WNK1 and OSR1 regulate the Na^+, K^+, $2Cl^-$ cotransporter in HeLa cells

Anthony N. Anselmo*, Svetlana Earnest*, Wei Chen*, Yu-Chi Juang*, Sung Chan Kim*†, Yingming Zhao*†, and Melanie H. Cobb*‡

Departments of *Pharmacology and †Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX 75390

Contributed by Melanie H. Cobb, June 2, 2006

Oxidative stress-responsive kinase (OSR) 1 and sterile20-related, proline-, alanine-rich kinase (SPAK) are Ste20p-related protein kinases that bind to the sodium, potassium, two chloride cotransporter, NKCC. Here we present evidence that the protein kinase with no lysine [K] (WNK) 1 regulates OSR1, SPAK, and NKCC activities. OSR1 exists in a complex with WNK1 in cells, is activated by recombinant WNK1 *in vitro*, and is phosphorylated in a WNK1-dependent manner in cells. Depletion of WNK1 from HeLa cells by using small interfering RNA reduces OSR1 kinase activity. In addition, depletion of either WNK1 or OSR1 reduces NKCC activity, indicating that WNK1 and OSR1 are both required for NKCC function. OSR1 and SPAK are likely links between WNK1 and NKCC in a pathway that contributes to volume regulation and blood pressure homeostasis in mammals.

blood pressure | kinase | osmotic | stress

Protein kinase cascades mediate cellular responses to extracellular signals and environmental change. A large group of protein kinases with remarkably complex and diverse functions are related to the yeast protein kinase Ste20p (1). Ste20p was identified as a component of a mitogen-activated protein kinase cascade in the yeast pheromone-induced mating pathway (2, 3). One substrate of Ste20p is Ste11p, the MAP kinase kinase kinase in the module; thus, Ste20p is the prototypical MAP4K. Ste20p also controls cytoskeletal reorganization required for budding in yeast. Ste20p kinases coordinate the activities of downstream molecules not only because of their catalytic activities but also because of their capacities to bind and organize upstream molecules, including receptors and adaptors, and downstream molecules, including cytoskeletal elements and other protein kinases.

More than 30 Ste20p-related kinases are encoded in the human genome in two structurally distinct subfamilies, the p21-activated kinase (PAK) subfamily and the germinal center kinase (GCK) subfamily (1). The PAKs bind to GTP-liganded forms of the Rho family small G proteins Rac and Cdc42 and are key regulators of cytoskeletal organization and cell motility (4). The germinal center kinase-related kinases all contain N-terminal catalytic domains and diverse C-terminal domains that were used to categorize them into eight subfamilies (1).

OSR1 and the sterile20-related, proline-, alanine-rich kinase (SPAK) are the only two mammalian protein kinases in the germinal center kinase-VI subfamily. Although it has not been shown to be sensitive to oxidative stress, OSR1 was named for its similarity to oxidative stress-responsive kinase SOK1 (Ste20/oxidant stress response kinase-1) (5). OSR1 does respond to osmotic stress and phosphorylates PAK1, inhibiting its responsiveness to Cdc42 (6). In addition to their similar kinase domains, OSR1 and SPAK share two conserved C-terminal regions, known as PF1 and PF2 domains. The PF1 domain lies immediately C-terminal to the protein kinase domain and is required for catalytic activity of OSR1, although not part of the consensus core of protein kinases (6). The PF2 domain mediates protein–protein interactions and shows some similarity to a key regulatory region in STRAD, a subunit of the activation complex for the tumor suppressor LKB1 (7). Two hybrid tests revealed that SPAK and OSR1 bind to the Na^+, K^+, $2Cl^-$ cotransporter (NKCC) 1 and NKCC2, members of the solute carrier (SLC) 12 family (8, 9). Coimmunoprecipitation experiments confirmed the association of SPAK with NKCC1 (9). Residues in the N-terminal cytoplasmic domain of NKCC1 are phosphorylated and increase its activity (10, 11). Although there is disagreement regarding the regulatory action of SPAK, one group of studies showed that overexpression of inactive SPAK blocked NKCC1 phosphorylation and activation (8, 12). Certain sites phosphorylated in NKCC1 are conserved in NKCC2 and the sodium chloride cotransporter, another SLC12 family member, and are known to be phosphorylated in NKCC2 in response to vasopressin (13). Both NKCC1 and NKCC2 are recognized by a phosphospecific antibody raised to the phosphorylated sequence from NKCC1 (11, 13).

Here we confirm that the kinase with no lysine [K] (WNK) 1 (14) is an upstream activator of OSR1 and SPAK. In addition, we show that decreased expression of either WNK1 or OSR1 through small interfering RNA (siRNA) reduces NKCC activity in HeLa cells, indicating that these kinases are required for NKCC activity. Through positional cloning and gene disruption studies, WNK1 has been shown to be an essential gene that has an important role in the control of blood pressure in mammals (15, 16). Regulation of NKCC provides a potential mechanism by which WNK1 influences volume regulation and ion homeostasis.

Results

OSR1 Binds to WNK1 Through Conserved C-Terminal Motifs. To understand more regarding how OSR1 is activated, we performed two-hybrid screens with full-length OSR1 and a C-terminal fragment of OSR1 that includes its noncatalytic regulatory PF2 domain. Several inserts from the screens encoded overlapping portions of the protein kinase WNK1 that spanned residues 1834–2020 of the longer human splice form (1579–1765 of the shorter rat splice form). Each of the WNK1 inserts contained the sequence GRFXV. The interacting region, residues 1834–2020, together contains three GRFXV motifs, all between residues 1855–1960 (1600–1705 of the rat splice form). The motifs are conserved in WNK1 across species. Other WNK family members each have one similar motif. The interactions of a group of deletion proteins derived from the C terminus of human WNK1 were tested with the kinase domain, residues 1–344, and the C terminus, residues 292–527, of OSR1 (Fig. 1*A*). The WNK1 fragments containing at least one of the GRFXV motifs (streaks 2, 10, and 12) interacted with the C terminus of OSR1 but not with the N terminus of OSR1 (streaks 1, 9, and 11). OSR1 and its homolog SPAK bind to NKCC1 and, presumably, NKCC2 through a similar motif also present in these proteins (9, 17).

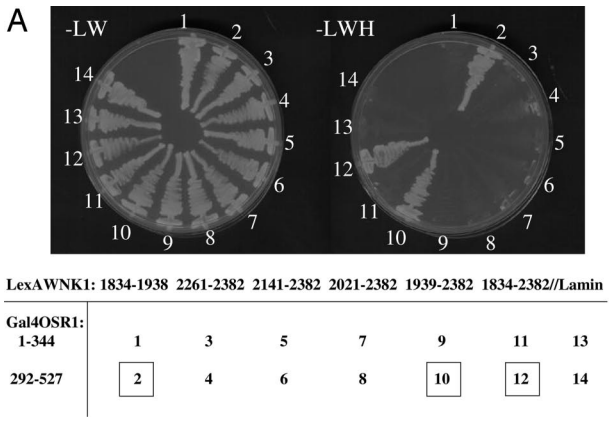
To confirm that the proteins interact in cells, OSR1 was expressed in HEK 293 cells and endogenous WNK1 was immuno-

BIOCHEMISTRY

Conflict of interest statement: No conflicts declared.

Abbreviations: NKCC, Na^+, K^+, $2Cl^-$ cotransporter; PAK, p21-activated kinase; SLC, solute carrier; SPAK, sterile20-related, proline-, alanine-rich kinase; WNK, with no lysine [K].

‡To whom correspondence should be addressed. E-mail: melanie.cobb@utsouthwestern.edu.



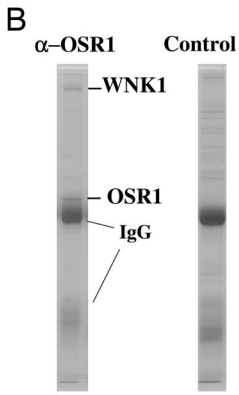


Fig. 1. Interaction of OSR1 with WNK1. (*A*) Two-hybrid analysis of the interaction of N-terminal and C-terminal domains of OSR1 with fragments of WNK1. The residues of fragments of WNK1 and OSR1 tested for interaction are as indicated. (*B*) Immunoprecipitation of OSR1 and mass spectrometric analysis of the major bands. The affinity-purified OSR1 antibody was used to immunoprecipitate OSR1 and associated proteins from HeLa lysates. Coomassie blue-stained proteins in one of three similar immunoprecipitations are shown. The protein stain of an experiment using an unrelated antibody is shown for comparison.

precipitated (Fig. 6, which is published as supporting information on the PNAS web site). OSR1 was found in the WNK1 immunoprecipitates but not in the preimmune controls. A strong band was detected in lysates of cells transfected with the larger amount of OSR1 DNA, and a faint band was detected with the smaller amount of OSR1 plasmid transfected. Sorbitol, a stimulus for both OSR1 and WNK1 (6, 18), did not change their association.

Antibodies to OSR1 were used to immunoprecipitate OSR1 and associated proteins from HeLa cell lysates. The Coomassie blue-stained gel of the immunoprecipitates from one such experiment (Fig. 1*B*) shows two major bands (in addition to IgG) and a number of minor bands. These two major bands are absent from an immunoprecipitation experiment with an unrelated antibody. The bands at ≈58 and ≈250 kDa were excised and analyzed by mass spectrometry. The smaller band is OSR1 and the larger band is WNK1. This result suggests that a significant fraction of the OSR1 in cells is constitutively associated with WNK1.

OSR1 Oligomerizes. The presence of a single PF2 domain in OSR1 suggested that it would interact with only a single partner at a time. Many Ste20p family members are oligomeric. We therefore tested the possibility that OSR1 self-associates, which would allow it to interact in a complex with multiple partners. Myc-tagged OSR1 full length (1–527) or fragments were cotransfected with Flag-tagged OSR1 (Fig. 7, which is published as supporting information on the PNAS web site). Full-length OSR1 coimmunoprecipitated with the full-length wild-type or kinase-dead (KR) OSR1 from cells lysed with or without detergent. As a control, ERK2 did not interact with OSR1 immunoprecipitated with antibody to either tag. Truncation of the PF2 domain in OSR1 1–433 had no effect on its self-association. The folded kinase domain, OSR1 1–344, was sufficient for its oligomerization. This result is consistent with the possibility that an OSR1 oligomer is associated with multiple partners through its PF2 domains.

WNK1 Phosphorylates OSR1 and SPAK and Stimulates Their Activity. OSR1 catalyzed the incorporation of little or no phosphate into either kinase-dead WNK1 1–491 or full-length WNK1 (data not shown). On the other hand, WNK1 catalytic fragments, residues 1–491 or 193–483, phosphorylated the catalytic domain of OSR1, clearly beyond the extent of OSR1 autophosphorylation (Fig. 2*A*). The stoichiometry achieved was >1 mol phosphate/mol OSR1. Neither of the WNK1 fragments used for *in vitro* reactions contained the more C-terminal interacting motifs; thus, phosphorylation occurred in the absence of stable binding of the PF2 domain to the GRFXV motifs.

We previously showed that OSR1 phosphorylated another Ste20p family member PAK1 on T84 in its regulatory domain (6). We used an N-terminal fragment of PAK1 as a substrate to measure OSR1 and SPAK activity. Phosphorylation by WNK1 193–483 increased the activity of OSR1 1–344, which was observed rapidly upon OSR1 phosphorylation (Fig. 2 *B* and *C*). OSR1 autophosphorylation in the absence of phosphorylation by WNK1 did not result in enhanced OSR1 activity. A WNK1 mutant, inactive because of mutation of the obligate autophosphorylation site (S382A), did not activate OSR1 (Fig. 2*D*). Quantitation of the difference in substrate phosphorylation by OSR1 with and without phosphorylation by WNK1 shows as much as a 25-fold activation of OSR1 by WNK1 and a >20-fold activation of SPAK 63–390. To determine whether OSR1 phosphorylates NKCC2, a fragment of

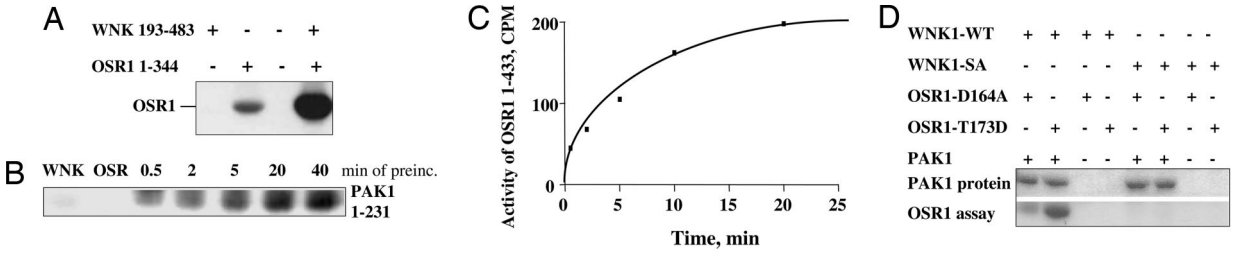


Fig. 2. Phosphorylation and activation of OSR1 1–344 by WNK1 193–483. (*A*) Phosphorylation of OSR1 with WNK1 *in vitro*. Phosphorylation reactions were in 30 μl of 1× kinase buffer (20 mM Hepes, pH 7.6/50 μM ATP (5 μCi of [γ-^{32}P]ATP)/10 mM $MgCl_2$/10 mM β-glycerol phosphate) at 30°C for 30 min. The autoradiogram is shown. (*B*) Activation of OSR1 by WNK1. OSR1 was incubated with WNK1 for the indicated times in the presence of 0.5 mM ATP, followed by the addition of PAK1 1–231 for an additional 15 min. The autoradiogram of substrate phosphorylation is shown. PAK1 1–231 was phosphorylated for 40 min by WNK1 alone in the first lane and by OSR1 alone in the second lane. (*C*) The time course of activation of OSR1 is plotted. (*D*) Activation of OSR1 requires the kinase activity of WNK1. Inactive WNK1 in which the obligate autophosphorylation site S382 has been mutated to alanine was used as the enzyme for comparison to active WNK1. OSR1 D164A is a catalytically inactive mutant. OSR1 T173D is an activation loop mutation without apparent effect on activity. (*D Upper*) The Coomassie-blue stained PAK1 1–231 in the assays. (*D Lower*) The autoradiogram of phosphorylated PAK1 1–231. *A–C* show one of five similar experiments. *D* shows one of two similar experiments.

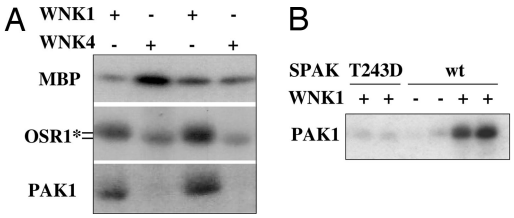


Fig. 3. Specificity of WNK1 and WNK4 toward OSR1 and SPAK. (*A*) WNK1 and WNK4 differentially activate OSR1. Equal amounts (lanes 1 and 2) and equal units of activity calculated by using myelin basic protein (MBP) as the substrate (lanes 3 and 4) of WNK1 and WNK4 were compared for their capacities to activate OSR1. (*A Top*) Kinase assays by using the model substrate MBP. (*A Middle*) OSR1 phosphorylation. A slight gel shift is associated with OSR1 activation. (*A Bottom*) The activity of OSR1 after preincubation with WNK1 or WNK4 by using PAK1 1–231 as substrate. Shown is one of three similar experiments. (*B*) Mutation of the essential activation loop threonine prevents activation of SPAK by WNK1. SPAK 63–390, either T243D or wild type, was incubated with WNK1, and the activity then was measured with PAK1 as indicated above. The autoradiogram shows PAK1 phosphorylation by SPAK and is one of two similar experiments.

NKCC2 containing the N-terminal phosphorylation sites was expressed and tested as substrate. After activation with WNK1, OSR1 also phosphorylated the NKCC2 fragment, as did SPAK (data not shown, see Fig. 4*A*).

WNK4 and WNK1 share >85% identity in their kinase domains; yet they do not have identical substrate specificities (19). We therefore compared effects of WNK1 and WNK4 on OSR1 kinase activity by using equal amounts of catalytic domain fragments of WNK1 and WNK4 and with equal units of WNK1 and WNK4 activity measured with the model substrate myelin basic protein (Fig. 3*A Top*). We found that OSR1 was phosphorylated to a lesser extent by WNK4 (Fig. 3*A Middle*). The phosphorylation that was observed did not cause increased OSR1 activity. Incubation with WNK1, but not WNK4, activated OSR1, which was detected by using PAK1 as the OSR1 substrate (Fig. 3*A Bottom*). Activation of OSR1 was associated with a small shift in its electrophoretic mobility. Only phosphorylation by WNK1 induced this shift; WNK4 did not cause this shift (Fig. 3*A Middle*). Because it seemed possible that WNK4 might show selectivity for the OSR1 homolog SPAK, which also was suggested by a recent study (20), we tested the capacity of WNK1 and WNK4 to activate SPAK. WNK1 phosphorylated full-length SPAK and induced a relatively large and complete gel shift visible in the Coomassie-stained gel (Fig. 8, which is published as supporting information on the PNAS web site). This shift was associated with a substantial activation of SPAK, which also recognized PAK1 as a substrate. On the other hand, WNK4 caused neither substantial phosphorylation nor a shift in mobility of SPAK under the same conditions. No increase in substrate phosphorylation by SPAK was detected. Thus, the two WNK family members, WNK1 and WNK4, display a marked difference in specificity in phosphorylating OSR1 and SPAK. The explanation for this specificity difference is currently unknown.

OSR1 and SPAK contain a serine and two threonine residues in its activation loop that might be sites of activating phosphorylation. Mutation of Thr-185 of OSR1 or Thr-243 of SPAK alone prevented activation of either kinase by WNK1 (data not shown and Fig. 3*B*), indicating that this site must be phosphorylated to activate the kinases and is consistent with an earlier report (21). Although mutation of this site to Asp caused a >3-fold increase in SPAK activity, the increase is much less than that after phosphorylation of the wild-type fragment by WNK1 (Fig. 3*B*). Because this site is poorly phosphorylated in kinase-dead mutants of OSR1, the role of OSR1 autophosphorylation in its activation mechanism remains to be determined.

We identified the smallest fragment of OSR1 that could be activated by WNK1. OSR1 and OSR1 truncations 1–443, 1–370, 1–343, and 1–323 were activated by WNK1, whereas 1–290, the minimal but inactive protein kinase domain, was not (data not shown). Residues 291–344 contain the PF1 domain, which is essential for OSR1 kinase activity and appears to be important for correctly folded protein. OSR1 1–323 is somewhat less stable than the longer fragment but retains the ability to be activated by WNK1.

OSR1 Activity Is Attenuated by Reduced Expression of WNK1. WNK1 and OSR1 are activated in response to osmotic stress in the form of nonpermeant osmolytes such as sorbitol (6, 18). To determine whether WNK1 is required for activation of OSR1, we examined the effect of reducing WNK1 expression in HeLa cells with siRNAs. siRNAs targeting human OSR1, human WNK1, or, as a control, rat WNK1, which does not target human WNK1, were used. Endogenous OSR1 was immunoprecipitated from unstimulated and sorbitol-stimulated cells, and its activity was measured by using an N-terminal fragment of NKCC2 as a substrate (Fig. 4*A*). Sorbitol-stimulated OSR1 activity as detected either by weak autophosphorylation or phosphorylation of the N terminus of NKCC2. Activation of WNK1 also was apparent from its behavior in gels; instead of a

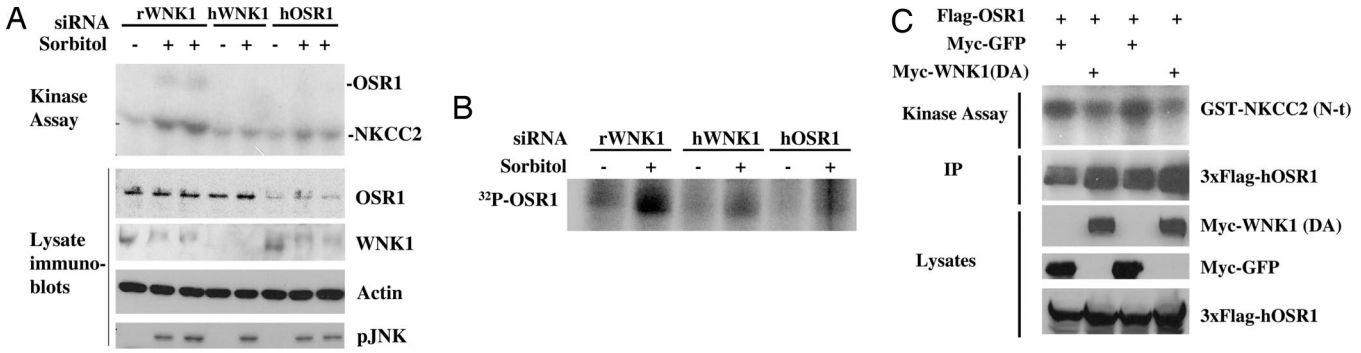


Fig. 4. WNK1 is required for OSR1 activation. (*A*) Endogenous OSR1 was immunoprecipitated from cells that previously had been transfected with siRNA directed against rat or human WNK1 or against human OSR1. Twenty-four hours after transfection with oligonucleotides, cells were placed in serum-free medium; after an additional 12 h, the cells were placed in medium with or without 0.5 M sorbitol for 30 min. Cells then were lysed, and the kinase activity of endogenous OSR1 (see *Materials and Methods*) was assayed by using a fragment from the N terminus of NKCC2, GST-SLC12-A1 1–175, as a substrate. Shown is one of three comparable experiments. (*B*) Methods were as in *A*, except that after 12 h of starvation, cells were placed in phosphate-free medium (Invitrogen) to which 0.125 mCi/ml ^{32}P was added. Endogeneous OSR1 was immunopurified and washed three times with 10 mM Hepes, pH 7.4/1 M NaCl/0.1% TX-100 and then once with 10 mM Hepes, pH 7.4. The autoradiogram is shown. (*C*) HEK 293 cells were transfected by using FuGENE with p3XFLAG-OSR1 and either pCMV5-mycGFP or pCMV5-mycWNK1(D368A) (kinase-dead) and treated as described in *A*, without sorbitol. The kinase activity of immunopurified 3× FLAG-OSR1 was assayed by using GST-SLC12A1(N terminus) as substrate. To detect 3× Flag-OSR1 in immunoprecipitates or lysates, anti-Flag antibody was used.

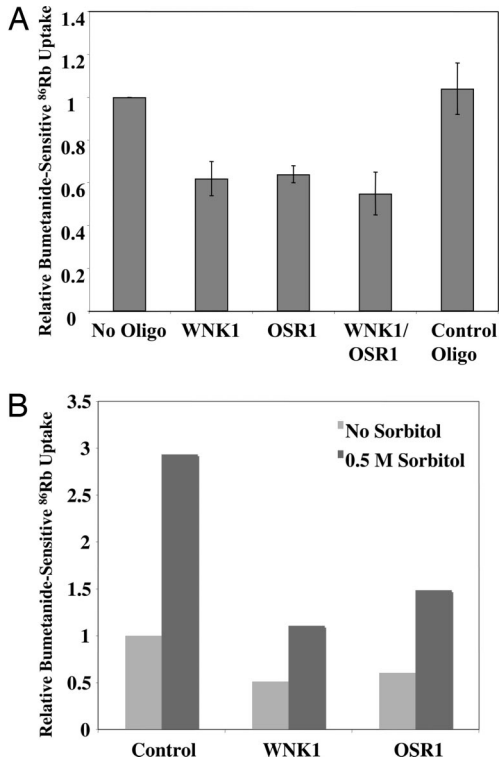


Fig. 5. Reduced expression of WNK1 or OSR1 inhibits endogenous NKCC1 activity. HeLa cells were treated with the indicated siRNAs for 48 h. ^{86}Rb influx assays, performed as described in *Materials and Methods*, were normalized to uptake under the control condition. (*A*) Average of six independent experiments. Error bars representing the standard error of the mean are shown. The control oligonucleotide is an siRNA that targets endogeneous RanBP2. (*B*) HeLa cells were treated with the indicated siRNAs for 48 h. Cells were treated for 30 min with medium minus or plus 0.5 M sorbitol. ^{86}Rb uptake was assayed. Shown is one of three similar experiments.

single band, upon activation, WNK1 separates into multiple, slower-migrating species. Knockdown of WNK1 expression, which was estimated by immunoblotting to reduce WNK1 expression by 80% or more, significantly reduced activation of OSR1 by sorbitol.

When dsRNA oligonucleotides targeting rat WNK1 were used as a control, no loss of OSR1 activation was observed in this human cell line nor was expression of WNK1 perturbed. Knockdown of OSR1 expression by using siRNA resulted in a 60–80% loss of OSR1 protein and a significant loss of OSR1 kinase activity in the immunoprecipitates as expected. The residual OSR1 was activated by sorbitol. As expected, activation of the stress-activated mitogen-activated protein kinase JNK by sorbitol was undisturbed by depletion of either WNK1 or OSR1 (Fig. 4*A Bottom*). We have shown that JNK activity is not linked to either OSR1 or WNK1 in mammalian cells (6, 14). Thus, WNK1 is at the top of a cascade leading to phosphorylation and activation of OSR1 that, in turn, phosphorylates its substrates.

To determine whether OSR1 is phosphorylated in cells, HeLa cells were transfected with dsRNAs as in the experiment above. After 48 h, cells were labeled with ^{32}P. OSR1 immunoprecipitated from cells treated with no oligonucleotide or with dsRNAs directed at rat WNK1 showed a 2-fold increase in ^{32}P content after stimulation with sorbitol (Fig. 4*B*). Depletion of WNK1 or OSR1 reduced the amount of ^{32}P recovered in immunoprecipitated OSR1.

To explore further the role of WNK1 in OSR1 activation, we considered the observation that exogenously expressed OSR1 displays constitutively high activity. By cotransfection of cells with OSR1 and a kinase-deficient mutant of WNK1 (DA), OSR1 activity was substantially reduced (Fig. 4*C*), roughly in proportion to the efficiency with which cells expressed the WNK1 mutant. Taken together, these results support the hypothesis that WNK1 is a regulator of OSR1 activity not only by osmotic stress but also in resting HeLa cells.

WNK1 and OSR1 Regulate NKCC Activity. To explore the impact of the WNK1-OSR1 cascade on NKCC activity, RNA interference (RNAi) was used to suppress expression of WNK1 or OSR1 as described above. NKCC activity in HeLa cells, most likely due to NKCC1, the widely expressed isoform, then was assayed by using uptake of isotopically labeled rubidium. Ouabain was included to block Na^+, K^+ ATPase activity that contributes to rubidium uptake. Under these conditions as expected (22), >90% of the rubidium uptake was inhibited by bumetanide, an NKCC1/2-selective inhibitor. The bumetanide-sensitive component was used as the measurement of NKCC activity. Reduced expression of OSR1 caused an ≈40% decrease in NKCC activity in a 5-min assay (Fig. 5*A*). Reduced expression of WNK1 caused a comparable decrease in NKCC activity, ranging from 40% to a nearly 50% decrease in six experiments. A similar effect was observed with a second oligonucleotide targeting a different sequence in WNK1 (data not shown). The combination of RNAi toward WNK1 and OSR1 caused a small further decrease that did not reach statistical significance.

Because NKCC activity is regulated by changes in osmolarity, we tested effects of knocking down WNK1 and OSR1 on NKCC activity in cells exposed to osmotic stress. Based on a time course in the presence of sorbitol, uptake was assayed after a 30-min exposure to sorbitol. Knocking down expression of either WNK1 or OSR1 caused substantial reduction in NKCC activity in the presence of sorbitol (Fig. 5*B*). These findings suggest that OSR1 and WNK1 also are involved in regulation of NKCC activity in response to osmotic stress.

Discussion

We found that the C terminus of OSR1 binds to a motif present in three copies in WNK1. Subsequent immunoprecipitation analysis of endogenous OSR1-associated soluble proteins indicates that WNK1 is the major protein bound to OSR1 in cells. In fact, these proteins are associated in a near-stoichiometric ratio. The apparent lack of effect of sorbitol on their binding indicates that this complex is present under both normal conditions and during osmotic stress in cells. In addition to the stable interaction between WNK1 and OSR1 through noncatalytic regions of each, the rapid activation of OSR1 by WNK1 is strongly indicative of the significance of their association in cells. Reduced OSR1 activation in cells in which WNK1 expression has been knocked down or in cells expressing a dominant negative WNK1 mutant demonstrates that regulation of OSR1 activity requires WNK1. These findings lead us to conclude that OSR1 is a bona fide WNK1 target and that these proteins function as a unit.

WNK1 activates not only OSR1 but also SPAK *in vitro*. OSR1 and SPAK are 88% identical in their kinase domains and 75% identical in their PF2 domains. Thus, it is not surprising that WNK1 also activates SPAK. However, our current data do not allow us to address the possibility that WNK1 and SPAK interact in cells. Antibodies that recognize nanogram quantities of recombinant SPAK did not detect any SPAK protein in HeLa cells expressing endogenous or siRNA-depleted amounts of OSR1. Thus, the extent to which SPAK also may form complexes with WNK proteins is unclear from these experiments. Nevertheless, our findings suggest that WNK1 is an upstream regulator of both OSR1 and SPAK *in vivo*.

Several years ago we isolated cDNAs encoding WNK1, the first of the four mammalian members of the WNK protein kinase subgroup identified (14). Pseudohypoaldosteronism type II (PHAII), a rare form of monogenic hypertension, is associated with mutations in either WNK1 or WNK4 (15). The disease also known

as Gordon's syndrome manifests itself as hyperkalemia and hyperchloremia, often many years before the onset of hypertension. The mutations in WNK4 were in its coding sequence; these mutations still have obscure effects on the protein. Mice in which the WNK1 gene has been disrupted do not complete embryonic development (16). The mutations found in WNK1, through positional cloning studies, were deletions in introns and were reported to increase its expression without changing its coding sequence. Thus, understanding its normal physiological functions should help to elucidate disease mechanisms. Mice retaining one wild-type WNK1 allele display reduced blood pressure, indicating that blood pressure is sensitive to WNK1 dosage. WNK1 is ubiquitously expressed (14); however, in kidney, mRNA encoding a splice form lacking a large portion of the protein kinase domain appears to predominate (23, 24). If protein expression in kidney mirrors transcript abundance, (which is not always so with WNK1; ref. 25), then in normal individuals WNK1 kinase-dependent functions may be performed largely outside kidney. Thus, expression of full-length WNK1 in kidney in PHAII may induce regulatory events, for example, activation of apical NKCC2, which may not be part of its normal physiological repertoire.

NKCC1, NKCC2, and sodium chloride cotransporter together with the four K^+ Cl^- cotransporters comprise the SLC12 family (26). NKCC2 (SLC12A1) is expressed predominantly in the thick ascending limb of the kidney, where it assists in the reabsorption of ions from its location in the apical membrane. NKCC1 and NKCC2 are inhibited by bumetanide, furosemide, and related diuretics. NKCC2 is the presumed therapeutic target because of its role in kidney. NKCC1 (SLC12A2) has a substantial function in regulatory events that impact ion balance and cell volume control. NKCC1 is expressed in secretory epithelia, including trachea, salivary glands, and inner ear. In these tissues, it is localized in the basolateral membrane and takes up ions to support chloride secretion by apical chloride channels into the lumen of these tissues (27, 28). NKCC1 is ubiquitously expressed in nonepithelial tissues, such as cardiac cells and fibroblasts, and influences volume regulation and ion balance through its sensitivity to changes in osmotic stress, intracellular chloride, and, directly or indirectly, cell volume. Animals lacking NKCC1 are deaf and display poor balance due to secretory defects in the inner ear (29). Loss of NKCC1 in mice results in low blood pressure and loss of vascular tone (30), as does loss of WNK1 (16). In recent studies dealing with forms of salt-sensitive hypertension, it was suggested that regulation of vascular tone is a second site that may contribute to development of disease (31). Thus, the effect of NKCC1 on blood pressure may be significantly due to its effects on venous resistance. Likewise, low blood pressure in WNK1+/− mice may result from its reduced action on NKCC1.

Given their apparent differences in expression in kidney, the relative paucity of information on WNK2 and WNK3, and the fact that several studies suggest that WNK1 and WNK4 may act antagonistically (32), a key issue is the specificity of WNK family members to regulate OSR1/SPAK and their cotransporter targets. Some ambiguity exists regarding functions of WNK4; it has been reported to inhibit NKCC1 function in a reconstituted system and to reduce membrane expression of sodium chloride cotransporter in *Xenopus* oocytes (32–34). On the other hand, recent overexpression studies suggest that both WNK4 and WNK3 stimulate NKCC1 (20, 35). Based on extensive mass spectrometric analysis of soluble proteins captured in OSR1 immunoprecipitates, no other WNKs were associated with OSR1 (S.C.K. and Y.Z., unpublished data). This result most likely reflects little expression of other WNK family members in these cells instead of, or perhaps in addition to, OSR1 binding specificity.

SPAK has been reported to interact with NKCC1 in cells (8), and both OSR1 and SPAK have been reported to interact through the PF2 domain with NKCC1 and NKCC2 through two-hybrid tests (9). Overexpression of kinase-dead SPAK and shark NKCC1 in HEK 293 cells reduced NKCC1 activation and its phosphorylation in response to low ionic strength (9), consistent with the view that phosphorylation of these sites activates cotransporter function. Phosphorylation sites identified in the N-terminal cytoplasmic domain of NKCC1, NKCC2, and sodium chloride cotransporter are phosphorylated by SPAK and OSR1 *in vitro* (36). Our findings suggest that WNK4 has little or no ability to activate OSR1 and SPAK. This specificity is surprising in view of the overexpression experiments showing that WNK4 can activate NKCC1 (20). It is important to note that significant control of many ion transporters occurs through regulated endocytosis and exocytosis. The impact of those processes on the function of SLC12 family members has not yet been fully defined and also may reveal differences and additional convergences in the functions of WNK family members.

Whereas other studies have shown the potential of WNK family members to stimulate SLC12 family transporters by using overexpression, our data with siRNA show that WNK1 is required to control endogenous NKCC activity in cultured cells. Based on current knowledge of expression patterns of the kinases and cotransporters, we speculate that WNK1 may modulate the activity of NKCC2 and, perhaps, other members of the SLC12 family, when it is overexpressed in PHAII. We suggest that one of its normal targets outside of the kidney is NKCC1. Additional work on the impact of phosphorylation by OSR1 and SPAK on function of these cotransporters and the actions of all WNK family members on them will be required to clarify the importance of these observations to the normal and pathophysiologic mechanisms of action of WNK protein kinases. Nevertheless, our findings clearly demonstrate that WNK1 is an OSR1 kinase sufficient to activate this and the related downstream protein kinase, is necessary for its basal activity and its activation in response to osmotic stress, and is required for NKCC1 function in cells.

Materials and Methods

Mutagenesis and Plasmids. The 1.6-kb RT-PCR product encoding full-length human OSR1 was cloned into pGEX-KG. This plasmid was used as the template for subsequent mutagenesis. Other OSR1 plasmids were as described in ref. 6. Mutants of OSR1 were created with the QuikChange kit (Stratagene). cDNAs encoding SPAK and NKCC2 (SLC12A1) were purchased from American Type Culture Collection. SPAK was subcloned into pHisParallel. A fragment of NKCC2 encoding residues 1–175 was subcloned into pGEX-KG. Constructs were transformed into the bacterial strain TG-1 and grown at 30°C to reduce the frequency of mutations. All constructs were sequenced to confirm correct amplification.

Proteins and Antibodies. GST fusion and His-6 tagged OSR1, WNK1, PAK1, and NKCC2 proteins were expressed in the strain BLR(DE3)pLys (Novagen). Cells were grown at 30°C to OD_{600} = 0.5–0.6 and induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside at 30°C for 4–6 h before harvest. SPAK was expressed in Rosetta (DE3) cells as above except at 37°C. Proteins were purified on glutathione-agarose or Ni^{2+}-NTA-agarose (6, 37). The monoclonal anti-Flag antibody was from Sigma and was used at 1:1,000 for immunoblotting and 1:500 for immunoprecipitation. The OSR1 antibody (U5438) was as described and used at 1:5,000 for immunoblotting and 1:500 for immunoprecipitation.

Cell Culture and Transfection. HEK 293 cells were grown in Dulbecco's modified Eagle medium containing 10% FBS, 1 mM L-glutamine, and 100 units/ml penicillin/streptomycin at 37°C in 10% CO_2. HEK 293 or HeLa cells were transfected with plasmid DNAs by using FuGENE 6 (Roche Applied Science) and with dsRNA oligonucleotides by using Oligofectamine (Invitrogen) immediately after subculturing. Oligonucleotides: rWNK1, sense, gcugaaaugcuaaagggucTT; antisense, gacccuuuagcauuucagcTT; hWNK1.1, sense, cagacagugcaguauucacTT; antisense, gugaauacugcacugucugTT; hWNK1.2, sense, ggaugauaucgaagagcugTT; antisense, cagcucuucgauaucauccTg; hOSR1.1, sense, guacug-

gaagggcuggaauTT; antisense, auuccagcccuuccaguacTT (all from Ambion); Ambion control oligonucleotide (catalog no. 4611G); and RanBP2 target, aatggacgtttccgattta (Dharmacon Research, Lafayette, CO).

Two-Hybrid Analysis. A Jurkat T cell cDNA library (from Mike White, University of Texas Southwestern Medical Center) was screened as described in ref. 38. Protein–protein interactions were tested by streaking cotransformants on medium lacking Leu, Trp, and His in addition to β-gal assays.

Immunoprecipitation and *in Vitro* Kinase Assays. For coimmunoprecipitation and kinase assays, lysate proteins (0.1–0.3 mg) were incubated with the indicated antibody for 1 h at 4°C, and then with 30 ml of a 50% slurry of protein A-Sepharose beads for 1 h. Beads were washed three times with detergent buffer (0.25 M Tris, pH 7.4/1 M NaCl/0.1% Triton X-100/0.1% sodium deoxycholate) and one time with 10 mM Hepes (pH 7.6). Beads or purified recombinant kinases were incubated with indicated substrates as described in the figure legends.

For immunoprecipitation of OSR1-associated proteins, cytosolic extracts were prepared from HeLa cells by the method described in Dignam *et al.* (39). One milliliter of S100 ($\approx$16 mg of protein in 20 mM Tris·HCl, pH 8.0/100 mM NaCl/1 mM EDTA/0.5% Nonidet P-40) was cleared by centrifugation at 20,000 $\times$ *g* for 20 min at 4°C. The protein was rotated with 20 μg of antibody, affinity purified by using the antigen OSR1 345–527 for 4 h at 4°C. The cleared solution was rotated with 40 μl of protein A-Sepharose beads (50% slurry) for 2 h at 4°C. The immunoprecipitates were washed five times with 20 mM Tris·HCl (pH 8.0)/100 mM NaCl/1 mM EDTA/0.5% Nonidet P-40. Proteins were eluted with 20 ml of 2$\times$ Laemmli sample buffer and subjected to SDS/PAGE (4–20% Tris/Glycine Gel; Invitrogen).

Nano-HPLC/Mass Spectrometry Analysis for Protein Identification. HPLC/MS/MS analysis was performed in an LCQ DECA XP ion-trap mass spectrometer (ThermoFinnegan, San Jose, CA) equipped with a nanoelectrospray ionization source. The source was coupled online to an Agilent 1100 series nano flow LC system (Agilent Technologies, Palo Alto, CA). Two microliters of the peptide solution in buffer A (2% acetonitrile/97.9% water/0.1% acetic acid, vol/vol/vol) was manually injected and separated in a capillary HPLC column (50 mm length $\times$ 75 μm internal diameter, 5-μm particle size, and 300 Å pore diameter) packed in-house with Luna C18 resin. Peptides were eluted from the column with a 5-min gradient of 5% to 80% buffer B (90% acetonitrile/9.9% water/0.1% acetic acid, vol/vol/vol) in buffer A. The eluted peptides were electrosprayed directly into the LCQ DECA XP ion-trap mass spectrometer. Normalized energy for collision-induced dissociation is 35%. Each MS/MS spectrum was obtained by averaging three microscans with maximum injection time of 110 ms for each microscan. The MS/MS spectra were acquired in a data-dependent mode, such that the masses and fragmentation patterns of the two strongest ions in each MS scan were determined. All spectra were acquired in centroid mode. Protein sequence database search and manual verification of the identified proteins were carried out as described in ref. 40.

NKCC1 Assay. Briefly, 100% confluent HeLa cells were washed twice with solution A (SA) (140 mM NaCl/5 mM KCl/1 mM $MgCl_2$/1 mM $CaCl_2$/10 mM Hepes (pH 7.4)/10 mM glucose/10 mM pyruvate/0.1% BSA) and then incubated for 30 min in the same solution at 37°C and 5% CO_2 (22). ^{86}Rb (5 mCi/ml; 1 Ci = 37 GBq) influx was measured for 5 min in assays with or without 10 mM bumetanide (Sigma) at 37°C and 5% CO_2. The reactions were stopped by three rapid washes with an ice-cold solution containing 0.1 M $MgCl_2$ and 10 mM Hepes, pH 7.4. The cells were lysed in 2% SDS, and total protein concentration was measured. To measure NKCC1/2 activity in response to hyperosmotic stress, HeLa cells were preincubated with 0.5 M sorbitol in medium for the indicated times. After aspirating the medium, the cells were quickly washed twice in SA plus 0.5 mM ouabain (SAO), followed by addition of SAO plus 1 mCi/ml ^{86}Rb with or without 10 mM bumetanide. Cells were washed, lysed, and assayed as previously described.

We thank Chou-Long Huang for valuable discussions; Xiaoshan Min for providing purified WNK4 (University of Texas Southwestern Medical Center); Shmuel Muallem, Joseph Albanesi, current and former members of the Cobb laboratory, Bing-e Xu, Malavika Raman, Lisa Lenertz, and Byung-Hoon Lee for suggestions about this work and manuscript; and Dionne Ware for administrative assistance. This work was supported by National Institutes of Health Grants DK34128 and GM53032 (to M.H.C.) and CA107943 (to Y.Z.) and Robert A. Welch Foundation Grants I1243 (to M.H.C.) and I1550 (to Y.Z.). This work is in partial fulfillment of the requirements for a Ph.D. (Y.Z.).

1. Dan, I., Watanabe, N. M. & Kusumi, A. (2001) *Trends Cell Biol.* **11,** 220–230.
2. Leberer, E., Dignard, D., Harcus, D., Thomas, D. Y. & Whiteway, M. (1992) *EMBO J.* **11,** 4815–4824.
3. Ramer, S. W. & Davis, R. W. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 452–456.
4. Bokoch, G. M. (2003) *Annu. Rev. Biochem.* **72,** 743–781.
5. Tamari, M., Daigo, Y. & Nakamura, Y. (1999) *J. Hum. Genet.* **44,** 116–120.
6. Chen, W., Yazicioglu, M. & Cobb, M. H. (2004) *J. Biol. Chem.* **279,** 11129–11136.
7. Baas, A. F., Boudeau, J., Sapkota, G. P., Smit, L., Medema, R., Morrice, N. A., Alessi, D. R. & Clevers, H. C. (2003) *EMBO J.* **22,** 3062–3072.
8. Dowd, B. F. & Forbush, B. (2003) *J. Biol. Chem.* **278,** 27347–27353.
9. Piechotta, K., Lu, J. & Delpire, E. (2002) *J. Biol. Chem.* **277,** 50812–50819.
10. Darman, R. B. & Forbush, B. (2002) *J. Biol. Chem.* **277,** 37542–37550.
11. Flemmer, A. W., Gimenez, I., Dowd, B. F., Darman, R. B. & Forbush, B. (2002) *J. Biol. Chem.* **277,** 37551–37558.
12. Piechotta, K., Garbarini, N. J., England, R. & Delpire, E. (2003) *J. Biol. Chem.* **278,** 52848–52856.
13. Gimenez, I. & Forbush, B. (2003) *J. Biol. Chem.* **278,** 26946–26951.
14. Xu, B., English, J. M., Wilsbacher, J. L., Stippec, S., Goldsmith, E. J. & Cobb, M. H. (2000) *J. Biol. Chem.* **275,** 16795–16801.
15. Wilson, F. H., Disse-Nicodeme, S., Choate, K. A., Ishikawa, K., Nelson-Williams, C., Desitter, I., Gunel, M., Milford, D. V., Lipkin, G. W., Achard, J. M., *et al.* (2001) *Science* **293,** 1107–1112.
16. Zambrowicz, B. P., Abuin, A., Ramirez-Solis, R., Richter, L. J., Piggott, J., BeltrandelRio, H., Buxton, E. C., Edwards, J., Finch, R. A., Friddle, C. J., *et al.* (2003) *Proc. Natl. Acad. Sci. USA* **100,** 14109–14114.
17. Darman, R. B., Flemmer, A. & Forbush, B. (2001) *J. Biol. Chem.* **276,** 34359–34362.
18. Lenertz, L., Lee, B. H., Min, X., Xu, B., Wedin, K., Earnest, S., Goldsmith, E. J. & Cobb, M. H. (2005) *J. Biol. Chem.* **280,** 26653–26658.
19. Lee, B. H., Min, X., Heise, C. J., Xu, B., Chen, S., Shu, H., Luby-Phelps, K., Goldsmith, E. J. & Cobb, M. H. (2004) *Mol. Cell* **15,** 741–751.
20. Gagnon, K. B., England, R. & Delpire, E. (2005) *Am. J. Physiol.* **289,** F1246–F1261.
21. Vitari, A. C., Deak, M., Morrice, N. A. & Alessi, D. R. (2005) *Biochem. J.* **391,** 17–24.
22. Whisenant, N., Khademazad, M. & Muallem, S. (1993) *J. Gen. Physiol.* **101,** 889–908.
23. Xu, Q., Modrek, B. & Lee, C. (2002) *Nucleic Acids Res.* **30,** 3754–3766.
24. O'Reilly, M., Marshall, E., Speirs, H. J. & Brown, R. W. (2003) *J. Am. Soc. Nephrol.* **14,** 2447–2456.
25. Xu, B., Lee, B. H., Min, X., Lenertz, L., Heise, C. J., Stippec, S. & Cobb, M. H. (2005) *Cell Res.* **15,** 6–10.
26. Hebert, S. C., Mount, D. B. & Gamba, G. (2004) *Pflugers Arch.* **447,** 580–593.
27. Haas, M. & Forbush, B., III (2000) *Annu. Rev. Physiol.* **62,** 515–534.
28. Delpire, E. & Mount, D. B. (2002) *Annu. Rev. Physiol.* **64,** 803–843.
29. Flagella, M., Clarke, L. L., Miller, M. L., Erway, L. C., Giannella, R. A., Andringa, A., Gawenis, L. R., Kramer, J., Duffy, J. J., Doetschman, T., *et al.* (1999) *J. Biol. Chem.* **274,** 26946–26955.
30. Meyer, J. W., Flagella, M., Sutliff, R. L., Lorenz, J. N., Nieman, M. L., Weber, C. S., Paul, R. J. & Shull, G. E. (2002) *Am. J. Physiol.* **283,** H1846–H1855.
31. Iwamoto, T., Kita, S., Zhang, J., Blaustein, M. P., Arai, Y., Yoshida, S., Wakimoto, K., Komuro, I. & Katsuragi, T. (2004) *Nat. Med.* **10,** 1193–1199.
32. Yang, C. L., Angell, J., Mitchell, R. & Ellison, D. H. (2003) *J. Clin. Invest.* **111,** 1039–1045.
33. Kahle, K. T., Gimenez, I., Hassan, H., Wilson, F. H., Wong, R. D., Forbush, B., Aronson, P. S. & Lifton, R. P. (2004) *Proc. Natl. Acad. Sci. USA* **101,** 2064–2069.
34. Kahle, K. T., Wilson, F. H., Leng, Q., Lalioti, M. D., O'Connell, A. D., Dong, K., Rapson, A. K., MacGregor, G. G., Giebisch, G., Hebert, S. C., *et al.* (2003) *Nat. Genet.* **35,** 372–376.
35. de los Heros, P., Kahle, K. T., Rinehart, J., Bobadilla, N. A., Vazquez, N., San Cristobal, P., Mount, D. B., Lifton, R. P., Hebert, S. C. & Gamba, G. (2006) *Proc. Natl. Acad. Sci. USA* **103,** 1976–1981.
36. Moriguchi, T., Urushiyama, S., Hisamoto, N., Iemura, S., Uchida, S., Natsume, T., Matsumoto, K. & Shibuya, H. (2005) *J. Biol. Chem.* **280,** 42685–42693.
37. Xu, B., Stippec, S., Lenertz, L., Lee, B. H., Zhang, W., Lee, Y. K. & Cobb, M. H. (2004) *J. Biol. Chem.* **279,** 7826–7831.
38. Bumeister, R., Rosse, C., Anselmo, A., Camonis, J. & White, M. A. (2004) *Curr. Biol.* **14,** 439–445.
39. Dignam, J. D., Lebovitz, R. M. & Roeder, R. G. (1983) *Nucleic Acids Res.* **11,** 1475–1489.
40. Kho, Y., Kim, S. C., Jiang, C., Barma, D., Kwon, S. W., Cheng, J., Jaunbergs, J., Weinbaum, C., Tamanoi, F., Falck, J., *et al.* (2004) *Proc. Natl. Acad. Sci. USA* **101,** 12479–12484.