WNK1, a Novel Mammalian Serine/Threonine Protein Kinase Lacking the Catalytic Lysine in Subdomain II*

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We have cloned and characterized a novel mammalian serine/threonine protein kinase WNK1 (with no lysine (K)) from a rat brain cDNA library. WNK1 has 2126 amino acids and can be detected as a protein of ~230 kDa in various cell lines and rat tissues. WNK1 contains a small N-terminal domain followed by the kinase domain and a long C-terminal tail. The WNK1 kinase domain has the greatest similarity to the MEKK protein kinase family. However, overexpression of WNK1 in HEK293 cells exerts no detectable effect on the activity of known, co-transfected mitogen-activated protein kinases, suggesting that it belongs to a distinct pathway. WNK1 phosphorylates the exogenous substrate myelin basic protein as well as itself mostly on serine residues, confirming that it is a serine/threonine protein kinase. The demonstration of activity was striking because WNK1, and its homologs in other organisms lack the invariant catalytic lysine in subdomain II of protein kinases that is crucial for binding to ATP. A model of WNK1 using the structure of cAMP-dependent protein kinase suggests that lysine 233 in kinase subdomain I may provide this function. Mutation of this lysine residue to methionine eliminates WNK1 activity, consistent with the conclusion that it is required for catalysis. This distinct organization of catalytic residues indicates that WNK1 belongs to a novel family of serine/threonine protein kinases.

The protein kinase superfamily contains over a thousand members that share a catalytic core of approximately 300 residues organized in two domains (1–3). Conserved structural motifs within the core sequence maintain the basic fold of the catalytic domain, and fewer than 10 highly conserved residues create the functional elements of the active site (4, 5). Prior to the solution of the three-dimensional structure of cAMP-dependent protein kinase (PKA)1 by Knighton et al. (4), several of the residues essential for the integrity of the structure and the active site were identified primarily by a combination of multiple sequence alignment (1, 6), chemical modifications (7), and alanine scanning mutagenesis (8). Among these a lysine residue near the N terminus of the kinase in protein kinase subdomain II (Lys27 in PKA); this residue has frequently been mutated to eliminate the catalytic activity of protein kinases (9). This lysine functions to anchor and orient ATP through interactions with the α and β phosphor groups (4, 5, 10). Until recently all members of the protein kinase family were found to contain a lysine following a short string of hydrophobic residues in this conserved position. Kinase suppressor of Ras (KSR) contains arginine in place of this lysine but has not yet been shown to catalyze phosphorylation of protein substrates (11, 12). Mutation of this arginine in KSR does impair its function in reconstitution assays, suggesting that it plays a significant role in KSR function (11). Structural analysis of the mitogen-activated protein (MAP) kinase ERK2 shows that substitution of the conserved lysine with arginine causes the phosphoryl groups of ATP to be rotated away from the position necessary for phosphoryl transfer (10). Thus, the function of arginine in this conserved position of KSR is uncertain.

We have identified a novel protein kinase WNK1 (with no lysine (K)), which contains cysteine in place of lysine at the usual conserved location but has kinase activity as deduced from its ability to autophosphorylate and to phosphorylate an exogenous substrate in vitro. We have investigated the basis for its catalytic activity using a structural model, mutagenesis, and protein expression.

WNK1 was isolated in a nested PCR cloning strategy aimed to identify novel members of the MAP/extracellular signal-regulated protein kinase (ERK) kinase (MEK) family. MAP kinases are a family of protein kinases that have been utilized to varying degrees to regulate or modulate almost all signal transduction pathways in cells (13, 14). These enzymes themselves are regulated by cascades of at least two upstream protein kinases, a MEK and a MEK kinase. Members of the MEK (or MKK) family display considerable selectivity for their particular MAP kinase targets, thereby contributing to signaling specificity (13). They activate MAP kinases by dual phosphorylation on a tyrosine and a threonine residue, and each MEK recognizes only a small subset of possible MAP kinase substrates.

After the purification and cloning of the first MEK family member, MEK1 (15, 16), others (MKK2, MKK3, MKK4, MKK5, MKK6, and MKK7) were discovered through low stringency or PCR screens rather than by purification (17–25). Not only did this approach streamline the definition of the components of...
known MAP kinase cascades, but it also uncovered new pathways. We continued to examine clones derived from the screen that led us to isolate cDNAs encoding MEK5 (22). As described in this report, one of these clones encoded the unusual protein kinase WNK1.

**MATERIALS AND METHODS**

**Isolation of cDNA Clones Encoding WNK1**—First strand cDNA isolated from nerve growth factor stimulated PC12 cells was used as the template in PCR reactions utilizing nested degenerate primers derived from MEK sequences (22). One PCR product (product 15) was used to screen a rat forebrain cDNA library (kindly provided by Jim Boulter) at low stringency, and a weakly hybridizing clone of approximately 900 base pairs (PC12 clone 2-3) was isolated that had a short region of identity to the PCR product. This PC12 clone 2-3 was then used to rescreen the rat forebrain cDNA library at high stringency, and a group of strongly hybridizing clones were isolated. Partial sequences that encoded the N terminus and the kinase domain of WNK1 were assembled from two of these clones. A 0.5-kb WNK1 probe was labeled with [α-32P]dCTP by random-priming (Amersham Pharmacia Biotech) and used to screen another rat brain cDNA library that contains longer inserts (also kindly provided by Jim Boulter). One of the clones isolated contained the complete 3' WNK1 sequence. The full-length WNK1

**FIG. 1. Sequence of WNK1.** A, the amino acid sequence of full-length WNK1. The kinase domain is *underlined*, and the N-terminal peptide used to raise the anti-WNK1 antibody is *doubly underlined*. B, schematic representation of WNK1 showing the location of the kinase domain within the protein. Two potential coiled-coil regions and two proline-rich regions are also shown. There are total of 24 potential SH3 domain binding motifs (PXXP) in WNK1 represented by *black lines* in the diagram. In addition, the two proline-rich regions contain 3 and 11 PXXP motifs, respectively. C, sequence alignment of the kinase domain of WNK1 and its orthologs. The *roman numerals* shown above the sequences indicate subdomains. The accession numbers for the sequences from top to bottom are WNK1 (AF227741), AJ242724, Z68296, AF080436, AL049659, and Z46636.

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cDNA was assembled from these clones.

Northern Blot Analysis—A rat adult multi-tissue Northern blot (CLONTECH) was hybridized with a random-primer (Amersham Pharmacia Biotech) 0.5-kb WNK1 5' probe according to the manufacturer’s suggestions. The same blot was stripped and reprobed with a 2.8-kb WNK1 3' probe. This Northern blot was stripped again and hybridized with a β-actin probe to confirm the presence of mRNA in each lane.

Plasmids, Mutagenesis, and Proteins—A pSK-WNK1 full-length construct was created from three overlapping cDNA clones and was used as the template for the subsequent subcloning. A 1.6-kb WNK1 fragment encoding residues 1–555 was amplified by PCR with an EcoRI site incorporated at the 5' end and a HindIII site incorporated at the 3' end, and this fragment was digested with EcoRI-HindIII and ligated into pGEX-KG or pCMV5-Myc vectors that had been digested with EcoRI and HindIII to create pGEX-KG-WNK1 (1–555) and pCMV5-Myc-WNK1 (1–555). To make a pGEX-KG-WNK1 full-length construct, pGEX-KG-WNK1 (1–555) was digested with HindIII, filled-in with Klenow, and then digested with ScaI; pSK-WNK1 was digested with SpeI, filled-in with Klenow, and then digested with ScaI. The 6.5-kb insert fragment from pSK-WNK1 was gel purified and ligated into the 4.8-kb vector backbone. All constructs and mutants were sequenced to confirm the identity of the inserted DNA and that the insert had been ligated correctly.

Site-directed mutagenesis was carried out using the Quikchange kit (Stratagene) according to the manufacturer’s suggestions. The same blot was stripped and reprobed with a 2.8-kb WNK1 3' probe. This Northern blot was stripped again and hybridized with a 2.8-kb probe, which was digested with EcoRI and HindIII; pSK-WNK1 was digested with HindIII, filled-in with Klenow, and then digested with ScaI. The 6.5-kb insert fragment from pSK-WNK1 was gel purified and ligated into the 4.8-kb vector backbone to create pGEX-KG-WNK1 (1–555) and pCMV5-Myc-WNK1 (1–555). To make a pGEX-KG-WNK1 full-length construct, pGEX-KG-WNK1 (1–555) was digested with HindIII, filled-in with Klenow, and then digested with ScaI. The 6.5-kb insert fragment from pSK-WNK1 was gel purified and ligated into the 4.8-kb vector backbone. All constructs and mutants were sequenced to confirm that the sequences were correct.

GST-WNK1 (full-length and 1–555) proteins were expressed in and purified from Escherichia coli strain BL21DE3 using the standard protocol (28). The induction conditions were: 40 μg/ml (for 1–555) or 400 μg/ml (for full-length) isopropyl-β-D-thiogalactopyranoside at 30 °C for 5 h. The protein concentration was estimated by comparing to serial dilutions of bovine serum albumin on the same gel stained with Coomassie Blue. Myelin basic protein (MBP) was purchased from Calbiochem.

RESULTS AND DISCUSSION

Isolation of the Rat Full-length cDNA Encoding WNK1—In an attempt to isolate novel mammalian MEKs, nested degenerate PCR primers designed based on sequences conserved among MEK family members were used to amplify products from first strand cDNA isolated from PC12 cells (22). One product of 150 base pairs was used to probe a rat forebrain cDNA library. A clone isolated from a low stringency screen was used to screen two rat brain cDNA libraries and several positive clones encoding a novel protein kinase named WNK1.
were isolated. The full-length WNK1 cDNA containing 7.2 kb was assembled from three overlapping clones. The sequence surrounding the ATG start codon matched the Kozak consensus sequence for translation initiation, and stop codons were present upstream in all three reading frames. Although there was no poly(A) track found downstream of the stop codon in the available WNK1 sequence, there is a polyadenylation signal sequence (AATAAA) at the end of the cDNA clone. The open reading frame encoded by the WNK1 cDNA contains 2126 amino acids with a serine/threonine protein kinase domain in the N-terminal 490 residues (Fig. 1A and B). A partial clone encoding a closely related kinase (WNK2) was also isolated.

Homologs of WNK1 exist in Caenorhabditis elegans, Phycomyces, Arabidopsis, and Oryza as well as other mammals (Fig. 1C). A human expressed sequence tag containing an open reading frame lacking the kinase domain is almost identical to the WNK1 C-terminal sequence, and two human open reading frames containing partial kinase domains show strong similarity to the WNK1 catalytic domain, indicating that they may encode parts of human WNK family members. WNK1 and its homologs are characterized by the absence of the catalytic lysine found in subdomain II of almost all known protein kinases (1). In WNK1, a cysteine lies in the position of the usual lysine. WNK1 shares several sequence features with the MEK
family, including the length of the activation loop (between subdomain VII and VIII), the position of potential activating phosphorylation sites (SFAKS; these sites are also conserved in the IκB kinases IKK1 and 2 (34, 35)), conservation in the region of protein substrate binding, and a pattern of conserved hydrophobic residues near the C terminus. However, the kinase...
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WNK1 Is a Serine/Threonine Protein Kinase—To examine the kinase activity of WNK1, Myc-tagged WNK1 was transfected in 293 cells and then immunoprecipitated from lysed cells with an anti-Myc antibody. The immunoprecipitate showed kinase activity toward MBP as well as itself (Fig. 5A). Although activity toward MBP was detected, autophosphorylation was more consistent than MBP phosphorylation in the immune complex kinase assay because of the high background contributed by other protein kinases. Wild type GST-WNK1 expressed in E. coli phosphorylated both itself and MBP. Mutation of the putative Mg\(^{2+}\) binding residue aspartate 368 to alanine generated a WNK1 protein with no detectable kinase activity. Phosphoamino acid analysis revealed that phosphorylation occurs mainly on serine residues, indicating that WNK1 is a serine/threonine kinase (Fig. 5B).

To test the endogenous activity of WNK1, the endogenous protein was immunoprecipitated with anti-WNK1 antibodies and assayed by its ability to autophosphorylate. Immunoblotting confirmed that WNK1 was immunoprecipitated (not shown). The ability of WNK1 to autophosphorylate was apparent from the incorporation of labeled phosphate into a band of approximately 230 kDa. In contrast, neither preimmune serum nor the unrelated anti-ERK1 antibody immunoprecipitated an autophosphorylating band of this size (Fig. 5C). To identify regulators of WNK1, we tested a number of agents and stimuli to determine whether they could increase WNK1 activity in 293 cells. Endogenous WNK1 was immunoprecipitated following cell treatment, and autophosphorylation was assayed. Among the stimuli tested, 0.5 M NaCl (Fig. 5D) and less so 0.5 M sorbitol (not shown) caused a reproducible increase in WNK1 autophosphorylation, suggesting that WNK1 may be involved in osmosensing pathways. No effects were detected with epi- dermal growth factor, the microtubule disrupting agent nocodazole, anisomycin, lysophosphatidic acid (Fig. 5D), serum, heat shock, phorbol ester, H\(_2\)O\(_2\), or okadaic acid (not shown).

The Known MAP Kinase Pathways Are Not Activated by Overexpression of WNK1 in 293 Cells—Because WNK1 was isolated as a possible MEK homolog and has modest similarity to the MEKK-like and Ste20p-like kinases within its catalytic domain, we examined the potential regulation of MAP kinase pathways by WNK1. The WNK1 kinase domain (residues 1–555) was expressed in mammalian cells and the activities of the known MAP kinases, HA-tagged forms of ERK2, ERK5, JNK1, or p38, were measured. Myc-tagged WNK1 (1–555), constructs, either wild type or kinase dead (D368A), were used in the majority of experiments because expression was to a much greater extent than for full-length WNK1. HA-tagged proteins were then immunoprecipitated with an anti-HA antibody and assayed using MBP, c-Jun, or ATF-2 as substrates. No obvious changes in activity of any of these kinases were observed with overexpression of either wild type or kinase-dead WNK1 (1–555) (Fig. 6) or with full-length WNK1 (not shown). In vitro, GST-WNK1 displayed neither MEK nor MEKK activity. It failed to phosphorylate recombinant ERK1, ERK2, ERK5, JNK1, or p38. WNK1 also did not phosphorylate MEK1, MKK2, MKK3, MKK4, MKK5, MKK6, or MKK7; nor did it phosphorylate I\(_k\)B, ribosomal protein S6, or fragments of MEKK1 (not shown). In addition, the two possible phosphorylation sites of WNK1 (SFAKS) that lie in the same relative positions as the activating sites of phosphorylation in the MEK family were mutated to aspartic acid (S\(_{78D}/S\(_{82D})\). Although comparable mutations increase the activity of some MEK family members (e.g. MEK1), there was no detectable effect of these mutations on WNK1 activity (not shown). In summary, these results suggest that WNK1 does not directly regulate the MAP kinase pathways tested.
WNK1 Lacks the Conserved Catalytic Lysine Residue in Subdomain II—Several residues involved in catalysis of phosphoryl transfer are highly conserved in all protein kinases, notably the catalytic lysine residue present in kinase subdomain II, which binds to ATP. Surprisingly, this apparently invariant lysine residue is replaced by a cysteine (Cys) in WNK1. The lack of lysine at this position was confirmed in multiple independent clones of rat WNK1. More striking, this unusual difference is conserved across diverse species, suggesting functional relevance. Two possible explanations for this deviation in WNK1 were either that the catalytic lysine was located at a different position in the structure or that the WNK1 catalytic mechanism was distinct from other protein kinases. To distinguish between these possibilities, we first created a structural model of WNK1 based on the coordinates of PKA (Fig. 7). Based on this model, several candidate lysine residues that might potentially function in ATP binding, Lys233, Lys256, and Lys259, were mutated to methionine. In addition, Cys250 was mutated to either alanine or lysine to determine whether it was required for catalysis. These mutants were expressed in bacteria as GST fusion proteins and assayed in vitro using MBP as substrate (Fig. 8). WNK1 C250K had greatly reduced kinase activity, suggesting that Cys250 may play some catalytic role. However, WNK1 C250A had the same activity as wild type protein. This result demonstrated that Cys250 is not required for WNK1 activity, but a lysine that has a larger and positively charged side chain may interfere with the folding or activity of the catalytic site. WNK1 K256M and K259M exhibited kinase activity similar to the wild type protein, indicating that Lys256 and Lys259 are not required for catalytic activity. In contrast, WNK1 K233M had no detectable kinase activity, indicating that Lys233, like Asp386, plays a critical role in kinase activity. This lysine residue, Lys233, is conserved in position in all the WNK homologs, consistent with its importance for catalytic activity. Interestingly, Lys233 replaces a glycine residue in the catalytic site structure can be preserved despite this significant sequence deviation. Interestingly, a key lysine present in GTPases and other nucleotidases often lies quite near the glycine string (37). Perhaps the altered organization of the catalytic residues in WNK1-like kinases reflects a yet-to-be-discovered regulatory or functional adaptation better served by this particular sequence arrangement.

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