EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 39, No.5, 565-573, October 2007

WNKs: protein kinases with a unique kinase domain

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Accepted 23 July 2007

Abbreviations: ENaC, epithelial sodium channel; IGF, insulin-like growth factor; KCC, potassium-chloride cotransporter; NCC, sodium-chloride cotransporter; Nedd4, neural precursor cell-expressed, developmentally down-regulated 4; NKCC, sodium-potassium-chloride cotransporter; OSR1, oxidative stress-responsive kinase; PHA II, pseudohypoaldosteronism type II; ROMK, renal outer medullary potassium channel; SGK, serum and glucocorticoid-inducible kinase; SLC12, solute carrier family 12; SPAK, sterile20-related, proline-, alanine-rich kinase; WNK, with-no-lysine (K)

Abstract

WNKs (with-no-lysine [K]) are a family of serine-threonine protein kinases with an atypical placement of the catalytic lysine relative to all other protein kinases. The roles of WNK kinases in regulating ion transport were first revealed by the findings that mutations of two members cause a genetic hypertension and hyperkalemia syndrome. More recent studies suggest that WNKs are pleiotropic protein kinases with important roles in many cell processes in addition to ion transport. Here, we review roles of WNK kinases in the regulation of ion balance, cell signaling, survival, and proliferation, and embryonic organ development.

Keywords: genome, human; hyperkalemia; hypertension; neoplasms; protein kinases; pseudohypoaldosteronisms; WNK1 protein, human

Introduction

WNKs (with-no-lysine [K]) are serine-threonine protein kinases that have unusual protein kinase domains due to the unique placement of the catalytic lysine relative to all other protein kinases (Xu *et al.,* 2000). For all other protein kinases, a conserved catalytic lysine is present in sub-domain II of protein kinase domain (Knighton *et al.,* 1991) (Figure 1A). In contrast, the catalytic lysine of WNK kinases is in sub-domain I. The amino acid of WNK1 corresponding to the catalytic lysine of PKA is cysteine (cysteine-250 in Figure 1A and 2). The three-imensional structure of the WNK1 protein kinase domain, however, is similar to other protein kinases (Min *et al.,* 2004) (Figure 2). The three-dimensional structure of the kinase domain of most protein kinases adopts a bi-lobar structure (Knighton *et al.,* 1991). The active site of the kinase domain resides in a cleft formed by the folding domains. The catalytic lysine of WNKs and other protein kinases, despite being localized in different parts of the kinase domain, both point toward the active site in the cleft (Figure 2). WNK kinases catalyze the phosphorylation of endogenous and exogenous substrates (Xu *et al.,* 2000, 2004; Lenertz *et al.,* 2005; He *et al.,* 2007). Thus, WNKs are *bona fide* protein kinases.

There are four mammalian WNKs (Xu *et al.,* 2000; Verissimo and Jordan, 2001; Wilson *et al.,* 2001). WNK1, the first member identified, is over 2100 amino acids long and contains a ~270 amino acid kinase domain located near the amino terminus (Xu *et al.,* 2000). WNK2, 3, and 4 are products of different genes and 1200 to 1600 amino acids in length (Xu *et al.,* 2000; Verissimo and Jordan, 2001; Wilson *et al.,* 2001) (Figure 1B). The four WNK kinases share a conserved kinase domain with 85-90% sequence identity. In addition to the protein kinase domain, WNKs contain an autoinhibitory domain that can suppress protein kinase activity, and numerous protein-interaction motifs, including 1-2 coiled-coil domains and multiple PXXP proline-rich motifs. Little sequence identity exists beyond the above conserved domains/motifs.

WNK proteins are ubiquitously expressed in tissues and at least some splice forms display tissue-specific expression (Xu *et al.,* 2000; Delaloy *et al.,* 2003; O'Reilly *et al.,* 2003). Numerous studies in the past few years have implicated that WNK kinases play important roles in regulation of ion balance, cell signaling, survival, and proliferation, and organ development. Here, we review these studies.

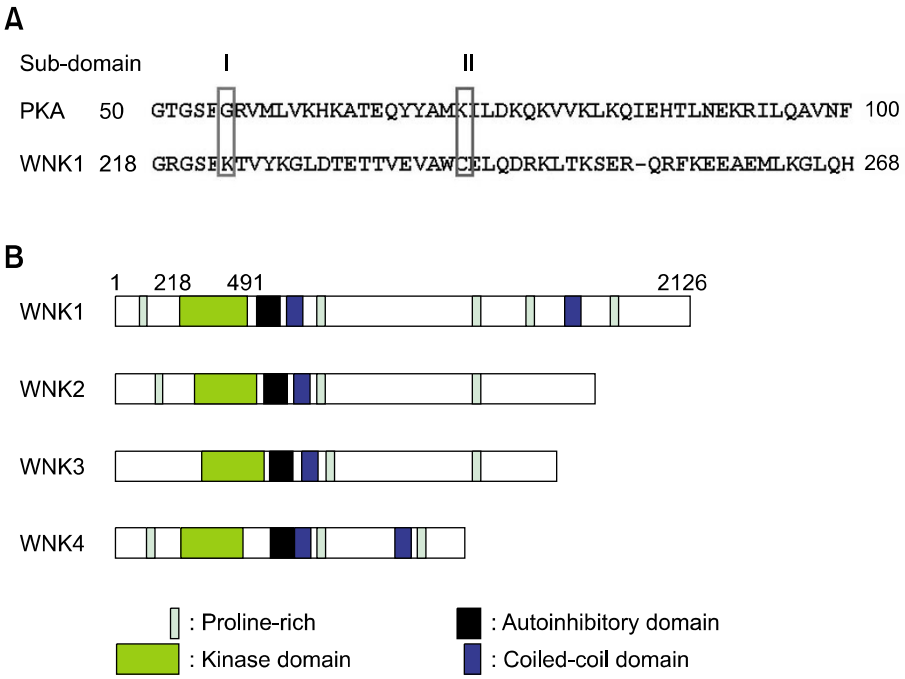

Figure 1. (A) Partial amino acid sequence of the kinase domain of PKA and WNK1. The amino acid of WNK1 corresponding to the catalytic lysine of PKA is cysteine-250 (indicated by red box). The catalytic lysine of WNK1 (lysine-233) is in the sub-domain I (indicated by green box). (B) Domain structure of WNK1-WNK4.

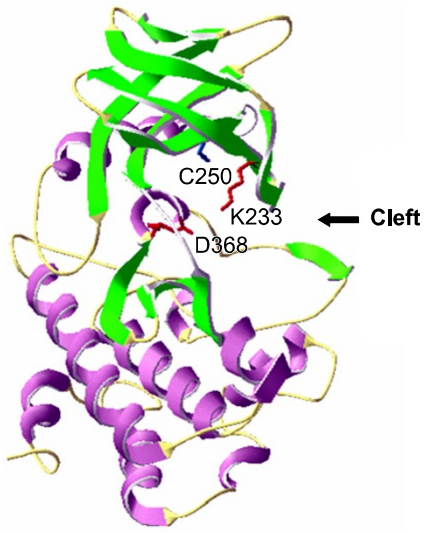

Figure 2. Crystal structure of the catalytic domain of WNK1. Ribbon representation was prepared using Deepview 3.7 based on the crystal structure solved by Min *et al.* (2004) (Protein Data BanK, accession code 1T4H). Cystine-250 (blue), lysine-233, and aspartate-368 (red) are shown in ball-and-stick representation. The cleft between the N-terminal lobe (green) and C-terminal lobe (magenta) is the catalytic active site.

Mutations of WNK1 and 4 cause a genetic hypertension syndrome

Pseudohypoaldosteronism type II (PHA II), also known as Gordon's syndrome, is an autosomal-dominant disease characterized by hypertension, hyperkalemia, hyperchloremia, and metabolic acidosis (Gordon, 1986). Positional cloning studies have identified mutations in either *WNK1* or *WNK4* as causes of PHA II (Wilson *et al.,* 2001). Disease-causing mutations in the *WNK1* gene are large deletions of the first intron thought to lead to increased expression of the wild type protein and in the *WNK4* gene are missense mutations in the coding sequence outside the protein kinase domain (Wilson *et al.,* 2001). Studies on how WNK1 and 4 mutations cause PHA II have revealed important roles of WNK1 and 4 in regulating ion transport.

Pathogenesis of hypertension in PHA II

Hypertension in PHA II is associated with low plasma serum rennin levels, indicating increased Na^+ retention and volume expansion (Gordon, 1986). The thiazide-sensitive Na-Cl co-transporter (NCC) in the distal convoluted tubule accounts for ~15% of sodium chloride reabsorption in the kidney. It was reported that wild type WNK4 inhibits the activity of NCC and disease-causing WNK4 mutants fail to inhibit NCC (Wilson *et al.,* 2003; Yang *et al.,* 2003). In addition, WNK4 phosphorylates claudins 1-4, the tight-junction proteins involved in the regulation of paracellular ion permeability (Kahle *et al.,* 2004; Yamauchi *et al.,* 2004). The paracellular chloride permeability is greater in cells expressing WNK4 mutants than in cells expressing wild type proteins. An increase in paracellular Cl^- reabsorp-

tion would lead to increased Na^+ reabsorption secondarily. These results suggest that hypertension in patients with *WNK4* mutations may be caused by increased NaCl reabsorption through NCC and the paracellular pathway. However, hypertension in patients with WNK4 mutations was estimated to be six times more sensitive to thiazide treatment than individual with essential hypertension (Mayan *et al.,* 2002). The increase in NaCl reabsorption through NCC may be relatively more important than that through the paracellular pathway. Recent studies using mice carrying a mutant WNK4 gene (created either by transgene integration or by gene knock-in) support the hypothesis that increased NCC activity is the primary cause of hypertension resulting from WNK4 mutations (Lalioti *et al.,* 2006; Yang *et al.,* 2007).

Expression of WNK1 abolishes inhibition of NCC caused by WNK4 in *Xenopus* oocytes (Yang *et al.,* 2003), suggesting that *WNK1* mutations cause hypertension by releasing WNK4-mediated inhibition of NCC in the distal convoluted tubule. However, hypertension in PHA II patients with WNK1 mutations is not particularly sensitive to thiazide diuretics (Disse-Nicodeme *et al.,* 2000). Other mechanism(s) may also be involved. Consistent with this observation, WNK1 activates SGK1 leading to activation of the epithelial Na^+ channel ENaC (Xu *et al.,* 2005a), a channel also responsible for Na^+ reabsorption in kidneys. Thus, hypertension in PHA II patients with *WNK1* mutations may be caused by increased activity of both ENaC and NCC.

Pathogenesis of hyperkalemia in PHA II

Potassium secretion by the kidney is the primary mechanism to excrete excess K^+ and to maintain a normal blood K^+ level. ROMK K^+ channels in the apical membrane of the distal renal tubule are predominantly responsible for the K^+ secretion in the baseline (Hebert, 1995). WNK1 decreases surface expression of ROMK channels by stimulating their endocytosis via clathrin-coated vesicles (Lazrak *et al.,* 2006; Wade *et al.,* 2006; He *et al.,* 2007). A decrease in renal K^+ secretion via ROMK1 may contribute to hyperkalemia in PHA II patients caused by WNK1 mutations.

Wild type WNK4 also decreases surface expression of ROMK1 and WNK4 mutants that cause disease exhibit a greater inhibition of ROMK (Kahle *et al.,* 2003; He *et al.,* 2007), suggesting that increased inhibition of ROMK1 by mutant WNK4 may also contribute to hyperkalemia in PHA II patients with WNK4 mutations. Recent studies using transgenic and knock-in mice models, however, reported no detectable difference in the expression of ROMK between wild type mice and mice carrying transgenic or knock-in WNK4 mutant (Lalioti *et al.,* 2006; Yang *et al.,* 2007). In the latter two reports, authors provide results to support the hypothesis that hyperkalemia in patients with WNK4 mutations is due to an increase in NaCl reabsorption via NCC, which causes a decrease in Na^+ reabsorption and secondarily impairs K^+ secretion at the more distal sites. Nevertheless, hyperkalemia in patients with WNK4 mutations precedes the development of hypertension (Mayan *et al.,* 2004). It is possible that inhibition of ROMK1 by mutant WNK4 contributes to hyperkalemia in the early stage before the onset of increased NCC activity and hypertension.

Full length vs kidney-specific WNK1

There are multiple splice forms of WNKs expressed in tissue-specific patterns (Delaloy *et al.,* 2003; O'Reilly *et al.,* 2003). The largest WNK1 transcript encodes a polypeptide of nearly 2400 residues. A kidney-specific or KS-WNK1 is produced by replacing the first 4 exons with an alternative 5' exon. Polypeptide encoded by this shorter WNK1 transcript lacks the amino terminal 437 residues of the full length form, and, as a result, has no protein kinase activity. It is expressed highly in the kidney but not elsewhere (Delaloy *et al.,* 2003; O'Reilly *et al.,* 2003). It was reported that the transcript encoding KS-WNK1 is more abundant than that encoding full length WNK1 in the kidney. The relative abundance of KS-WNK1 vs full length WNK1 proteins in the kidney has not been determined. Whether the large deletion of intron 1 in *WNK1* in PHA II patients affects expression of KS-WNK1 is unknown. The function of KS-WNK1 has been examined. KS-WNK1 by itself has no effects on NCC and ROMK1, but antagonizes the full length WNK1-mediated regulation of NCC and ROMK (Lazrak *et al.,* 2006; Subramanya *et al.,* 2006; Wade *et al.,* 2006). As a key pathway for renal K^+ secretion, ROMK1 is regulated by dietary K^+ intake (Chu *et al.,* 2003). Dietary K^+ restriction increases and decreases the expression of full length WNK1 and kidney-specific WNK1 in rat kidneys, respectively (Lazrak *et al.,* 2006). The reciprocal effects on full-length and KS-WNK1 would amplify the inhibition of K^+ secretion via ROMK1, which is an expected adaptive response to reduced dietary K^+ intake. These results suggest that KS-WNK1 functions as a physiological antagonist of full length WNK1 with respect to its regulation of renal K^+ transport.

Regulation of SLC12 cation-chloride cotransporters by WNKs

The *SLC12* (solute carrier family 12) gene family comprises Na-K-Cl cotransporters (NKCC1 and NKCC2), K-Cl cotransporters (KCC1-KCC4), and the thiazide-sensitive NCC (Gimenez and Forbush, 2003). In addition to the regulation of NCC as described above, WNK1, 3 and 4 increase ion transport mediated by NKCC1 and NKCC2 (Rinehart *et al.,* 2005; Vitari *et al.,* 2005; Anselmo *et al.,* 2006; Gagnon *et al.,* 2006). WNK3 and 4 also inhibit KCCs (Vitari *et al.,* 2005; Gagnon *et al.,* 2006; Garzon-Muvdi *et al.,* 2007).

While NCC is present only in the distal convoluted tubule of kidney and responsible for a large portion of NaCl reabsorption, members of NKCC and KCC subfamily are widely present throughout the body. NKCCs mediate Cl^- entry together with Na^+ and K^+. In contrast, KCCs mediate Cl^- exit together with K^+. By controlling the intracellular concentrations of Cl^- and companion cations, NKCC and KCC play essential roles in the regulation of neuronal excitability, cell volume, and blood pressure (Meyer *et al.,* 2002; Kahle *et al.,* 2005). The actions of WNKs on members of the SLC12 family in addition to NCC, both in the kidney and in other tissues, may also contribute to symptoms of PHA II. For example, NKCC1 is essential for vascular smooth muscle contraction (Meyer *et al.,* 2002). Activation of NKCC1 by WNK1 may lead to vasoconstriction and hypertension. Moreover, increased Na^+ reabsorption via NKCC2 in the thick ascending limb of kidney may contribute to hypertension in patients of PHA II with WNK1 mutations.

Mechanisms of regulation by WNKs

One mechanism for WNK regulation of ion transporters is by alteration of their activity. The regulation of NKCCs by WNK1 and 4 involves two related Ste20p family protein kinases SPAK and OSR1. These protein kinases are thought to interact with and phosphorylate NKCC1, NKCC2, and NCC at their N-terminus (Piechotta *et al.,* 2002; Gimenez and Forbush, 2003). Phosphorylation of NKCC1 is correlated with increased transporter activity. OSR1 (and SPAK) forms a tight complex with WNK1 and is directly activated by WNK1 through a catalytic mechanism (Moriguchi *et al.,* 2005; Vitari *et al.,* 2005, 2006; Anselmo *et al.,* 2006). WNK4 also binds and regulates OSR1 and SPAK (Gagnon *et al.,* 2006). The role of SPAK/OSR1 in the regulation of KCCs by WNK3 has not been examined. The regulation of paracellular Cl^- permeability by WNK4 involves phosphorylation of the tight junction proteins claudins (Yamauchi *et al.,* 2004).

WNKs also regulate surface expression of membrane proteins. WNK1 activates ENaC by preventing its endocytosis associated with a Nedd4-mediated ubiquitination (Xu *et al.,* 2005a). WNK1 and WNK4 inhibit ROMK by stimulating endocytosis of the channel via clathrin-coated vesicles (CCV) (Kahle *et al.,* 2003; Lazrak *et al.,* 2006; Wade *et al.,* 2006). One unique feature of WNK proteins is that they contain multiple PXXP motifs known for binding SH3 domains. A recent study (He *et al.,* 2007) reports that WNK1 and 4 stimulate CCV-mediated endocytosis of ROMK by recruiting intersectin, a multimodular endocytic scafford protein containing five SH3 domains (Sengar *et al.,* 1999). The effects of WNK1 and 4 on NCC also occur through changes of membrane expression (Yang *et al.,* 2003; Cai *et al.,* 2006). The precise mechanism of regulation of surface expression of NCC, though, remains unknown.

In aggregate these studies suggest that, in addition to regulating the activities of some of their transporter targets via phosphorylation, WNKs also generally act on the machinery that controls endocytosis and exocytosis of membrane transporters. WNK1 partially co-localizes with and phosphorylates synaptotagmin 2, a protein involved in vesicle exocytosis and endocytosis (Lee *et al.,* 2004). Phosphorylation by WNK1 changes the requirement for calcium to induce binding of synaptotagmin 2 to phospholipid vesicles. These studies suggest the potential for modulation of endocytic and/or exocytic pathways by WNKs. In support of this idea, two WNK family members, WNK2 and WNK4, were identified in an RNAi screen of human protein kinases required for clathrin- and caveolin-dependent and independent endocytosis (Pelkmans *et al.,* 2005). The capacity to modulate membrane turnover may be essential for the breadth of transport mechanisms apparently affected by WNKs. If a more general action on membrane assembly and turnover can be substantiated, it seems likely that membrane transport and receptor systems throughout the body may be influenced by WNKs.

Role of kinase domain of WNKs in the regulation of effectors

Mutation of the catalytic lysine (lysine-233; K233) or a conserved aspartate (aspartate-368; D-368) in the kinase domain prevents WNK1 regulation of ROMK1 (Lazrak *et al.,* 2006; Wade *et al.,* 2006). Effects of these mutations may be due to loss of

kinase activity and/or alteration of structure of WNKs. The crystal structure of the kinase domain of WNK1 reveals that K233 and D368 are in close proximity (Min *et al.,* 2004) (Figure 2). He *et al.* (2007) reported that a WNK1 mutant carrying charge conserved substitution of lysine-233 (to arginine; K233R) regulates ROMK1 similar to the wild type WNK1. A double mutant of WNK1 carrying charge reversal mutations of K233 and D368 (K233D/D368K) also regulates ROMK1 as well as wild type WNK1 does. Both single mutant K233R and double mutant K233D/D368K lack catalytic activity. These results suggest that electrostatic interactions between K233 and D368 are critical for regulating endocytosis of ROMK1 and that kinase activity of WNK1 is not necessary for the regulation. In the same study, the authors further provide evidence supporting that a stable structure of the kinase domain (involving the interaction between K233 and D368) is critical for WNK1 to recruit intersectin through binding to PXXP motifs (He *et al.,* 2007).

Upstream regulators of WNK kinases

While the downstream targets of WNK kinases are being unraveled, their upstream regulators remain much less understood. At the moment, there are two potential physiologically important regulators of WNK1. One is hypertonicity (Xu *et al.,* 2000; Lenertz *et al.,* 2005). Hypertonicity stimulates autophosphorylation of WNK1 on serine-382, activating WNK1 to phosphorylate SPAK and OSR1 (Anselmo *et al.,* 2006; Zagorska *et al.,* 2007). SPAK and OSR1 kinase activate NKCC1 and NKCC2 by causing phosphorylation on the transporters (Moriguchi *et al.,* 2005; Vitari *et al.,* 2005, 2006; Anselmo *et al.,* 2006). Hyperotonicity also induces a rapid redistribution of WNK1 from cytosol to vesicular structures (Zagorska *et al.,* 2007). The activation of NKCC via WNK1 and SPAK/OSR1 may be important for regulatory volume increase of cells in response to hypertonicity. In hypertonic extracellular solutions, cells shrink as water leaves. Activation of NKCC increases net chloride and cation entry into cells, raising cell osmolarity and driving water reuptake.

Insulin/insulin-like growth factors (IGF) have also been implicated as upstream regulators of WNK1 (Vitari *et al.,* 2004; Jiang *et al.,* 2005; Xu *et al.,* 2005b). The threonine-58 of WNK1 is a phosphorylation site for Akt/protein kinase B, a downstream kinase of insulin/IGF pathway (Vitari *et al.,* 2004; Xu *et al.,* 2005b). Insulin and IGF1 cause phosphorylation of endogenous WNK1 at the threonine residue (Vitari *et al.,* 2004: Jiang *et al.,* 2005). Activation of ENaC by WNK1 via serum and glucocorticoid-inducible kinase (SGK1) depends on basal phosphorylation of threonine-58 (Xu *et al.,* 2005b). The activation of ENaC by Akt-WNK1 pathway may underlie the mechanism of sodium retention by insulin.

Role of WNK1 in organ development

WNK1 is ubiquitous (Xu *et al.,* 2000; Delaloy *et al.,* 2003; O'Reilly *et al.,* 2003), raising the question of whether WNK1 plays any role in other organ systems. Support that WNK1 is involved in other organ systems comes from a recent study of mice in which the *WNK1* gene has been disrupted (Zambrowicz *et al.,* 2003). *WNK1* heterozygous mice have low blood pressure. Mice homozygous for disruption of the *WNK1* gene die during embryonic development before embryonic day 13. The timing of death suggests defects in the cardiovascular development. Consistent with this possibility, Delaloy *et al.* (2006) reported that WNK1 expresses in the developing and adult heart and blood vessels in mice. The expression during embryonic development occurs as early as embryonic day 9.5-10.5. Analyzing embryos of time-mated WNK1-null mice, we recently found that WNK1 is indeed essential for cardiovascular development (Xie *et al.,* 2007). These findings raise interesting questions as to whether effects of WNK1 overexpression on the cardiovascular system may contribute to hypertension in PHAII patients with WNK1 mutations. The mechanism and signaling pathways for regulation of development by WNK1 remains to be investigated.

Role of WNK kinases in cell signaling, proliferation, survival, and oncogenesis

Functions of WNK proteins in signaling, survival, and proliferation have been reported. The fly WNK (CG7177) was among molecules identified in an RNAi screen for proteins required for the survival of cultured *Drosophila* cells (Boutros *et al.,* 2004). The same protein was also a strong inhibitor of signaling through the Wnt pathway, which is critical for embryonic development as well as in the adult (DasGupta *et al.,* 2005). WNK1 activates the ERK5 MAPK signaling pathway in response to low concentrations of EGF (Xu *et al.,* 2004). ERK5 is implicated in proliferative as well as developmental pathways, notably in cardiovascular development (Nicol *et al.,* 2001; Yan *et al.,* 2003). WNK3 pro-

motes cell survival by inhibiting caspase-3 (Verissimo *et al.,* 2006). In addition, WNKs modulate TGFβ-Smad signaling (Lee *et al.,* 2007). There are two effects of WNKs on TGFβ-Smad signaling. One is to increase the cellular Smad2 level. The other is to decrease the accumulation of phosphorylated Smad2 in the nucleus, causing inhibition of Smad2-mediated transcription. The dual actions of WNKs on TGFβ-Smad signaling may be relevant to the fact that TGFβ signaling can lead to both tumor suppression and progression depending the microenvironment (Derynck *et al.,* 2001; Massague and Gomis, 2006).

Large-scale cancer genomic sequencing have identified point mutations of *WNK1* in breast and colon cancer (Stephens *et al.,* 2005; Sjoblom *et al.,* 2006), *WNK2* in lung cancer (Davies *et al.,* 2005), and *WNK1-WNK4* in a spectrum of solid tumors (Greenman *et al.,* 2007). A recent epigenome scan has revealed that decreased *WNK2* expression links to the genesis of adult infiltrative gliomas (Hong *et al.,* 2007). Epigenome scan revealed a significant increase of aberrant methylation in contiguous CpG islands of the promoter of *WNK2* in adult glioma tissues. The aberrant methylation in gliomas is associated with a reduced expression of *WNK2* compared to the normal brain. Restoration of WNK2 in gloma cells by forced expression of exogenous WNK2 suppresses colony formation. Thus, WNK2 functions as a tumor suppressor for glioma cells and *WNK2* silencing contributes to genesis of gliomas. Given their roles in cell survival and proliferation, connections between WNKs and oncogenesis can be anticipated to unfold in the future.

Conclusion

WNKs are expressed widely. Our knowledge of biology of WNKs and their physiological roles are limited at the present time. In the next few years the mass of information that has been and will be gathered on WNKs should help to elucidate their mechanisms of action in normal physiology and disease. In addition to the kinase domain, WNK proteins contain multiple domains for protein-protein interactions. Regulation of protein function by WNKs occurs by both catalytic and non-catalytic mechanisms. As in the above example of the role of the kinase domain of WNK1 in the regulation of ROMK1 (He *et al.,* 2007), the role of the kinase activity of WNKs in the regulation of protein function should be carefully evaluated in each case, using experimental approaches beyond the simple tool of the kinase-dead K233 or D368 mutant.

Acknowledgments

We thank Dr. Michel Baum for critical reading and comments on the manuscript. Work from the authors' laboratories was supported by grants from the National Institutes of Health (GM53032 to MHC and DK54368 and DK59530 to CLH) and the Welch Foundation (I1243 to MHC).

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