Calcium-binding protein 39 facilitates molecular interaction between Ste20p proline alanine-rich kinase and oxidative stress response 1 monomers

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Submitted 27 August 2012; accepted in final form 25 September 2012

Ponce-Coria J, Gagnon KB, Delpire E. Calcium-binding protein 39 facilitates molecular interaction between Ste20p proline alanine-rich kinase and oxidative stress response 1 monomers. Am J Physiol Cell Physiol 303: C1198–C1205, 2012. First published October 3, 2012; doi:10.1152/ajpcell.00284.2012.—X-ray crystallography of the catalytic domain of oxidative stress response 1 (OSR1) has provided evidence for dimerization and domain swapping. However, the functional significance of dimer formation or domain swapping has yet to be addressed. In this study, we used nine glutamine residues to link the carboxyl end of one SPAK (related Ste20 kinase) monomer to the amino end of another SPAK monomer to assess the role of kinase monomers versus dimers in Na-K-2Cl cotransporter 1 (NKCC1) activation. Transport studies in Xenopus laevis oocytes show that forcing dimerization of two wild-type SPAK molecules results in cotransporter activation when calcium-binding protein 39 (Cab39) is coexpressed, indicating that the presence of Cab39 can bypass the upstream phosphorylation requirement of SPAK normally associated with kinase activation. We determined that monomers are the functional units of the kinase as concatamers consisting of an active and various inactive monomers were still functional. Furthermore, we found that two different nonfunctional SPAK mutants could be linked together in a concatamer and activated, presumably by domain swapping, indicating that dimerization and domain swapping are both important components of kinase activation. Finally, we demonstrate rescue of a nonfunctional SPAK mutant by domain swapping with wild-type OSR1, indicating that heterodimers of the two Ste20-related kinases are possible and therefore potentially relevant to the regulation of NKCC1 activity.

AMONG THE 30 PROTEINS that belong to the family of mammalian Ste20p-like kinases (3), a subset of them display an unusual characteristic, namely the exchange of the P + 1 loop domain of their activating segments between monomers. Domain swaps were evidenced upon resolution of the crystal structures of Chk2 (22), DAPK3, SLK, LOK/STK10 (26), and for oxidative stress response 1 (OSR1) [(16, 28); Fig. 1]. Three-dimensional domain swapping, which has been demonstrated in a relatively large number of proteins (for review, see Ref. 18), is a mechanism by which two or more proteins form a dimer or higher-molecular oligomer by exchanging identical structural elements. To date, the functional significance of dimer formation and/or domain swapping between Ste20p/Sps1 proline alanine-rich kinase (SPAK) and OSR1 kinases has not been addressed. In fact, as it was determined that the domain swapping initially observed in several proteins occurred upon conditions that were artificial or nonphysiological (e.g., truncated proteins) (18), it is important to provide independent support for the swapping of the P + 1 loop domain between/within these Ste20-related kinases.

OSR1 is a kinase that is found from protist (Capsaspora, NCBI accession no. EFW42229) to human (NCBI accession no. NP_005100). The OSR1 gene duplicated during late vertebrate evolution to give rise to another gene, STK39, which encodes SPAK, a protein found exclusively in terrestrial vertebrates (i.e., mammals, birds, and possibly reptiles) (5). SPAK and OSR1 bind and regulate ion transport mechanisms involved in renal salt reabsorption (15, 19, 27, 32), modulation of inhibitory synaptic neurotransmission (14), and cell volume control (2, 11).

In the present study, we engineered several kinase concatamers by linking two monomers head-to-tail with short polyglutamine bridges to assess the functional difference of monomers versus dimers on cotransporter activation. Our laboratory has previously engineered several mutations within SPAK monomers that rendered the kinase either constitutively active (T243E-S383D) or inactive (K104R, T243A, T247A) (10, 12). We have incorporated these mutants into concatamers to demonstrate that although monomers are the functional units of the kinase, dimer formation facilitates monomer activation. We also demonstrate that SPAK and OSR1, which are often coexpressed in cells (14, 15, 19), can form functional heterodimers.

MATERIALS AND METHODS
cDNA constructs. To create SPAK-SPAK concatamers, the 3′-end of wild-type or constitutively active SPAK was modified to remove the stop codon, include a sequence that encodes nine glutamine residues, and insert unique in-frame MfeI and XhoI restriction sites. Our original SPAK cDNA contained a unique in-frame EcoRI site located upstream of the translation initiation codon (ATG) allowing creation of concatamers by digesting the original SPAK (or mutant) cDNAs with EcoRI and XhoI and ligating the cDNA fragments into the MfeI and XhoI sites of the modified SPAK cDNAs. The identity of the concatamers was confirmed through DNA sequencing. However, as the internal sequencing primer was able to bind both monomers, visual inspection of the chromatograms was necessary to confirm the presence of distinct codons within each monomer.

cDNA transcription. cDNAs subcloned into the Xenopus oocyte expression vector pBF were linearized with MluI, purified using a PCR purification kit (Qiagen, Valencia, CA), and transcribed into cRNA using Ambion’s mMESSAGE mMACHINE SP6 transcription system (Ambion, Austin, TX). After purification using Qiagen RNeasy mini-columns, the cRNA was eluted in diethyl pyrocarbonate (DEPC)-treated water and its integrity was tested by agarose gel electrophoresis. The cRNA was quantitated by measuring the absorbance at 260 nm.

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Isolation and injection of Xenopus laevis oocytes. All experiments using female Xenopus laevis frogs were approved by the Vanderbilt Institutional Animal Care and Use Program. A detailed isolation method can be found in Ref. 6. Briefly, Xenopus frogs were anesthetized with tricaine (1.7 g/l) buffered with NaHCO₃ (3.4 g/l), ovarian protein concentrations. For the denaturing gel, equivalent amounts of Bradford protein assay (Bio-Rad, Hercules, CA) to determine total mer and concatamer cRNA were homogenized by passing them day 5.

cotransport expression and function, respectively, were assessed on OSR1 monomers (orange monomer on the structure. Hydrophobic residues that form the P₁₈₉ loop threonine residue (T₁₈₉) (CPK representation) from monomer 1 which was not resolved is depicted as a dashed line. The P₁₈₉ helix that gradually returns toward monomer 1, and L₂₃₆ from monomer 2 are represented as a ribbon. Hydrophobic residues that form the P₁₈₉ pocket (P₁₉₀, M₁₉₃ from monomer 1, and L₂₃₆ from monomer 2) are highlighted (licorice representation). AL, activation loop.

RESULTS

To confirm the in vivo presence of a SPAK dimer, we injected groups of oocytes either with water (negative control) or cRNA that encoded for monomers of wild-type SPAK, monomers of constitutively active SPAK, or concatamers of wild-type SPAK (positive control). Two days postinjection, we mechanically lysed the oocytes and separated proteins in the absence of SDS (native gel). In Fig. 2, no signal was observed in water-injected oocytes; two major bands and one minor band were observed running between 75 kDa and 250 kDa in lanes containing oocyte samples injected with wild-type or constitutively active SPAK cRNA; only the largest major band was observed in lanes containing oocytes injected with the con-

Fig. 1. Cartoon of oxidative stress response 1 (OSR1) crystal structure illustrating domain swapping. Graphical representation of portions of two OSR1 monomers (orange monomer on the left and cyan monomer on the right) illustrating domain swapping. The αAL helix, P + 1 loop, and αEF helix of monomer 1 are highlighted in dark orange. Sequence between G174 and V187 of monomer 1 which was not resolved is depicted as a dashed line. The P + 1 loop threonine residue (T189) (CPK representation) from monomer 1 is located deep inside monomer 2. Residues that follow T189 and form the αEF helix that gradually returns toward monomer 1 are represented as a ribbon structure. Hydrophobic residues that form the P + 1 pocket (P190, M193 from monomer 1, and L236 from monomer 2) are highlighted (licorice representation). AL, activation loop.

Fig. 2. Evidence for native Ste20p/Sps1 proline alanine-rich kinase (SPAK) dimers in Xenopus laevis oocytes. Shown is a Western blot analysis of a 9% acrylamide native or nonadenaturing gel loaded with lysates from water-injected oocytes, or oocytes injected with cRNA encoding monomers of wild-type SPAK, constitutively active SPAK, or concatamers of wild-type SPAK. SDS was absent from sample buffer, running gel, stacking gel, and running buffer. Monomers are illustrated above each lane as a blue shaded oval. Concatamer is illustrated as a blue shaded oval (monomer 1) and a orange shaded oval (monomer 2) linked by 9-glutamine residues (solid line). Membranes were probed with rabbit anti-SPAK polyclonal antibody.
between groups determined with one-way ANOVA (Fig. 3, bar 8 vs. bar 7) that Cab39 also induced activation of the kinase, as indicated by the significant increase in NKCC1 function. We then created a concatamer consisting of a constitutively active monomer SPAK(T243E-S383D) linked to a wild-type monomer. The presence of only one constitutively active monomer was enough to confer activation of the cotransporter (Fig. 4, bar 2). However, whether NKCC1 activation was actually due to the constitutively active monomer stimulating the wild-type monomer in the concatamer, or the constitutively active monomer alone was sufficient to activate NKCC1, was unclear. To address this question, we linked a catalytically inactive mutant monomer, SPAK(T243A), to a constitutively active SPAK(T243E-S383D) monomer with the rationale that if both monomers needed to be functional, this new concatamer would not be able to activate NKCC1. As shown in Fig. 4 (bar 4), the SPAK(T243E-S383D)–SPAK(T243A) concatamer was able to stimulate NKCC1 function. Note that, in both conditions, constitutive monomer linked to wild-type or T243A monomer, coexpression of Cab39 slightly enhances the kinase activation of the cotransporter, although this did not reach statistical significance (Fig. 4, bars 3 and 5). As Cab39 binds to a WEF motif on SPAK, which is located close to the S383 residue, and Cab39 activates a SPAK(T243E) mutant as a monomer (12), we wondered whether Cab39 would also enhance the activity of the SPAK(T243E-S383D) mutant injected as a monomer. As seen in Fig. 4 (bar 7), coinjection of Cab39 cRNA with the

Fig. 3. Functional expression of SPAK concatamer in Xenopus laevis oocytes. Ouabain-resistant unidirectional (⁸⁶Rb) K⁺ fluxes were measured in groups of oocytes injected with combinations of Na-K-2Cl cotransporter 1 (NKCC1) cRNA, SPAK monomers, SPAK concatamers, with-no-lysine kinase 4 (WNK4), and calcium-binding protein 39 (Cab39; see legend under bars). Cartoons of the concatamers above the bars indicate the identity of the two monomers involved: monomer 1 in blue and monomer 2 in orange, with residues T243 and S383 labeled in the semicircles and residue T247 labeled in the center spheres. A zig-zag line represents a 9-glutamine residue linker connecting two monomers. Fluxes are expressed as nmol K⁺/oocyte h⁻¹. Bars represent means ± SE (n = 20–25 oocytes). Lines indicate significance between groups determined with one-way ANOVA (P < 0.001). *Additional significant differences within concatamer groups with Cab39 (P < 0.001).

Fig. 4. Cab39 expression enhances constitutively active SPAK monomer. Ouabain-resistant unidirectional (⁸⁶Rb) K⁺ fluxes were measured in groups of Xenopus oocytes injected with NKCC1 cRNA alone or in combination with concatamers of a constitutively active SPAK monomer (shaded blue) linked to either a wild-type or a mutant SPAK(T243A) monomer (shaded orange) and Cab39. Residues T243 and S383 are labeled in the semicircles and residue T247 is labeled in the center spheres. A zig-zag line represents the 9-glutamine residue linker connecting the two monomers. Note that last two bars represent NKCC1 activity in the presence of constitutively active SPAK without and with Cab39. Fluxes are expressed in nmol K⁺/oocyte h⁻¹. Bars represent means ± SE (n = 20–25 oocytes). Lines indicate significance between concatamer groups determined with one-way ANOVA (P < 0.001). *Additional significant differences within concatamer groups with Cab39 (P < 0.001).
FUNCTION OF SPAK/OSR1 MONOMERS AND DIMERS

constitutively active SPAK(T243E-S383D) cRNA significantly enhanced kinase stimulation of NKCC1. Analysis of the crystal structure of OSR1 revealed that residue T189 (corresponding to SPAK T247) from one monomer is clearly embedded within the structure of monomer 2 (Fig. 1 and Ref. 16). Previous mutagenesis studies have shown that alanine substitution of the P + 1 loop domain residue T247 completely prevents both kinase autoprophosphorylation as well as trans-phosphorylation and activation of NKCC1 (10). Therefore, we hypothesized that we should be able to rescue an inactive monomer containing a T247A mutation through dimerization and exchange of a wild-type T247 residue. We first had to confirm that SPAK(T243E-T247A-S383D) as a monomer fails to activate NKCC1 function. In fact, expression of the SPAK(T243E-T247A-S383D) monomer had a dominant-negative effect on NKCC1 activity (Fig. 5, bar 2), similar to what was previously observed with SPAK(K104R), SPAK(T243A), or SPAK(247A) (9, 11). Interestingly, this dominant-negative effect could be ameliorated in the presence of Cab39 (Fig. 5, bar 3). More importantly, when we coinjected Xenopus oocytes with cRNA encoding for a concatamer consisting of a SPAK(T243E-T247A-S383D) monomer linked to a wild-type SPAK monomer, we only observed significant activation of NKCC1 in the presence of Cab39 (Fig. 5, bars 4 and 5), indicating that the adaptor protein facilitates the exchange of domains and the reconstitution of a functional SPAK(T243E-T247-T383D) kinase monomer. Note that this “rescue” of kinase activity was not observed when the same kinases were injected as monomers (Fig. 5, bars 6 and 7). If all that was required from monomer 2 to reconstitute an active monomer was the P + 1 loop exchange and a wild-type T247, we should also see activation with a catalytically inactive monomer 2. Therefore, we tested a concatamer consisting of a SPAK(T243E-T247A-S383D) monomer linked to a SPAK(T243A) monomer and also observed activation of the cotransporter (Fig. 5, bars 8 and 9).

We have already shown in Fig. 4 that an active monomer is not affected by being linked to an inactive monomer. We therefore asked the question of whether a monomer would even exchange its P + 1 loop domain with another monomer if it was already active. To answer this question, we tested a concatamer consisting of a constitutively active monomer attached to a monomer where T247 was mutated into an alanine. Upon Cab39 interaction, if the active monomer were to swap part of its activation segment and exchange its wild-type T247 residue with an alanine, we would observe inactivation of the kinase. If on the other hand monomer 1 was already in an active conformation that prevented domain exchange, we would see no inhibition of NKCC1 function upon Cab39 expression. Figure 5 (bars 10 and 11) shows stimulation of NKCC1 function by the SPAK(T243E-S383D) monomer linked to SPAK(T247A) monomer and greater stimulation upon Cab39 expression. This latter result is consistent with the significant increase in cotransporter activity observed when Cab39 was coexpressed with the constitutively active SPAK(T243E-S383D) monomer (see Fig. 4, bars 6 and 7).

Next, we addressed the question of whether SPAK and OSR1 can interact through heterodimerization and help each other’s activation. Because we cannot distinguish between the effect of SPAK and OSR1 on NKCC1 function, we decided to test whether the native T189 in OSR1 could rescue a constitutively active SPAK monomer containing a T247A mutation in the presence of Cab39. Consistent with the high degree of conservation in their catalytic domains, native OSR1 monomer was able to partially rescue the mutant SPAK, indicating that the two kinases are able to domain swap their P + 1 loop segments (Fig. 5, bars 12 and 13).

Finally, to verify sizes and protein expression of our concatamers, we isolated protein lysates from frog oocytes injected with SPAK monomer cRNA or with different SPAK concatamer cRNAs. Lysates were separated by SDS-PAGE and immunoblotted with a polyclonal anti-SPAK antibody. Fig. 6, left, and in agreement with the nature of the concatamers, we observed a major band at twice the molecular size of the SPAK monomer (60 kDa). Although expressed at much lower levels, another protein band four times the molecular size of the SPAK monomer was also observed (Fig. 6, left). Interestingly, this higher-molecular size band was only observed when two constitutively active SPAK monomers were linked to each other. All other SPAK concatamers demonstrated the presence of the dimer only (Fig. 6, center). Note that the SPAK-OSR1 concatamer (center, lane 2) is slightly smaller because of the missing PAPA box in the OSR1 monomer. This was confirmed by stripping the membrane and reprobing with a polyclonal anti-OSR1 antibody (Fig. 6, right, truncated to show only the first two lanes).

DISCUSSION

The regulation of ion transport mechanisms is complex and involves a series of signaling steps mediated by intracellular kinases, phosphatases, and other proteins. For instance, Na+ reabsorption in the renal distal convoluted tubule is mediated by the thiazide-sensitive NaCl cotransporter (13). Activation of this carrier by SPAK phosphorylation of specific threonine

![Fig. 5. Heterodimeric domain swapping rescues kinase function in Xenopus laevis oocytes.](image-url)
residues (19, 23, 27, 32) is further under the control of WNK4 and WNK1, two kinases that act upstream of SPAK (11, 20, 29). The WNK kinases also act independently of SPAK by affecting the trafficking of the cotransporter to the plasma membrane (30, 33). The exact details of this complicated regulation are still being investigated.

When the crystal structure of OSR1 was resolved at 2.2 Å (16, 28), it revealed an unusual mode of dimerization characterized by the swapping of P + 1 loop segment domains. Our study was designed to address three questions: 1) is dimerization an intrinsic property related to the function of the kinase?; 2) are monomers or dimers the functional units of SPAK?; and 3) do SPAK and OSR1 form functional heterodimers?

Several other Ste20p-like kinases require dimerization for activation segment autophosphorylation (26). Although in vitro reactions demonstrated that SPAK was able to autophosphorylate and trans-activate NKCC1, several molecular approaches suggested absence of monomer interaction and intermolecular phosphorylation (9). Forcing dimerization of two wild-type SPAK monomers with a nine-glutamine linker did not, by itself, result in kinase activation. However, when we coinjected cRNA encoding for Cab39, a scaffold protein that binds to SPAK (7), we observed a significant activation of the cotransporter. This, by itself, is an important result because it demonstrates that neither the presence of the nine-glutamine linker nor the “head-to-tail” configuration impairs kinase function. Furthermore, these data indicate that when SPAK molecules are able to form a dimer and acquire an active conformation, they can stimulate NKCC1. These data, however, cannot differentiate between intra- versus intermolecular auto-phosphorylation, i.e., between one monomer self-phosphorylating versus one monomer phosphorylating its counterpart in a dimer. However, we do have another piece of evidence showing that kinase phosphorylation does occur intramolecularly. When we coexpressed Cab39 with a wild-type SPAK monomer linked to a catalytically inactive monomer, we still observed kinase activation of the cotransporter. The only plausible explanation is that domain swapping of the P + 1 loop T247 residue between monomers maintained the integrity of monomer 1, which in these conditions was then able to bind to NKCC1 and self-activate. These data are consistent with in vitro phosphorylation studies we published in 2006 where we showed that an active kinase, while still able to auto-phosphorylate, was unable to phosphorylate a catalytically dead (K104R) mutant (9).

The next obvious question is why do we not see this same activation when we coexpress a wild-type SPAK “monomer” and Cab39 in oocytes? One possible explanation is that SPAK molecules do not “find” each other and do not dimerize in oocytes unless tethered to one another. Although unable to stabilize individual SPAK monomers, Cab39 might be able to stabilize the tethered dimer allowing kinase activation in the absence of upstream phosphorylation by WNK4. Alternatively, the nature of the dimers formed by two tethered monomers versus two individual monomers might be slightly different (e.g., free energy difference). Our Western blot analysis of a native or nondenaturing gel has identified in vivo dimer formation in lysates obtained from frog oocytes after we injected cRNA encoding for monomers of both wild-type and constitutively active SPAK. On the basis of our finding of native dimerization, it is therefore more likely that dimerization and domain swapping leading to kinase activation are actually two separate events. Thus, instead of Cab39 facilitating dimer formation, binding of Cab39 could actually stabilize a SPAK monomer and help it reach an active conformation, and/or facilitate domain swapping between the monomers thereby allowing them to reach an active conformation. Indeed, binding of Cab39 to SPAK is believed to occur at the WEF sequence (WEF-like motif) (7), which is located only 10 residues downstream of the PF1 serine residue (S383). In support of this hypothesis are the mutagenesis experiments that demonstrated that alteration of many residues surrounding S383 also led to the activation of the kinase (8), and an experiment in which NKCC1 function was readily activated by a SPAK(T243E) mutant in the presence of Cab39 (12). Therefore, in this scenario, Cab39 binding to the WEF motif affects the structure of the PF1 domain in a manner similar to phosphorylation of S383 and allows the kinase to attain an active conformation. This mechanism of action is also consistent with Cab39 activating SPAK kinase activity in vitro (7). Another possibility, besides Cab39 stabilization or promoting domain swapping within dimers, is that the binding of WNK4 (or some another protein) is necessary before the individual monomers have the
proper conformation for P + 1 loop domain swapping and kinase activation.

When kinase concatamers consisting of both active and inactive monomers were tested, they were able to activate the cotransporter. This observation implies that the functional unit of SPAK is the monomer. Our Western blot analysis of all the SPAK concatamers demonstrated a predominant band at the expected size of the dimer. However, the concatamer consisting of two constitutively active SPAK monomers also presented a ~240 kDa band, suggesting the formation of a higher molecular weight, such as a tetramer. Whether or not this tetrameric complex occurs in vivo or has any physiological significance is currently unknown and requires further investigation.

The possibility that the monomer is the functional kinase unit does not preclude a role for the dimer. In fact, our experiments using SPAK(T247) mutants indicate that dimerization facilitates monomer activation. A highly conserved residue of particular interest is the P + 1 loop threonine (T247) which is part of a key catalytic triad (D-K-T) (16). It is found in all 31 GeneBank OSR1 sequences (from protists and plants, to human), the 11 GeneBank SPAK sequences, all mammalian Ste20 kinases excluding STRAD, the 4 WNK kinases, and as other examples, in PKA and CAMKIIα (Fig. 7). Mutation of this threonine into an aspartic acid (8). These observations support our first argument that the functional unit of SPAK is the monomer and supports our second argument that dimerization and P + 1 loop domain swapping are two separate mechanistic events by which inactive monomers can autoactivate through intermolecular interactions (Fig. 8).

In protein kinase A, the hydroxyl group present on the side chain of the threonine corresponding to the SPAK T247 residue forms a hydrogen bond from the carboxylate of a conserved catalytic loop aspartic residue (21, 31). In fact, and this might be an important addition to our understanding of SPAK activation, Yang and coworkers (31) noticed that kinase structures only show the hydrogen bond between the threonine and the aspartic acid residues when a peptide substrate occupies the P + 1 hydrophobic pocket, whereas the distance between the two residues does not permit interaction when the substrate is absent. Although intuitive, this observation indicates that kinases can only reach their final active conformation upon substrate binding.

An indication that exchange of the P + 1 loop segments might not occur when the kinase is in an active conformation comes from our observation that a T247A mutation in monomer 2 did not inactivate a constitutively active monomer 1 in the presence of Cab39. In fact, we observed more NKCC1 activity than with the concatamer alone. One possible explanation for this result is that the presence of Cab39 resulted in a domain swap which instead of inactivating monomer 1, reconstituted a native SPAK monomer 2, which when phosphorylated is capable of stimulating NKCC1 activity better than a SPAK monomer mutated to mimic phosphorylation (i.e., T243E-S383D). In fact, phosphorylation is mimicked only when T243 is substituted to a glutamic acid, as we have shown that phosphorylation is not mimicked when mutating the threonine residue into an aspartic acid (8). These observations support our first argument that the functional unit of SPAK is the monomer and supports our second argument that dimerization and P + 1 loop domain swapping are two separate mechanistic events by which inactive monomers can autoactivate through intermolecular interactions (Fig. 8).

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**Fig. 7.** Conservation of key P + 1 loop threonine in S/T kinases. Alignment of activation loop, P + 1 loop, and beginning of αEF helix in protist-, plant-, human-OSR1, human SPAK, 28 mammalian Ste20 kinases, all WNK kinases, PKA, and CAMKIIα shows extremely high degree of conservation belaying the importance of this region. Note the conservation of the P + 1 loop threonine residue (boxed).

**Fig. 8.** Mechanisms of SPAK activation. I: semicircle cartoon represents a full-length SPAK kinase with the activation loop residue T243 and PF1 residue S383 labeled in their respective amino-terminal catalytic and carboxyl-terminal regulatory domains. The P + 1 loop residue (T247) is hidden owing to the inactive kinase conformation. II: two inactive monomers are capable of forming dimers. III: WNK4 binding to the RFXV motif in the PF2 domain results in phosphorylation of T243 and S383 residues. Phosphorylation induces a conformational change making domain swapping of the P + 1 loop threonine (T247) residue possible. IV: the appearance of WNK4 is “faded” to indicate that WNK4 may or may not still be bound to activated SPAK at the PF2 region. V: Cab39 binds to the PF1 region of the native SPAK dimer and induces a conformational change unmasking the P + 1 loop segment. VI: domain swapping of the P + 1 loop threonine residue and phosphorylation of activation loop threonine (T243) result in an activated kinase. Again, whether Cab39 remains bound to the dimer is unknown, so it is depicted in a faded form. Note that NKCC1 (not shown) may be bound at some or all of these intermediate steps.
Several studies have shown an overlap of SPAK and OSR1 expression in various tissues (e.g., choroid plexus, renal epithelial cells, neurons) (24, 25). We previously demonstrated through semiquantitative Western blot analysis that SPAK and OSR1 expression was equivalent in dorsal root ganglion sensory neurons (14). Each kinase contributed to −50% of cotransporter activation, and a direct correlation existed between the level of SPAK + OSR1 expression and the level of NKCC1 activity. This redundant behavior is, however, not characteristic of all cells, as disruption of SPAK through T243A knock-in (27) or knockout (15, 19, 32) has a primary renal distal convoluted tubule phenotype, whereas a kidney-driven OSR1 knockout (17) has a primary renal thick ascending limb phenotype. Thus, in kidney, the two kinases seem to have distinct functions based on the nephron segment. Interestingly, in the SPAK knockout, but not the SPAK(T243A) knock-in mice, NKCC2 is hyperphosphorylated (15, 19, 32), indicating that, in wild-type animals, SPAK somehow affects OSR1 function. Coinjection of SPAK and OSR1 cRNA in oocytes results in additive activation of the cotransporter. However, in this experiment, the participation of SPAK/OSR1 heterodimers in NKCC1 activation cannot be distinguished from the effect of SPAK-SPAK and/or OSR1-OSR1 homodimers. Therefore, we utilized our concatamer strategy to address the possibility that SPAK and OSR1 might form functional heterodimers. Our data showing that native T189 residues from OSR1 can rescue a constitutively active SPAK monomer harboring a T247A mutation indicate that the two kinases are able to form catalytic heterodimers. This represents the first demonstration that the two kinases can influence each other’s function through direct interaction.

In the present study, we have determined three new pieces of information relevant to SPAK function and NKCC1 activation: 1) the monomeric form of SPAK is the functional unit; 2) dimerization is not an artifact of crystallization and facilitates monomer activation; and 3) SPAK and OSR1 are able to form functional heterodimers.

**GRANTS**
This work was supported by National Institutes of Health National Institute of General Medical Sciences Grant GM074771 (to E. Delpire).

**DISCLOSURES**
No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**
J.P.-C., K.B.G., and E.D. conceived and designed the research; J.P.-C. and E.D. performed the experiments; J.P.-C., K.B.G., and E.D. analyzed the data; E.D. performed the experiments; J.P.-C., K.B.G., and E.D. edited and revised the manuscript; E.D. drafted the manuscript; J.P.-C., K.B.G., and E.D. approved the final version of the manuscript; K.B.G. and E.D. prepared the figures.

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