thus syndapin binding with

WNK1 may not be critical for the regulation of ROMK, but it may be involved in the regulation of other proteins. The sequences of WNK1 and WNK4 are characterized by clustered PRDs, however, the binding partner and function of those PRDs are not known, but it will be of interest for the future study of WNK kinase.

During this study, we identified additional functional domain in the C-terminus of WNK4. Our lab has data showing the C-terminal functional domain may be also proline-rich domains. It is also reported that SGK phosphorylation prevented WNK4-mediated endocytosis of ROMK by phosphorylation on a serine residue close to the previously identified PHAII mutation site (R1164C). [Ring AM, 2007]. The mechanism for SGK antagonism is not well understood and still need confirmation in "in vivo" study. If the data is correct, it may suggest that the C-terminus may play as a regulatory domain of the PRD.

A general role of WNKs in the regulation of endocytosis

WNK1 is ubiquitously expressed and WNK4 is widely distributed in epithelia cells. Study of the function of WNK1 and WNK4 in endocytosis in general may yield new insights into their function. A recent siRNA-based kinome-wide screen using virus entry as a measure of clathrin- and caveolin-mediated endocytosis identifies WNK4 as a target [Pelkmans L, 2005]. WNK1 also affect clathrin-mediated endocytosis in general, though with a lower effect. In my study, I also confirmed the effect of WNK4 on clathrin-mediated endocytosis using the transferrin receptor as a model. siRNA knockout of WNK4, but not WNK1, significantly reduced the uptake of rhodamine-labeled transferrin under fluorescent microscopy. In another experiment using flow cytometry to quantitate the uptake of Alexar 564 labeled LDL, knockout of WNK1 partially inhibited the endocytosis of the LDL receptor, while siRNA knockout of intersectin 1s dramatically decreased the uptake of LDK by clathrin-mediated endocytosis (data not shown).

There are other examples suggesting WNK1 and WNK4 may regulate many type of endocytosis. Dr. Cha SK in the lab found that WNK1 and WNK4 also inhibited TRPV5 by caveolin-mediated endocytosis. It was also reported that WNK1 and WNK4 decreased CFTR the surface abundance, possibly by stimulating the endocytosis of CFTR [Yang CL, 2007; Dorwart MR, 2007]. A study of the function of WNK kinase in regulating both clathrin- and caveolin-mediated endocytosis could be important to understand the mechanism for WNK kinase in regulating the endocytosis of various structurally distinct ion channels and transporters.

In some other related reports, over-expression of WNK1 and WNK4 enhanced the EGF signaling pathway, while knockdown of WNK1 blocked EGF signaling pathway [Sun X, 2006; Shaharabany M, 2008]. The mechanism is likely due to the involvement of WNK1 in activation of the mitogen-activated protein (MAP) kinase ERK1/2 and/or ERK5 pathways. In some other reports, blockage of EGF receptor endocytosis impaired EGF signaling suggesting that internalization of EGF receptor is important for its signaling [Sigismund S, 2008]. Notably, intersectin, the binding partner of WNK kinase in this study, is found to be important for EGF receptor internalization [Martin NP, 2006]. Thus, it raised an interesting question to whether WNK1 and WNK4 kinases are involved in EGF receptor endocytosis and thus contribute to EGF signaling pathway. EGFR and WNK1 kinase are both highly expressed in cancer cell lines [Lenertz LY, 2005]. EGFR has been an important theraputical target for cancer treatment. The study of WNK and EGF receptor internalization may reveal novel signaling pathway and theraputical targets for cancer research.

Oligomerization, palmitoylation, oxidative stress and the function of KS-WNK1

In this study, I find that KS-WNK1 forms high molecular weight oligomer in both HEK cell and transgenic animals, but not in bacteria. Mutations in the cysteines abolished oligomerization, and addition of H_2O_2 facilitate the oligomerization. The nature and significance of KS-WNK1

oligomerization still need further studies. The binding affinity of different oligomer to WNK1 may vary and the oligomerization process may be a regulatory mechanism to modulate its binding to WNK1 and plasma membrane.

The role of palmitoylation in KS-WNK1 function is mysterious and needs further study. Due to technical difficulties, we haven't biochemically identify palmitoylation of KS-WNK1. However, the palmitoylation inhibitor 2-BP decreased the binding of KS-WNK1 to WNK1 and abolished the antagonism by KS-WNK1. Oxidative stress during K+ deficiency may function as endogenous inhibitor of KS-WNK1, since H_2O_2 abolished the effect of 4a domain. I speculate that H_2O_2 inhibits palmitoylation of the 4a domain and regulate KS-WNK1 function; however, it needs further experiments to be proved.

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