Regulation of WNK1 by an Autoinhibitory Domain and Autophosphorylation*

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WNK family protein kinases are large enzymes that contain the catalytic lysine in a unique position compared with all other protein kinases. These enzymes have been linked to a genetically defined form of hypertension. In this study we introduced mutations to test hypotheses about the position of the catalytic lysine, and we examined mechanisms involved in the regulation of WNK1 activity. Through the analysis of enzyme fragments and sequence alignments, we have identified an autoinhibitory domain of WNK1. This isolated domain, conserved in all four WNKs, suppressed the activity of the WNK1 kinase domain. Mutation of two key residues in this autoinhibitory domain attenuated its ability to inhibit WNK kinase activity. Consistent with these results, the same mutations in a WNK1 fragment that contain the autoinhibitory domain increased its kinase activity. We also found that WNK1 expressed in bacteria is autophosphorylated; autophosphorylation on serine 382 in the activation loop is required for its activity.

Protein kinases are essential regulators of numerous cellular processes ranging from cell cycle control to differentiation. WNKs (with no lysine [K]) comprise a newly described protein kinase subfamily implicated in controlling the ion permeability of epithelia (1–3). A unique feature in this kinase subfamily is that the lysine necessary for positioning ATP for phosphorylation is located in an atypical position in the catalytic domain. WNKs, distant relatives of the Ste20p family of protein kinases, have been found in numerous metazoan organisms, including plants, Caenorhabditis elegans, rodents, and humans (1–5). Although WNKs have not been found in Saccharomyces, a WNK homolog in Phycomyces was the first reported WNK sequence; because Phycomyces is a filamentous fungus, it cannot be concluded with certainty that WNKs function in unicellular organisms (4).

Rat WNK1 was the first mammalian member of this protein kinase family cloned and characterized (1). It contains an N-terminal kinase domain, two coiled-coil domains, and 24 PXXP motifs, which could potentially interact with SH3 domains of other proteins. Partial clones of WNK1 and WNK2 were isolated from cancerous prostate tissue and pancreatic cells, respectively (6, 7). Four WNK family members have been identified in humans with high sequence identity within their kinase domains (3).

Mutations in WNK1 and WNK4 have been shown to cause pseudohypopaldosteronism type II, a form of familial hypertension resulting from activation of a single gene (2). Northern analysis indicates that WNK1 is widely expressed in different tissues, whereas WNK4 is expressed primarily in kidney (1–3). The site of action of these kinases that leads to hypertension is proposed to be the cortical collecting duct of the kidney (2). Mutations found in WNK1 are deletions in the first intron, causing increased WNK1 expression. Mutations in WNK4 are in the coding sequence near the coiled-coil domains; these mutations are presumed to enhance WNK4 function. The known mutations are located in regions highly conserved among members of the WNK family but not common to other protein kinases. The broad distribution of WNKs suggests that they will impact the function of epithelial tissues throughout the body.

Protein kinase activity may be regulated by several mechanisms individually or in combination. Many protein kinases contain an autoinhibitory domain outside their catalytic domain, which suppresses kinase activity until some activating signal displaces it from its inhibitory site. Removal of the autoregulatory sequences will increase kinase activity as long as other modifications are not required to release the active conformation. cAMP-dependent protein kinase has been a model for the structural analysis of this mechanism (8, 9). Its regulatory subunit inhibits the activity of an otherwise active catalytic subunit (10). Most protein kinases that employ this mechanism contain an autoregulatory domain within the same protein chain. The first of these to be characterized directly was that in twitchin, a myosin light-chain kinase relative, in which intrasteric inhibition is relieved by the binding of calmodulin (11). The Ste20p-like kinase PAK1 can be activated by the small G proteins Rac or Cdc42; they bind to a region on PAK1 near the autoinhibitory domain promoting disinhibition (12, 13). Point mutations have been identified within the PAK regulatory domain that relieve intrasteric suppression of activity (12, 13). Harrison’s group solved the crystal structure of PAK1 in the autoinhibited conformation (14). They showed that inactive PAK1 exists as a dimer, and the autoinhibitory sequence makes extensive contacts with the active site. Binding of small

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The abbreviations used are: PAK1, p21-activated kinase 1; MBP, myelin basic protein; GST, glutathione S-transferase; MAPK, mitogen-activate protein kinase; ERK, extracellular signal-regulated kinase; MS, mass spectrometry; MEK, MAPK/ERK kinase; TAK1, TGFβ-activated kinase 1.

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G-proteins cause conformational changes that relieve autoinhibition (15). In this report, we present findings supporting the conclusion that WNK1 is regulated in part through an autoinhibitory mechanism. Additional experiments to probe the regulatory mechanisms of WNK1 have led to the identification of an autophosphorylation site in the activation loop; phosphorylation of this residue in the activation loop is a common mechanism for control of protein kinase activity. We also tested whether the position of the catalytic lysine in the phosphate anchor ribbon is consistent with activity in another protein kinase.

MATERIALS AND METHODS

Constructs, Mutagenesis, and Proteins—pGEX-KG-WNK1 and pGEX-KG-WNK1-(1–555) were as described previously (1). To make pGEX-KG-WNK1-(1–491) and pCMV5-Myc-WNK1-(1–491) constructs, a 1.5-kb DNA fragment encoding WNK1-(1–491) was amplified by PCR using pSK-WNK1 as the template and subcloned into pGEX-KG and pCMV5-Myc. To make pGEX-KG-WNK1-(1–639), a 1.9-kb DNA fragment encoding WNK1-(1–639) was amplified by PCR also using pSK-WNK1 as the template and subcloned into pGEX-KG. pGEX-KG-WNK1-(485–555) and pGEX-KG-WNK1-(485–639) plasmids were constructed in a similar way. To make His6-WNK1-(1–491) and His6-WNK1-(189–491), a PCR product encoding either WNK1-(1–491) or (189–491) was subcloned into a pParallel-His6 vector. pCMV5-Myc-ERK2, pCMV5-RasV12, and pCMV5-MAK1-R4F were as described previously (16). Site-directed mutagenesis was performed using the QuickChange kit (Stratagene) according to the manufacturer’s instructions. All mutations were confirmed by double-stranded DNA sequencing.

GST-WNK1, GST-WNK1-(1–491), GST-WNK1-(1–555), GST-WNK1-(1–639), GST-WNK1-(485–555), GST-WNK1-(485–639), GST, GST-PAK1-(1–231), His6-ERK2 and His6-WNK1-(1–491) were expressed in E. coli strain BL21 using standard purification protocols. His6-WNK1-(189–491) was expressed in the Rosetta strain (Novagen). Myelin basic protein (MBP) was from Sigma.

Cell Culture, Immunoblotting, and Immunoprecipitation—HEK 293 cells were maintained, transfected, and harvested as described previously (17). The anti-Myc antibody was obtained from the National Cell Culture Center. The anti-WNK1 antiserum 2562 was as described previously (1). For immunoblotting, cell lysates were separated by SDS-PAGE and electrotransferred to nitrocellulose paper. Blots were probed with the indicated antibodies, and then developed using enhanced chemiluminescence. Proteins were immunoprecipitated from 0.2 ml of cell lysate with 2 μl of the specified antibody and 40 μl of protein A-Sepharose beads for a total of 2 h. Precipitates were washed three times with 20 mM Tris-HCl (pH 7.4), 1 M NaCl, and once with 10 mM Hepes (pH 8.0) and 10 mM MgCl₂. In Vitro Kinase Assays—Kinase assays were performed in 30 μl of 1× kinase buffer at 30 °C as described (1). Reactions were stopped by adding 7.5 μl of 5× SDS sample buffer followed by boiling for 2 min. Reactions (20 μl) were analyzed by SDS-PAGE and autoradiography.

Mass Spectrometry—100 pmol of WNK1-(189–491) was purified from Rosetta cells was digested with 5 pmol of trypsin (Promega) at 37 °C for 1 h. The digested peptide was split into two equal aliquots. One aliquot was treated with Lambda protein phosphatase (New England BioLabs) at 30 °C for 1 h. Each digest was diluted to ~5 pmol/ml in 50 mM ammonium bicarbonate. Approximately 1 ml of diluted sample was mixed with 2.5-, 3-dihydroxybenzoic acid matrix solution (Agilent Technologies, Palo Alto, CA), then spotted onto a 96-well metal plate (PerSeptive Biosystems, Framingham, MA). Experiments were performed on a QSTAR Pulsar i quadrupole time-of-flight tandem mass spectrometer (MD SCIEX, Toronto, Canada) equipped with a matrix-assisted laser desorption ionization ion source. For MS scans, laser intensity was set at 55 μJ. For MS/MS experiments, laser intensity was adjusted high enough to obtain a sufficient fragmentation signal, the Q1 resolution was set to unit resolution, and argon was used as the collision gas in Q2. All the data were acquired in the manual tune mode, and the accumulation time was set to obtain sufficient signal yield.

RESULTS

Activity of GST-WNK1 Fragments—Protein kinases often contain an autoinhibitory domain outside of the kinase catalytic domain. The removal of the autoinhibitory domain usually results in a constitutively active kinase. To begin to define the mechanisms that regulate the activity of WNK1, we wished to determine if it contains such an autoinhibitory domain. Several GST-tagged WNK1 fragments were expressed in bacteria, purified and tested for their kinase activities (Fig. 1). GST-WNK1-(1–491) had readily detectable kinase activity when MBP was used as its in vitro substrate. However, GST-WNK1-(1–555) showed only 1–2% of the activity, suggesting that the additional residues in this fragment were suppressing the catalytic activity of WNK1. GST-WNK1-(1–639), which includes the first coiled-coil domain not present in the other two fragments, was intermediate in activity (~45% of 1–491). GST-WNK1-(159–491) also had significantly greater activity than GST-WNK1-(159–555) (data not shown).

GST-WNK1-(485–555) Inhibits the Activity of His6-WNK1-(1–491) in Vitro—Based on the relative activities of these fragments, we tested residues present in WNK1-(1–555) but not in WNK1-(1–491) as a possible autoinhibitory region. We expressed GST-WNK1-(485–555) in bacteria and tested its ability to inhibit the activity of His6-WNK1-(1–491). We also expressed a GST fusion protein containing residues 485–639, with the idea that inclusion of the coiled-coil domain might moderate the effect of an autoinhibitory domain, based on the observation above that increasing the length of the WNK1 fragment was able to restore activity significantly. As shown in Fig. 2A, GST-WNK1-(485–555) reduced WNK1 kinase activity in a dose-dependent manner; 50% inhibition was achieved at a concentration of the isolated intrasteric domain of ~0.4 μM. On the other hand, GST-WNK1-(485–639) had a significantly reduced ability to inhibit WNK activity compared with GST-WNK1-(485–555), consistent with the results above. As expected, GST alone had little effect on the activity of His6-WNK1-(1–491).

The Ste20p-like protein kinase PAK1 contains a well-defined autoinhibitory domain near its N terminus (18). To examine the specificity of the autoregulatory domain toward WNK1, we compared the ability of GST-PAK1-(1–231), which encompasses the PAK1 autoinhibitory domain, to influence the activity of His6-WNK1-(1–491). Although GST-WNK1-(485–555) exhibited a strong inhibitory effect on His6-WNK1-(1–491), the
WNK1 contains an autoinhibitory region just C-terminal to its kinase domain. A, GST alone, GST-WNK1-(485–555), or GST-WNK1-(485–639) proteins were examined for their ability to inhibit the kinase activity of His6-WNK1-(1–491) toward MBP. Top, autoradiogram of the kinase assay. Bottom, plot as percentage of 32P incorporation. One of three similar experiments. B, GST-PAK1-(1–231) protein was compared with GST-WNK1-(485–555) for its ability to inhibit the kinase activity of His6-WNK1-(1–491) toward MBP. Top, autoradiogram of the kinase assay. Bottom, plot as percentage of 32P incorporation. One of three similar experiments. C, GST alone was compared with GST-WNK1-(485–555) for its ability to inhibit the kinase activity of His6-ERK2 toward MBP. Top, autoradiogram of the kinase assay. Bottom, plot as percentage of 32P incorporation. One of two similar experiments.

autoinhibitory domain of PAK1 was a very poor WNK1 inhibitor (Fig. 2B) at concentrations that completely block PAK activity (not shown) (12). To test the specificity of this autoinhibitory domain toward other protein kinases, we examined the ability of GST-WNK1-(485–555) to inhibit ERK2 and p38 MAPKs. ERK2 was inhibited strongly by the WNK1 autoinhibitory domain (Fig. 2C), whereas the autoinhibitory domain had little effect on p38 (data not shown).

Point Mutations in the WNK1 Intrasteric Inhibitory Domain Impair Its Capacity to Suppress WNK1 Activity—Within their kinase domains WNK family members display almost 90% identity across mammalian species. The sequence identity outside the kinase core is limited; however, the region corresponding to WNK1 residues 485–555 is well conserved. A sequence alignment of this region among rat WNK1, human WNKs 1–4, and C. elegans WNK revealed two highly conserved phenylalanine residues, Phe-524 and Phe-526 in rat WNK1 (Fig. 3A). We mutated these two residues to alanine to determine whether the mutations would affect the ability of GST-WNK1-(485–555) to inhibit WNK1 activity. The F524A/F526A mutant showed a much reduced inhibitory effect compared with wild type GST-WNK1-(485–555), suggesting an 80–90% reduction in inhibitory potency (Fig. 3B). These results implicate these two phenylalanine residues in autoinhibition.

Assuming that Phe-524 and Phe-526 play important roles in WNK1 autoinhibition, mutation of these residues to alanine should increase the kinase activity of GST-WNK1-(1–555), although we expect that the inhibitory domain will still exert a significant effect within a single protein chain. To test this hypothesis, we expressed GST-WNK1-(1–555)-F524A/F526A and compared its kinase activity to wild type WNK1-(1–555). Indeed, the F524A/F526A mutant showed significantly enhanced activity (−2.5-fold relative to wild type). We calculated an estimated relative concentration of the autoinhibitory domain within the full-length protein to be in the range of 0.9–3 mM, based on the assumption of a 20-residue linker between the autoinhibitory site and the kinase core. With an IC50 of −0.4 μM and an effective concentration 3–4 orders of magnitude higher in the intact protein, the 2.5-fold increase in activity of GST-WNK1-(1–555) caused by the F524A/F526A mutations indicates a substantial effect of these residues on inhibition of the catalytic domain.

Mutation of WNK1 Autophosphorylation Site Reduces Its Kinase Activity—We showed previously that WNK1, like many protein kinases, autophosphorylates on serine residues (1). Autophosphorylation may be involved in the activation mechanism or may serve other purposes, such as creation of binding sites for other proteins. In the kinase family, autophosphorylation sites usually lie either within the activation loop or outside the catalytic core. WNK1 contains two serine residues, serine 378 and serine 382, within its activation loop that we expected would be phosphorylated due to the similarity of their positions to regulatory phosphorylation sites within other protein kinases, e.g. MAPKs and MAP kinase kinases. Because WNK1 expressed in bacteria has significant kinase activity, any modifications required for this activity should be self-catalyzed. Thus, we examined the possibility that WNK1 autophosphorylated on Ser-378 and/or Ser-382 and that these phosphorylations might be important for WNK1 activity. Ser-378 and Ser-382 were mutated singly and in combination to alanine or aspartic acid in GST-WNK1-(1–491) (Fig. 4A). The resulting mutants were tested for kinase activity (Fig. 4B). The single mutant S378A had ~50% of the activity of the wild type WNK1 fragment, suggesting that it is dispensable but contributes to maximal WNK1 activity. The double mutant and the single mutant S382A, on the other hand, had very little kinase activity (from 1–3% of wild type) toward MBP. The single mutant S382D and the double mutant also had very little activity. These results indicate that phosphorylation of S382 is essential for WNK1 activity and that aspartate cannot mimic phosphoryserine at this position. On the other hand, the single mutant S378D had 3- to 4-fold more activity than the wild type WNK1 fragment, suggesting that aspartate acid at this position can mimic the phosphorylated state of S378 and that this site also contributes to WNK1 activity.

To test whether WNK1 activation loop sites might be phosphorylated in mammalian cells, we transfected 293 cells with Myc-tagged WNK1-(1–491) wild type or the double mutant S378A/S382A. Cells were untreated or treated with 0.5 mM NaCl 15 min prior to harvest. Myc-WNK1-(1–491) was immunoprecipitated from cell lysates with an anti-WNK1 antibody fol-
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Fig. 3. Identification of two key residues in the WNK1 autoinhibitory domain. A, sequence alignment of the corresponding regions on human WNK1–4 and C. elegans WNK with rat WNK1 residues 514–537. Identical residues are shown in black boxes, and conserved residues are shown in gray boxes. The two key phenylalanine residues are shown with asterisks at the top. The consensus sequence is shown at the bottom. B, GST-WNK1(485–567) and F524A/F526A mutant protein was tested for its inhibition on His-WNK1(1–491) activity toward MBP. The -fold incorporation is shown. C, autoradiogram of the kinase assay using MBP as the substrate. The single S382A mutant behaved much like the double mutant (data not shown). We previously showed that NaCl activates endogenous WNK1 (1). NaCl caused a small but reproducible change in autophosphorylation of WNK1(1–491) but not of the double mutant. NaCl also caused a decrease in electrophoretic mobility of wild type and mutant WNK1(1–491). Although the shift in the wild type protein was not as obvious as for the double mutant in this experiment, the mobility of WNK1(1–491) has decreased consistently in NaCl-treated cells. WNK1 Is Autophosphorylated on S382—The molecular mass of His<sub>6</sub>-WNK1(198–491) determined by mass spectrometry was 80 Da higher than the calculated mass, suggesting that it was phosphorylated as isolated from bacteria. Following tryptic digestion, an aliquot of the protein was treated with phosphatase and compared with untreated protein. The MS scan of the tryptic digests showed a peak absent in the phosphatase-treated but present in the untreated sample (not shown). This peak corresponded to the peptide SVIGTPEFMAPEMYEEK in the activation loop. To determine which of the three potential phosphorylation sites in this peptide was phosphorylated, we measured the collision-induced dissociation MS of the parent ion that corresponded to the phosphopeptide p(SVIGTPEFMAPEMYEEK) (Fig. 4D). The py17 and py17a ions were identified, indicating that the phosphate is on the first serine of the peptide, serine 382. Additional analyses revealed some phosphorylation of serine 378 as well (data not shown).

ERK2 Is Active If the Catalytic Lysine Is Placed in the Phosphate Anchor Ribbon—Protein kinase catalytic domains have been characterized as containing twelve conserved subdomains that are located in well conserved structural elements of protein kinases (19, 20). Several key residues are absolutely required for catalysis and are thus considered invariable. Among these, the catalytic lysine, which binds phosphoryl groups of ATP, is found in subdomain II, which is located on beta strand 3 of the kinase core. A unique feature shared among WNKs is that this catalytic lysine is located not on beta strand 3, but instead on beta strand 2 in subdomain I, which contains another conserved element, the glycine-rich loop of the phosphate anchor ribbon. Beta strand 2 has a superficial position on the kinase core, making this catalytic residue of WNKs more surface-exposed than the functionally equivalent lysine of all other protein kinases.

The fact that all other kinases contain this essential lysine in beta strand 3 suggests the possibility that the catalytic lysine of protein kinases cannot function in any other position except in the WNK subfamily. To determine if the catalytic lysine of another protein kinase might be functional from the phosphate anchor ribbon, we mutated glycine 35 to lysine (G35K) and another protein kinase might be functional from the phosphate anchor ribbon. We previously showed that NaCl activates endogenous WNK1 (1). NaCl caused a small but reproducible change in autophosphorylation of WNK1(1–491) but not of the double mutant. NaCl also caused a decrease in electrophoretic mobility of wild type and mutant WNK1(1–491). Although the shift in the wild type protein was not as obvious as for the double mutant in this experiment, the mobility of WNK1(1–491) has decreased consistently in NaCl-treated cells.
G35K/K52A showed comparable activity to wild type ERK2 (data not shown). It is puzzling that this position of the active site lysine yields an active kinase in enzymes other than WNKs.

The WNK1 Mutant That Has the Normal Catalytic Lysine Placement Is Kinase-deficient—We then wanted to determine if the normal catalytic lysine placement could function in WNK1. To test this possibility, we constructed a WNK1 double mutant K233G/C250K, which eliminates the catalytic lysine from the phosphate anchor ribbon and instead places a lysine in the expected location for the catalytic residue in beta strand 3 (Fig. 6A). As shown before, both the K233M and C250K single mutants were completely kinase-deficient (1). One possibility for the lack of activity of C250K is that the bulk of the two lysine residues results in steric hindrance, preventing either lysine from carrying out normal catalytic function. Thus, we replaced the phosphate anchor ribbon lysine in WNK1 with the residue typically in that position in other kinases, a glycine. Nevertheless, WNK1 K233G/C250K did not exhibit detectable kinase activity, showing that the normal catalytic lysine placement in WNK1 could not restore its activity (Fig. 6B). This is in sharp contrast with the results indicating that the WNK catalytic
lysine placement retains kinase activity in ERK2. Other features of the WNK active site must dictate the unique lysine position.

DISCUSSION

We can now propose key elements of the regulatory mechanism of the WNK protein kinases. WNK1 contains an autoinhibitory sequence just C-terminal to the catalytic domain that is conserved in WNKs across species. This autoinhibitory domain suppresses the activity of the kinase domain, which is active once this domain is removed. Activation of WNKs requires autophosphorylation of at least one serine residue, serine 382 in WNK1, within the WNK activation loop. Phosphorylation of serine 378 also promotes increased activity. In addition, WNK1 is probably further activated by phosphorylation on other sites by one or more other protein kinases upon cell stimulation. This is suggested by the fact that S378A/S382A WNK1 consistently undergoes shifts in electrophoretic mobility, typical of quantitative changes in phosphorylation state, when cells are stimulated. Because this mutant has little catalytic activity on its own, its phosphorylation is almost certainly caused by one or more other protein kinases.

Many protein kinases contain phosphorylation sites within the activation loop. In some cases these are autophosphorylation sites, and in others these sites are phosphorylated by regulatory enzymes. WNK1 contains one essential and one more minor autophosphorylation site in this loop. WNK1 autophosphorylation occurs exclusively on serine residues (1). The S378A mutant autophosphorylates as well as wild type WNK1; S382 is highly phosphorylated in WNK1 because it is purified from bacteria; and there are no other serine residues in the activation loop. Thus, we conclude that the autophosphorylation that is detected must occur outside the activation loop, presumably in the serine-rich region N-terminal to the kinase domain. We note that replacement of the essential serine residue in the activation loop with aspartate does not enhance WNK1 activity (data not shown). Substitution of acidic residues for phosphorylated residues creates activated kinases in some cases, e.g. MEK1, but not others, e.g. ERK2 or TAK1 (21–23).

Interestingly, a GST-WNK1-(1–639) fusion protein retained considerable activity, although it also contains the autoinhibitory domain. Preliminary experiments indicate that WNK forms tetramers due to the coiled-coil domain C-terminal to the autoinhibitory domain. Gel filtration of cell lysates reveals that endogenous WNK1 is a tetramer. Recombinant fragments expressed in mammalian cells or in bacteria that retain the coiled-coil domain are also largely tetrameric. Smaller fragments of WNK1 behave as a monomer. At this time, we can only speculate on the mechanism of action of this domain. It seems likely that the formation of tetramers may enhance autoactivation of WNK1. One way in which oligomerization might enhance WNK1 activity is if autophosphorylation of WNK1 is a bimolecular event; intermolecular autophosphorylation controls the activity of several tyrosine kinases (e.g. see Ref. 24). This appears not to be so in the case of WNKs, because a kinase-dead WNK1 fragment is not a substrate for an active WNK1 fragment (data not shown). The finding that inclusion of the coiled-coil domain with the isolated autoinhibitory segment decreases autoinhibition of the WNK1 catalytic domain suggests that tetramerization suppresses the function of the autoinhibitory domain. Perhaps the coiled-coil domain interacts with the kinase domain to promote its autoactivation by some other mechanism.

Comparing the WNK autoinhibitory domain sequences reveals that conservation is strongest in a ~50-residue stretch (residues 515–569), which contains two phenylalanine residues that play key roles in autoinhibition. The FXF motif is reminiscent of one type of ERK2 docking domain found in several proteins (25). Interestingly, the WNK1 autoinhibitory domain can also inhibit ERK2. This is perhaps not surprising, because...
it has been previously reported that peptides containing FXF motifs act as inhibitors of ERK2 (25, 26). On the other hand, this inhibitory domain has little effect on the p38 MAPK, which is not reported to interact with FXF motifs or on the catalytic subunit of cAMP-dependent protein kinase (not shown). Mutating these two residues to alamines decreases inhibition caused by the isolated autoinhibitory domain and increases the activity of the auto-inhibited WNK1 fragment. We do not yet know if these residues primarily dock the autoinhibitory domain onto the kinase core or if they are involved in the folding of this small domain.

The most striking feature of the WNK protein kinase family is that they have the catalytic lysine within the glycine string in beta strand 2 instead of in a more buried position in beta strand 3 (1). We were able to show here that engineering this unique catalytic residue arrangement into ERK2 could result in an active kinase. Interestingly, only the ERK2 G35K/K52A mutant had kinase activity, whereas the G35K/K52R mutant was still kinase-deficient (data not shown). These results suggest that a large side chain at residue 52 will interfere with the function of K35 as the catalytic lysine within the glycine string, exhibited no detectable kinase activity. It is possible that the side chain of Trp-249, which just precedes the lysine in this mutant, is incompatible with the proper ATP interactions with Lys when it is placed at residue 250. We are suggesting that a large side chain at residue 52 will interfere with the unique catalytic residue arrangement into ERK2 could result in an active kinase. Interestingly, only the ERK2 G35K/K52A mutant had kinase activity, whereas the G35K/K52R mutant was still kinase-deficient (data not shown). These results suggest that a large side chain at residue 52 will interfere with the function of K35 as the catalytic lysine in ERK2. In contrast, the possibility that the side chain of Trp-249, which just precedes the lysine in this mutant, is incompatible with the proper ATP interactions with Lys when it is placed at residue 250. We are currently exploring the role of this catalytic site arrangement in the regulation of WNK1 activity.

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References
1. Xu, B., English, J. M., Wilsbacher, J. L., Stippec, S., Goldsmith, E. J., and Cobb, M. H. (2000) J. Biol. Chem. 275, 16795–16801
2. Wilson, F. H., Disee-Nicodeme, S., Choate, K. A., Ishikawa, K., Nelson-Williams, C., Desitter, I., Gunel, M., Milford, D. V., Lipkin, G. W., Achard, J. M., Feely, M. P., Dussol, B., Berland, Y., Unwin, R. J., Mayan, H., Simon, D. B., Farfel, Z., Jeunemaitre, X., and Lifton, R. P. (2001) Science 293, 1107–1112
3. Verissimo, F., and Jordan, P. (2001) Oncogene 20, 5562–5569
4. Ruiz-Perez, V. L., Murillo, F. J., and Torres-Martinez, S. (1995) Curr. Genet. 28, 309–316
5. Murakami-Kojima, M., Nakamichi, N., Yamashino, T., and Mizuno, T. (2002) Plant Cell Physiol. 43, 675–683
6. Moore, T. M., Garg, R., Coptcoat, M. J., Ridley, A. J., and Morris, J. D. (2000) J. Biol. Chem. 275, 4311–4322
7. Ito, M., Shichijo, S., Tsuda, N., Ochi, M., Harashima, N., Saito, N., and Itoh, K. (2001) Cancer Res. 61, 2038–2046
8. Knighton, D. R., Zheng, J., Ten Eyck, L. F., Xung, N.-H., Taylor, S. S., and Sowadski, J. M. (1991) Science 253, 414–429
9. Knighton, D. R., Pearson, R. B., Sowadski, J. M., Means, A. R., Ten Eyck, L. F., Taylor, S. S., and Kemp, B. E. (1992) Science 256, 130–135
10. Brandon, E. P.; Idzerda, R. L., and McKnight, G. S. (1987) Curr. Opin. Neurobiol. 7, 397–403
11. Hu, S. H., Parker, M. W., Lei, J. Y., Wilce, M. C., Benian, G. M., and Kemp, B. E. (1994) Nature 369, 581–584
12. Frost, J. A., Khokhlatchev, A., White, M. A., and Cobb, M. H. (1998) J. Biol. Chem. 273, 28293–28299
13. Zhao, Z.-S., Manier, E., Chen, Q., Chong, C., Leung, T., and Lim, L. (1998) Mol. Cell. Biol. 18, 2153–2163
14. Lei, M., Lu, W., Meng, W., Parrini, M. C., Eck, M. J., Mayer, B. J., and Harrison, S. C. (2000) Cell 102, 387–397
15. Parrini, M. C., Lei, M., Harrison, S. C., and Mayer, B. J. (2002) Mol. Cell 9, 73–83
16. Xu, B., Stippec, S., Robinson, F. L., and Cobb, M. H. (2001) J. Biol. Chem. 276, 20559–20565
17. Xu, B., Trisler, J. L., and Cobb, M. H. (1999) J. Biol. Chem. 274, 34029–34035
18. Johnson, C. A., Akamine, P., Radzio-Andzelm, E., Madhusudan, M., and Taylor, S. S. (2001) Chem. Rev. 101, 2243–2270
19. Hanks, S. K., Quinn, A. M., and Hunter, T. (1988) Science 241, 42–52
20. Knighton, D. R., Zheng, J., Ten Eyck, L. F., Ashford, V. A., Xung, N.-H., Taylor, S. S., and Sowadski, J. M. (1991) Science 253, 407–413
21. Robbins, D. J., Zhen, E., Owaki, H., Vanderbilt, C., Ebert, D., Geppert, T. D., and Cobb, M. H. (1993) J. Biol. Chem. 268, 5979–5986
22. Mansour, S. J., Mattes, W. T., Herrmann, A. S., Candia, J. M., Rong, S., Fukasawa, K., Vande Woude, G. F., and Ahn, D. B., Farfel, Z., Jeunemaitre, X., and Lifton, R. P. (2001) Science 293, 5562–5569
23. Kishimoto, K., Matsumoto, K., and Ninomiya-Tsuji, J. (2000) J. Biol. Chem. 275, 34035–34035
24. Cobb, M. H., Sang, B.-C., Gonzalez, R., Goldsmith, E., and Ellis, L. (1989) J. Biol. Chem. 264, 18701–18706
25. Jacobs, D., Hsu, P. C., Gonzalez, R., Goldsmith, E., and Ellis, L. (1999) J. Biol. Chem. 274, 7359–7364
26. Sharrocks, A. D., Sang, Y. H., and Galanis, A. (2000) Trends. Biochem. Sci. 25, 448–453