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Functional Insights into the Activation Mechanism of Ste20-related Kinases

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Key Words
Na-K-2Cl cotransport • SPAK • OSR1 • Ion fluxes • Cab39/Mo25 • Kinase activation • Chimera

Abstract
Mammalian Ste20-related kinases modulate salt transport and ion homeostasis through physical interaction and phosphorylation of cation-chloride cotransporters. Identification of a sea urchin (*Strongylocentrotus purpuratus*) ortholog of the mouse Oxidative Stress Response 1 (OSR1) kinase prompted the cloning and testing of the functional effect of a non-mammalian kinase on a mammalian cotransporter. Heterologous expression of sea urchin OSR1 (suOSR1) cRNA with mouse WNK4 cRNA and mouse NKCC1 cRNA in *Xenopus laevis* oocytes activated the cotransporter indicating evolutionary conservation of the WNK4-OSR1-NKCC signaling pathway. However, expression of a suOSR1 kinase mutated to confer constitutive activity did not result in stimulation of the cotransporter. Using a chimeric strategy, we determined that both the mutated catalytic and regulatory domains of the suOSR1 kinase were functional, suggesting that the tertiary structure of full-length mutated suOSR1 must somehow adopt an inactive conformation. In order to identify the regions or residues which lock the suOSR1 kinase in an inactive conformation, we created and tested several additional chimeras by replacing specific portions of the suOSR1 gene with complimentary mouse OSR1 sequences. Co-expression of these chimeras identified several regions in both the catalytic and regulatory domain of suOSR1 which possibly prevented the kinase from acquiring an active conformation. Interestingly, non-functional suOSR1 chimeras were able to activate mouse NKCC1 when a mouse scaffolding protein, Cab39, was co-expressed in frog oocytes. Sea urchin/mouse OSR1 chimeras and kinase stabilization with mouse Cab39 has provided some novel insights into the activation mechanism of the Ste20-related kinases.

Introduction

Human oxidative stress response kinase (hOSR1) was first identified by large-scale DNA sequencing of a genomic region on chromosome 3p22-p21.3 [1]. This serine/threonine kinase is part of a family of Ste20-related kinases which regulates multiple cellular processes

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including apoptosis, stress responses, development, growth, cell cycle, and cellular volume sensing [2-5]. Studies by the van Aalten and Goldsmith laboratories independently resolved the crystal structure of the catalytic domain of hOSR1 to 2.15 and 2.25 angstroms, respectively [6-7]. In each study, the authors determined that the inactive conformation of hOSR1 exists as a 'domain-swapped dimer', sharing the P+1 loop and EF alpha helix between dimer-related monomers. However, neither group was able to resolve the structure of the full-length kinase, citing the high flexibility of the connecting region between the catalytic domain and the conserved carboxy terminal (CCT) domain. Our laboratory recently demonstrated that double mutation of the residues targeted by mWNK4 [8], rendered both mouse SPAK and OSR1 kinases constitutively-active and therefore able to stimulate mouse NKCC1 (mNKCC1) without upstream activation by mWNK4 [9]. The publication of the purple sea urchin genome [10] provided a unique opportunity to further characterize the mechanism of Ste20 kinase activation. In this study, we identified, cloned, and expressed the sea urchin ortholog of OSR1 (suOSR1) with mNKCC1 in *Xenopus laevis* and performed radioactive tracer flux studies. We found that although wild-type suOSR1 (with mWNK4) is capable of substituting for mOSR1 in the activation of mNKCC1, mutation of a threonine residue into a glutamic acid (T197E) and a serine residue into an alanine (S338A) in the sea urchin kinase (to confer constitutive activity) was unable to stimulate cotransporter function.

In a recent study, the mouse calcium binding protein 39 (mCab39 - also named MO25) was demonstrated to stabilize the STRAD pseudokinase in a conformation which could activate downstream substrates [11]. When we co-expressed mCab39 with the suOSR1 (T197E, S338A) mutant and mNKCC1 in Xenopus laevis oocytes, both kinase activity was rescued and cotransporter function was restored. Interestingly, the restoration of cotransporter function was not enhanced by the addition of mWNK4 cRNA. Furthermore, co-expression of mCab39 with a single catalytic domain mutation (T243E) of mSPAK also resulted in full activation of mNKCC1.

In this study, we have confirmed the evolutionary conservation of the WNK4-OSR1-NKCC1 signaling pathway by substitution of wild-type mouse OSR1 with sea urchin OSR1. Furthermore, our discovery that the suOSR1 (T197E, S338A) mutant was not 'constitutively active' provided an opportunity to elucidate novel insights into the mechanism of Ste20-related kinase activity. Multiple sea urchin/mouse chimeric constructs have identified specific regions within the catalytic and regulatory domains important to the activation of OSR1. Finally, the stabilization/activation of both the suOSR1 (T197E, S338A) mutant and the mSPAK (T243E) mutant by mouse Cab39 suggests that the regulation of ion homeostasis by the cation-chloride cotransporters might involve a much larger complex of proteins than previously realized.

Materials and Methods

Animals

Live *Strongylocentrotus purpuratus* specimens were acquired from Friday Harbor Marine Labs (courtesy of Dr. Megan Dethier, University of Washington) and maintained in a 80 L glass aquarium with artificial sea water at 4°C, until euthanasia. Gonad, intestine, and esophagus tissue were isolated and frozen in liquid nitrogen. *Xenopus laevis* pigmented female frogs were housed as previously described [12]. All frog procedures and experiments were approved by the Vanderbilt University Institutional Animal Care and Use Committee.

RNA isolation and reverse-transcription

Tissues (1g) were homogenized in 7 ml of a solution containing 4 M guanidine thiocyanate, 22 mM Na-acetate, and 2 mM mercaptoethanol. The homogenates were layered onto 4 ml of a 5.7 M CsCl, 24 mM Na-acetate solution in polyallomer ultracentrifuge tubes (Beckman) and spun at 32,000 rpm at 20°C overnight. The supernatants were then discarded and the RNA pellets resuspended into di-ethyl-pyrocarbonate (DEPC)-treated water and precipitated with 3 M Na-acetate and ethanol. Total RNA from each tissue was reverse-transcribed at 37°C for 1 h. The 40 µl reactions contained 5 µg RNA, 8 µl 5 x buffer, 2 µl random hexamers (0.4 mg/ml), 4 µl 100 mM DTT, 2 µl 10 mM dNTPs, 2 µl RNAsin and 2 µl SuperScript II (Invitrogen, Carlsbad, CA).

Cloning of the sea urchin OSR1

Sense and antisense oligonucleotide primers were designed to PCR amplify from intestine cDNA two overlapping fragments of the open reading frame of sea urchin OSR1 kinase. The four primers contained restriction sites for directional subcloning into several vectors. After separation of the PCR reaction using 1% agarose gel electrophoresis, we gel extracted (Qiagen, Valencia, CA) 5' and 3' fragments (907 and 741 bp, respectively) and ligated them into the TA cloning vector pGEM-Teasy (Invitrogen). A sequenced-verified full-length OSR1 was created and inserted into the amphibian oocyte expression vector pBF using SpeI and XhoI.

Mutagenesis of sea urchin and mouse OSR1

We previously determined that mutation of a threonine residue (T185E) and a serine residue (S325A) in mouse OSR1 resulted in a constitutively-active kinase [9]. We therefore used

the full-length sea urchin OSR1 cDNA sub-cloned into pGEM as a template for QuikChange Mutagenesis (Stratagene, La Jolla, CA) with complementary sense and antisense oligonucleotides to mutate a homologous threonine residue (T197) into a glutamic acid residue and a homologous serine residue (S338) into an alanine residue to create a constitutively-active sea urchin OSR1. The mutations were verified by DNA sequencing and the full-length mutated OSR1 was moved back into pBF with SpeI-XhoI restriction enzymes.

Construction of mouse-sea urchin OSR1 Chimeras

We moved fragments of the mouse OSR1 and sea urchin OSR1into pBluescript as templates for QuikChange Mutagenesis (Strategene, La Jolla, CA, USA). Using complementary sense and antisense oligonucleotides, unique restriction sites already present in mouse OSR1 were introduced into sea urchin OSR1 (and vice versa) to allow swapping of specific sections of each molecule with the other (see Table 1). We also used site-directed mutagenesis to change mouse OSR1 specific residues into sea urchin OSR1 residues within the chimeric constructs. All mutations were verified by DNA sequencing and then the full-length mouse-sea urchin chimeras were inserted into pBF with SpeI-XhoI restriction enzymes.

Cloning of mouse calcium binding protein 39

Sense and anti-sense oligonucleotide primers were designed to PCR amplify (Expand Long Range, Roche) from mouse brain cDNA the entire open reading frame of the calcium binding protein 39. The primers contained EcoRI and XhoI restriction sites for directional subcloning into several vectors. After separation of the PCR reaction using 1% agarose gel electrophoresis, the 1026 bp fragment was gel extracted and ligated into pGEM-Teasy. A sequenced-verified full-length clone was created and inserted into the amphibian oocyte expression vector pBF using EcoRI and XhoI.

cRNA Transcription

Each cDNA in pBF, an amphibian expression vector, was grown in Luria-Bertani broth (LB) with 0.1 mg/ml ampicillin and the DNA was purified using a plasmid midiprep kit (Qiagen, Valencia, CA) and quantitated by measuring DNA absorbance at 260 nm. DNA (20 µg) was linearized overnight with MluI and purified using a QIAquick PCR purification kit (Qiagen). Linearized DNA (2 µg) was then transcribed into cRNA using Ambion's mMESSAGE mMACHINE SP6 transcription system (Ambion, Austin, TX). cRNA quality was verified by gel electrophoresis (1% agarose/0.693% formaldehyde), and quantitated by measurement of absorbance at 260 nm.

Isolation of Xenopus laevis oocytes

Stages V-VI *Xenopus laevis* oocytes were isolated from 10 different frogs as previously described [12] and maintained at 16°C in modified L15 medium (Leibovitz's L15 solution diluted with water to a final osmolarity of 195-200 mOsM and supplemented with 10 mM HEPES and 44 µg gentamicin sulphate). Oocytes were injected one day post isolation (day 2) with 50 nl water containing 15 ng mouse NKCC1 cRNA and on day 3 with 50 nl water containing 10 ng of mouse or sea urchin kinase (OSR1, Cab39, WNK4) cRNA. K^+ uptake measurements were performed using ^{86}Rb as a tracer on day 5 post-isolation.

Figure	Chimera	Mouse	Sea Urchin	Restriction sites
4	1	M^1 - K^{287}	S^{300} - S^{516}	AflII
	2	H^{288} - S^{527}	M^1 - K^{299}	AflII
5	1	M^1 - T^{173}	G^{186} - S^{516}	AgeI
	2	M^1 - L^{134}	D^{147} - S^{516}	XhoI
	3	M^1 - E^{87}	I^{100} - S^{516}	BglII
6	1	E^{135} – G^{158}	M^1 - L^{146}, T^{171} – S^{516}	XhoI, BamHI
	2	S^{99} – K^{287}	M^1 - G^{110}, S^{300} – S^{516}	BamHI, AflII
	3	G^{174} – K^{287}	M^1 - T^{185}, S^{300}– S^{516}	AgeI, AflII
	4	E^{135} – L^{247}	M^1 - L^{146}, D^{260}– S^{516}	XhoI, XhoI
	5	E^{135} –I^{218}	M^1 - L^{146}, E^{231}– S^{516}	XhoI, SacI
7	1	R^{327} –S^{527}	M^1 - G^{339}	AatII
	2	K^{287} - G^{326}	M^1 - K^{299}, R^{340} – S^{516}	AflII, AatII
	3	K^{287} - Q^{356}	M^1 - K^{299}, G^{369} – S^{516}	AflII, MfeI

Table 1. Segments of mouse and sea urchin used to construct OSR1 chimeras. Fragments are delineated by their beginning and ending amino acid and corresponding numbers for their respective species. The last column indicate the restriction sites used to join the different fragments.

K^+ uptakes in Xenopus laevis oocytes

Groups of 20 oocytes in a 35-mm dish were washed once with 3 ml isosmotic saline (96 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES buffered to pH 7.4, 200 mOsM) and preincubated for 15 min in 1 ml same isosmotic saline containing 1 mM ouabain. The solution was then aspirated and replaced with 1 ml isosmotic flux solution containing 5 µCi ^{86}Rb. Two - 5 µl aliquots of flux solution were sampled at the beginning of each ^{86}Rb uptake period and used as standards. After 1 h uptake, the radioactive solution was aspirated and the oocytes were washed 4 times with 3 ml ice-cold isosmotic solution. Single oocytes were transferred into glass vials, lysed for 1 h with 200 µl 0.25N NaOH, neutralized with 100 µl glacial acetic acid, and ^{86}Rb tracer activity was measured by β-scintillation counting. NKCC1 flux is expressed in nanomoles K^+/oocyte/h. Previous studies have demonstrated that ~90% of the ^{86}Rb uptake is bumetanide-sensitive and therefore mediated by the cotransporter [13].

Statistical Analysis

K^+ uptake was measured in individual oocyte by scintillation counting. Each experimental group was represented by twenty to twenty-five oocytes (n = 20-25). Differences between groups were tested using one-way ANOVA, followed by multiple comparisons using the Student-Newman-Keuls, Bonferroni, and Tukey's post-hoc tests. $P > 0.05$ was considered to be non-significant, whereas $P < 0.001$ was considered to be very significant.

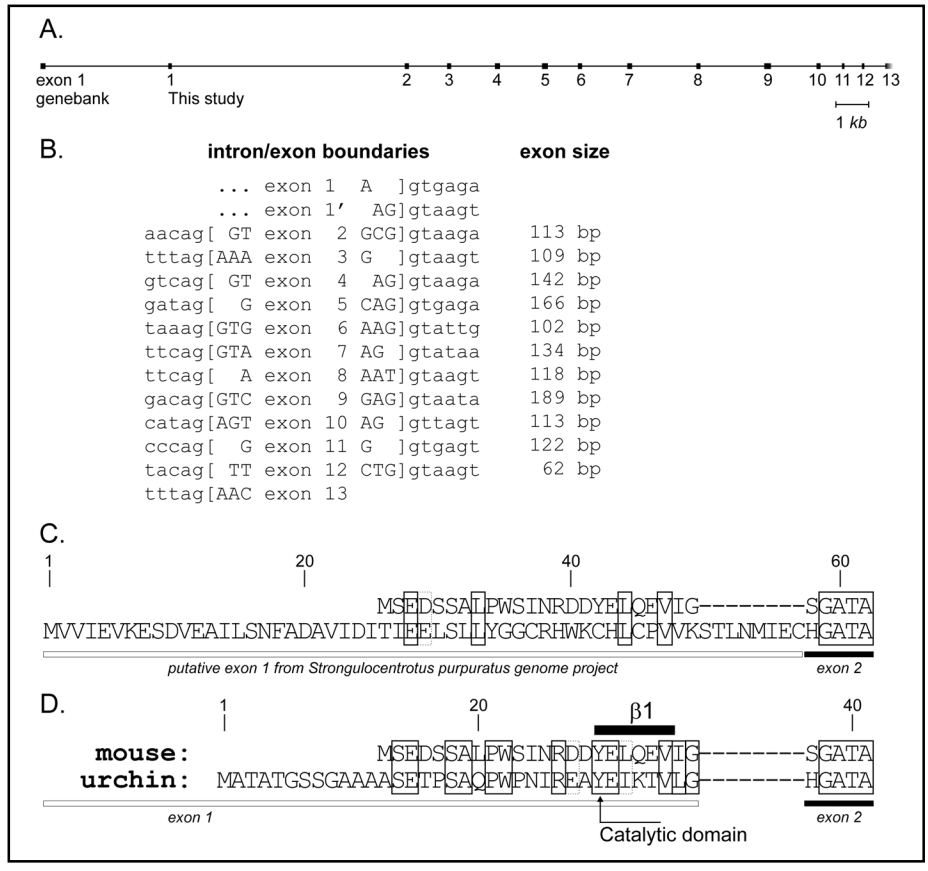

Fig. 1. Cloning of sea urchin OSR1. A) Genomic organization of sea urchin OSR1 with 13 exons spanning 25 kb. B) Intron/exon boundary sequences for the 13 exons of sea urchin OSR1. C) Open reading frame of Sea Urchin Consortium OSR1 illustrating absence of conserved sequence which constitutes the β1 sheet of the kinase catalytic domain of mOSR1. D) Open reading frame of sea urchin OSR1 with alternative exon 1 located downstream of the consortium exon 1 aligned with mOSR1 containing the five conserved β1 residues.

Results

In 2006, the Sea Urchin Genome Sequencing Consortium published the 814-megabase genome of the purple sea urchin *Strongylocentrotus purpuratus*. A transcript was annotated as mRNA similar to STE20/SPS1-related proline-alanine rich protein kinase. Analysis of the sequence revealed that this gene is an ortholog to the human oxidative stress response (hOSR1) kinase. Sea urchin OSR1 (suOSR1) consists of 13 exons spanning some 25 kb of genomic sequence (Fig. 1A &1B). Analysis of the open reading frame of the published suOSR1 revealed the absence of a conserved sequence that constitutes the β1 sheet of the catalytic domain of the kinase (Fig. 1C). To determine if the first exon was misidentified, we translated 40 kb of genomic sequence upstream of exon 2 in three frames and aligned the first 25 amino acids of mOSR1 against all three translated sequences. We identified a sequence which when translated, encodes for an alternative beginning of suOSR1 and contains 11 out of 25 conserved residues with mOSR1, including the critical residues of β1 sheet (Fig. 1D). Based on this alternative exon 1, we designed sense and antisense primers to PCR amplify the kinase from sea urchin tissues (see Material and Methods). After

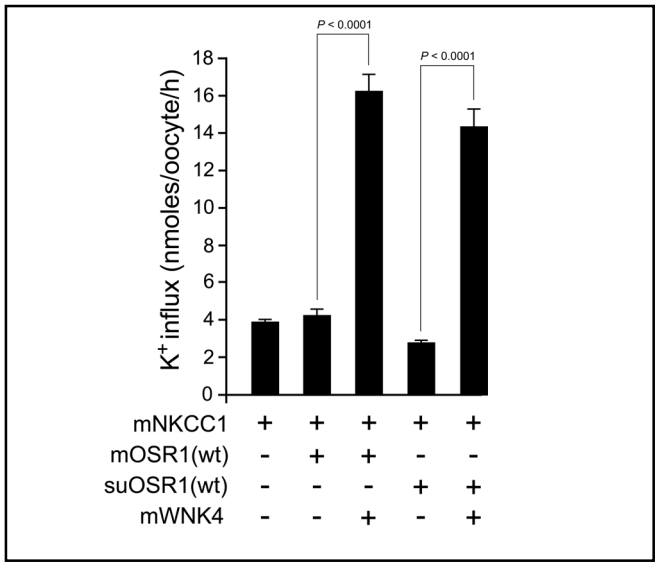

Fig. 2. K^+ influx in oocytes expressing wild-type mouse and sea urchin OSR1 kinases. Wild-type mouse and sea urchin OSR1 co-expressed with mouse NKCC1 in the absence and presence of mWNK4 were tested by ^{86}Rb (K^+) flux in *Xenopus laevis* oocytes. Water (50 nl) containing 15 ng of mouse cotransporter cRNA was injected on day 2, and 10 ng of each mouse and sea urchin kinase cRNA on day 3. K^+ flux was measured on day 5 in isosmotic (195 mosmol/l) conditions. Bars represent mean S.E.M. (n = 20 - 25 oocytes). *P* values were obtained from one way ANOVA.

sequencing and assembly of the kinase, we co-injected suOSR1 cRNA, mWNK4 cRNA, and mNKCC1 cRNA in *Xenopus laevis* oocytes and performed tracer fluxes with ^{86}Rb as a congener for K^+. Consistent with our previous studies with mSPAK [14], co-expression of wild-type sea urchin kinase activated the cotransporter in the presence of mWNK4 (Fig. 2). In contrast to the constitutively-active mOSR1 (T185E, S325A or S325D) which stimulates mNKCC1 regardless of the presence of mWNK4, conserved mutations in suOSR1 (T197E, S338A or S338D) did not confer constitutive kinase activity and failed to stimulate the cotransporter (Fig. 3).

In order to resolve this lack of suOSR1 (T197E, S338) activation of mNKCC1, we used a chimeric strategy and replaced specific portions of the inactive suOSR1 with the orthologous portion from constitutively-active mOSR1 to identify the regions of the mutated suOSR1 which inhibits kinase function. In Fig. 4, we have swapped the catalytic and regulatory domains of suOSR1 (T197E, S338A) with constitutively-active mOSR1 and co-expressed each of these chimeras in *Xenopus laevis* oocytes with mNKCC1. ^{86}Rb tracer influx experiments show that replacement of the suOSR1 (T197E, S338A) regulatory domain with the constitutively-active regulatory domain of mOSR1 stimulated mNKCC1 activity in *Xenopus laevis* oocytes to the same level as that of full-length constitutively-active mOSR1. Surprisingly, the constitutively-active catalytic domain of mOSR1 combined with the regulatory domain of suOSR1 (T197E, S338A), also fully stimulated mNKCC1 activity in frog oocytes.

To further delineate the catalytic region of mOSR1 that confers activity to the sea urchin kinase, we created another chimera where we reduced the portion of mOSR1 to only the first half of the catalytic domain and again demonstrated activation of mNKCC1 (Fig. 5A). We further reduced the portion of mOSR1 (Chimeras 2 and 3) and demonstrate in Fig. 5B that a minimum of 100 amino acids (N-lobe) of the mOSR1 catalytic domain are necessary to restore activity to the suOSR1 (T197E, S338A) mutant and stimulate mNKCC1 function. The residues necessary to maintain sea urchin kinase activity are outlined by a dashed red box in Fig. 5A and illustrated in the space filling and ribbon diagrams of the catalytic domain in Fig. 5C. For completeness, we also created a chimera which complements chimera 1 in Fig. 5A, and observed that replacing the second half of the sea urchin catalytic domain with its mouse counterpart also resulted in an active kinase (Fig. 6, chimera 1). Regions involved in preventing suOSR1 (T185E, S338A) activation of

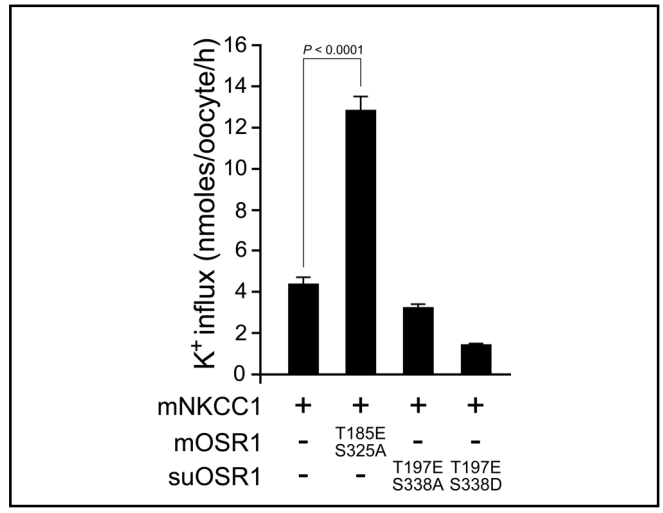

Fig. 3. K^+ influx in oocytes expressing sea urchin OSR1 kinase harboring mutations previously shown to render mouse OSR1 kinase constitutively-active. T-loop threonine and PF1 serine-mutated mouse and sea urchin OSR1 kinases were co-expressed with mouse NKCC1 and tested by ^{86}Rb (K^+) flux in *Xenopus laevis* oocytes. Water (50 nl) containing 15 ng of mouse cotransporter cRNA was injected on day 2, and 10 ng of mouse and sea urchin kinase cRNA on day 3. K^+ flux was measured on day 5 in isosmotic (195 mosmol/l) conditions. Bars represent mean S.E.M. (n = 20 - 25 oocytes). *P* values were obtained from one way ANOVA.

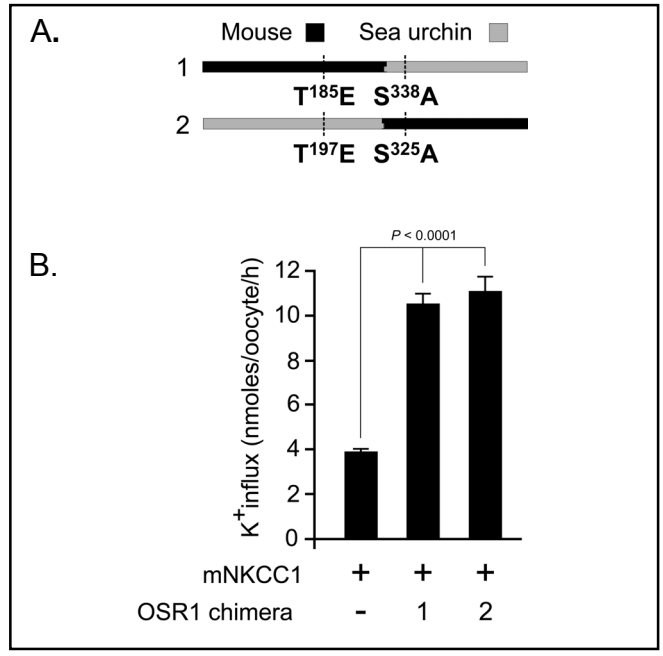

Fig. 4. K^+ influx in oocytes expressing mOSR1-suOSR1 chimeras demonstrate both mutated catalytic and regulatory domain of sea urchin OSR1 are functional. A) Cartoon of constitutively-active mOSR1 and suOSR1 chimeras. B) Mouse/sea urchin chimeras were tested by ^{86}Rb (K^+) flux in *Xenopus laevis* oocytes. Bars represent mean S.E.M. (n = 20 - 25 oocytes). *P* values were obtained from one way ANOVA.

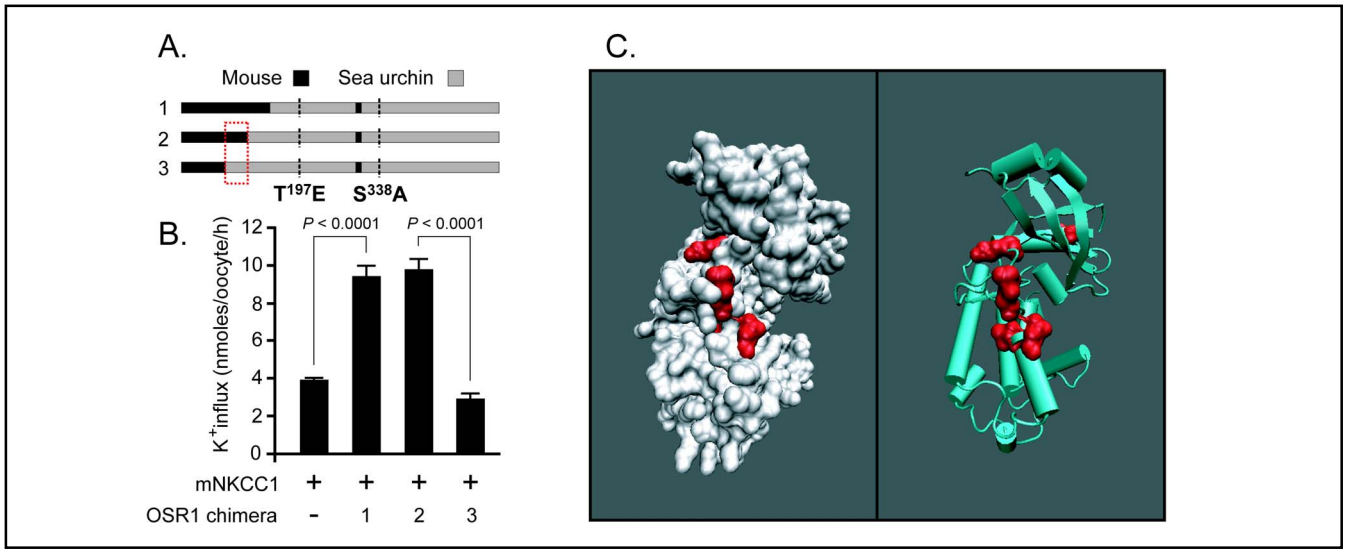

Fig. 5. K^+ influx in oocytes expressing additional N-terminal mOSR1-suOSR1 chimeras demonstrate minimal region of mouse catalytic domain that is required for a functional kinase. A) Cartoon of constitutively-active mOSR1-suOSR1 chimeras. B) Mouse/sea urchin chimeras were tested by ^{86}Rb (K^+) flux in *Xenopus laevis* oocytes. Bars represent mean S.E.M. (n = 20 - 25 oocytes). *P* values were obtained from one way ANOVA. C) Structure of the catalytic domain of OSR1 using Protein Data Bank (PDB) identification number: 3DAK. Left panel: Surface-exposed unique residues within the overlap of chimera #2 and #3 (see red dashed box in #1) colored in red. Right panel: Ribbon diagram illustrating beta sheets and alpha helices with unique residues within the overlap of chimera #2 and chimera #3 colored in red.

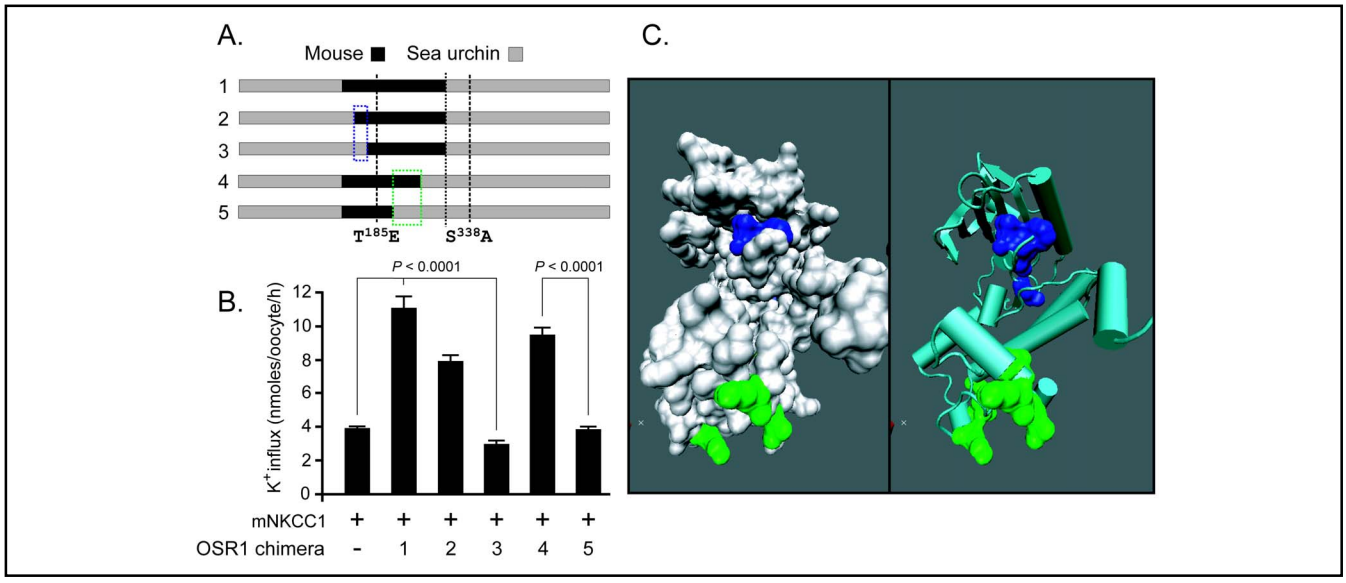

Fig. 6. K^+ influx in oocytes expressing mOSR1-suOSR1 chimeras identifies another region of the mouse catalytic domain conferring activity. A) Cartoon of constitutively-active mOSR1-suOSR1 chimeras. B) Mouse/sea urchin chimeras were tested by ^{86}Rb (K^+) flux in *Xenopus laevis* oocytes. Bars represent mean S.E.M. (n = 20 - 25 oocytes). *P* values were obtained from one way ANOVA. C) Structure of the catalytic domain of OSR1 using Protein Data Bank (PDB) identification number: 3DAK. Left panel: Surface-exposed unique residues within the overlap of chimera #2 and #3 (see blue dashed boxes in A) and chimeras #4 and #5 (see green dashed box in A). Right panel: Ribbon diagram illustrating beta sheets and alpha helices with unique residues within the overlap of chimeras #2 and #3 colored blue, and chimeras #4 and #5 colored green.

mouse NKCC1 were identified by reducing the size of the C-lobe segment from both the 5' and 3' ends (Fig. 6A and 6B). Dashed blue and green boxes in Fig. 6A outline regions necessary to maintain sea urchin kinase activity

and cotransporter stimulation. These same regions are illustrated in the space filling and ribbon diagrams of the catalytic domain in Fig. 6C. Site-directed mutagenesis to alter individual residues within these overlapping regions did not restore kinase activation or cotransporter stimulation (data not shown).

We next substituted two different portions of the regulatory domain of mOSR1 into the suOSR1 (T197E, S338A) mutant. The first involved replacing the latter two-thirds of the sea urchin kinase with the complementary region from mOSR1. The second involved inserting the mutated PF1 (S325A) region from mOSR1 into the full-length suOSR1 (T197E, S338A) mutant. Because neither of these chimeras was capable of stimulating mNKCC1 activity, we created a third chimera by extending the PF1 region with an additional 26 amino acids from mOSR1. This chimeric construct was able to stimulate mNKCC1 cotransport activity to the same level as constitutively-active mOSR1 (Fig. 7). Alignment of the overlapping region between the second and third chimeras identified several non-conserved residues which we altered by site-directed mutagenesis and tested for activation of mNKCC1. However, as with the C-lobe residues, none of the individual point mutations were able to rescue suOSR1 kinase activity and restore NKCC1 function (data not shown).

When we co-injected wild-type suOSR1 with mWNK4 in oocytes expressing mNKCC1, we observed cotransporter stimulation similar to that when mOSR1 was utilized (Fig. 2), but failed to observe cotransporter stimulation with the suOSR1 (T197E, S338A) mutant. Therefore, we decided to test the effect of mWNK4 on the mutant suOSR1 kinase. Unexpectedly, our functional studies produced inconsistent results ranging from dominant negative to full stimulation of the mouse cotransporter (Fig. 8, columns 3-5) between and within experiments. As the oocyte system is typically reproducible and reliable, this variability suggested the action of an additional factor. Given that the mouse calcium binding protein 39 (mCab39) was recently shown to regulate mouse SPAK/OSR1 [15], we cloned mCab39 and tested its effect on suOSR1 modulation of mNKCC1 function. We observed that co-injection of mCab39 rescued suOSR1 (T97E, S338A) kinase activity and fully stimulated the cotransporter. Interestingly, co-injection of mWNK4 with mCab39 and suOSR1 (T197E, S338A) did not increase cotransporter stimulation, when compared to mCab39 and suOSR1 (T197E, S338A) alone. In order to make sure that mCab39 was not 'recruiting' other upstream kinases, we co-expressed mCab39 alone, with

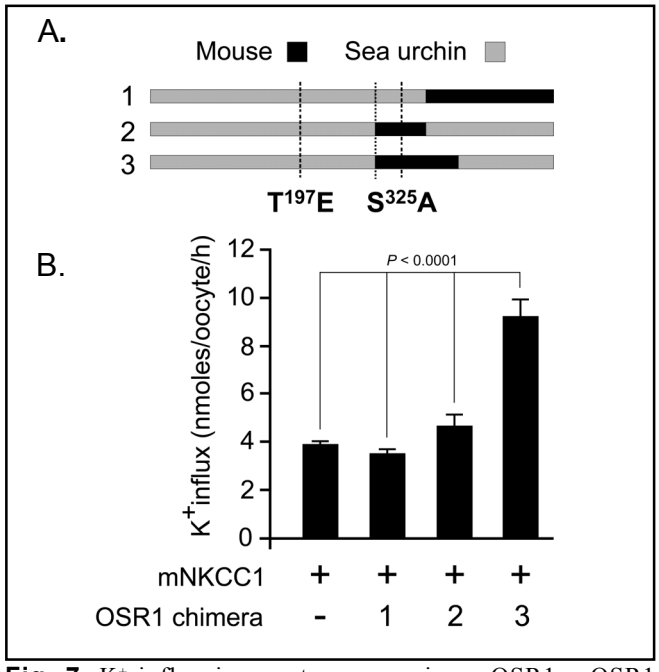

Fig. 7. K^+ influx in oocytes expressing mOSR1-suOSR1 chimeras demonstrate importance of the PF1 region of the regulatory domain of mouse OSR1. A) Cartoon of constitutively-active mOSR1-suOSR1 chimeras. B) Mouse/sea urchin chimeras were tested by ^{86}Rb (K^+) flux in *Xenopus laevis* oocytes. Bars represent mean S.E.M. (n = 20 - 25 oocytes). *P* values were obtained from one way ANOVA.

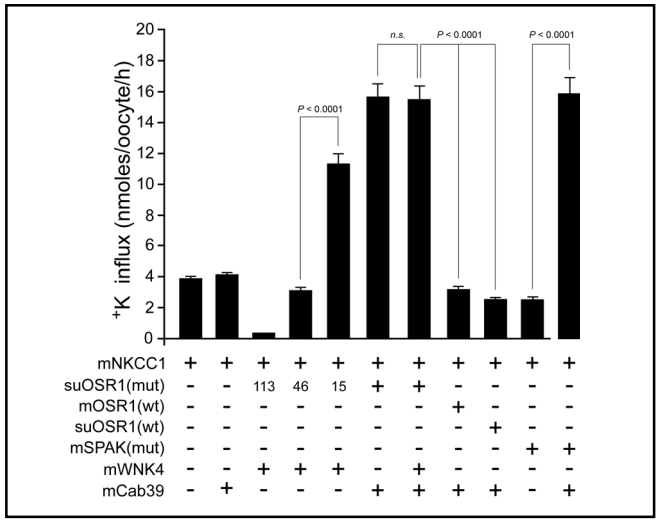

Fig. 8. K^+ influx in oocytes expressing mouse Cab-39 rescues T-loop threonine and PF1 serine-mutated suOSR1 activity. Wild-type and mutant forms of mOSR1, mSPAK, and suOSR1 in the presence and absence of mWNK4 and mCab-39 were tested by ^{86}Rb (K^+) flux in *Xenopus laevis* oocytes. Legend: suOSR1(mut) = T197E, S338A; mOSR1(wt) = wild-type; suOSR1(wt) = wild-type; mSPAK (mut) = T243E; mCab39 = mouse calcium binding protein 39. Numeric values for suOSR1 (mut) indicate the number of oocytes per bar. Bars represent mean S.E.M. (n = 20-25 oocytes). *P* values were obtained from one way ANOVA.

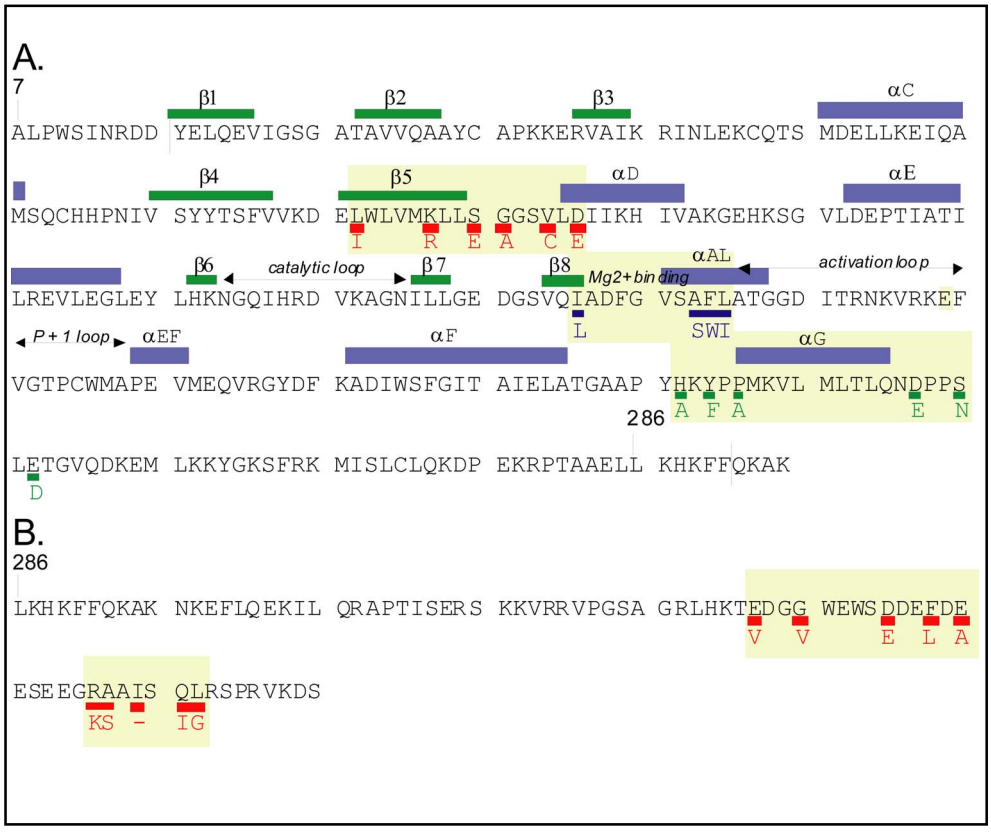

Fig. 9. Features of the OSR1 catalytic domain. A) The amino acid sequence the mouse OSR1 catalytic domain with α-helices (blue boxes) and β-strands (green boxes) is shown. The structural features are based on the human OSR1 crystal structure (PDB ID: 3DAK, [6]). The catalytic domain is defined by vertical lines at residues position Ala7 and Phe286. The position of the catalytic-, Mg^{2+}-binding-, activation-, and P+1 loops are also indicated. The red, blue, and green shaded boxes and residues highlight the non-conserved regions between suOSR1 and mOSR1 that are likely involved in locking the suOSR1 kinase in an inactive conformation. B) Amino acid sequence of the mouse OSR1 PF1 domain highlights the non-conserved regions between suOSR1 and mOSR1 highlighted by red shaded boxes and residues.

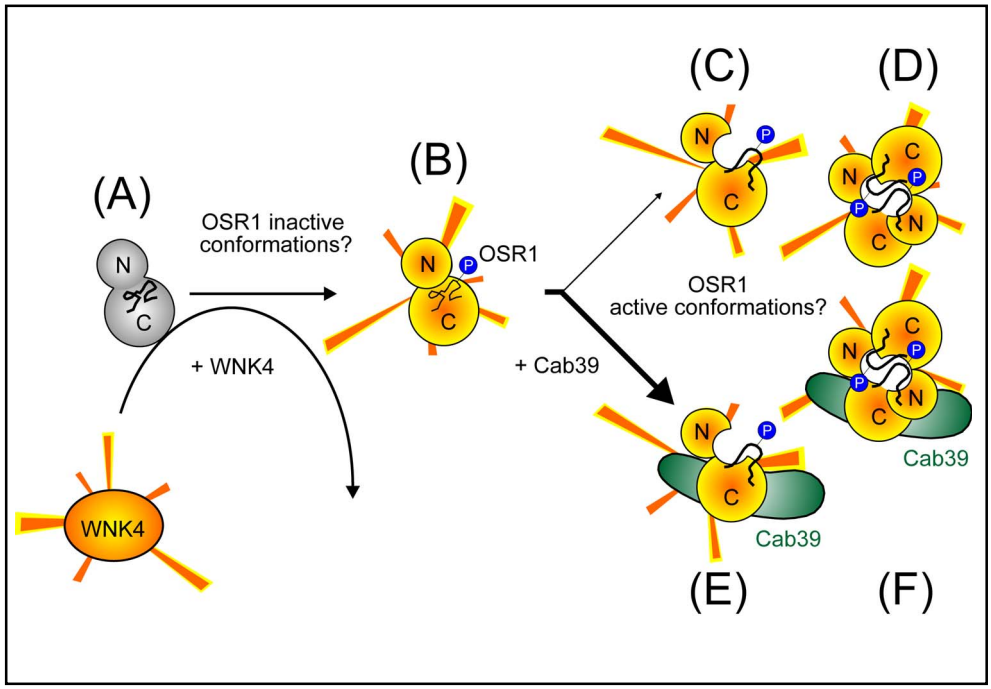

Fig. 10. Working model of OSR1 kinase activation and the role of Cab39. N and C represent the N- and C-lobes of the catalytic domain of OSR1, respectively. The regulatory domain is not shown. (A) Active WNK4 phosphorylates a T-loop threonine in the C-lobe and a serine in the PF1 region of the regulatory domain. (B) Phosphorylation or activation is indicated by the addition of a Phosphate (P) and a change in color from grey to orange. The phosphorylation event leads to the kinase acquiring an active conformation, either as a monomer (C) or a dimer (D). The presence of Cab39 stabilizes and facilitates the active conformation of individual (E) or dimer-swapped (F) kinases.

wild-type mOSR1, and with wild-type suOSR1 and observed no stimulation of the mouse cotransporter. Based on these new findings, we decided to revisit the mutagenesis requirement for activation of mOSR1 and mSPAK. We had previously shown that mutation of the T-loop threonine into glutamic acid alone was insufficient

to render the kinases constitutively-active [9]. Interestingly, now co-expression of mCab39 with the mSPAK (T243E) mutant rescued kinase activation and stimulated mNKCC1 cotransport (Fig. 8).

Discussion

The sequencing of the echinoderm *Strongylocentrotus purpuratus* (purple sea urchin) genome identified over 7000 orthologous genes to the chordate *Homo sapiens* (human) genome [10]. Analysis of an annotated mRNA transcript revealed a gene orthologous to human oxidative stress response kinase (hOSR1), a member of the Ste20-related proline-alanine rich protein kinase family. Regulation of cellular processes such as development, growth, apoptosis, and cell cycle control underlies the importance of this serine/threonine kinase family. Despite the highly dissimilar environment, the high degree of conservation prompted us to clone and functionally compare suOSR1 activity with mOSR1 activity in *Xenopus laevi*s oocytes. Interestingly, a conserved sequence at the beginning of the β1 sheet of the catalytic domain of the sea urchin kinase could not be found suggesting the possibility that the first exon of the published open reading frame of suOSR1 was misidentified. This was a likely possibility as we had previously found that the first exon of the sea urchin Na-K-2Cl cotransporter had been misidentified by the Sea Urchin Genome Sequencing Consortium (unpublished data). Our analysis of 40 kb of the suOSR1 genome upstream of exon 2 identified an alternative exon 1 which contained 11 out of 25 conserved amino acid residues with mOSR1, including the five conserved residues within the first β-sheet of the catalytic domain. Misidentification of the first exon in 2 proteins out of 2 indicates the unreliability of using automated routines to identify the 5' ends of mRNAs and proteins.

Despite the high degree of protein conservation between the sea urchin and mammalian kinase, it is still remarkable that OSR1 from sea urchin is 1) able to be activated by mouse WNK4; and 2) able to stimulate the mouse Na-K-2Cl cotransporter. This conservation across species is indicative of an ancient protein complex maintained by evolutionary pressure because of its critical physiological role in the regulation of apoptosis, development, response to physiological stress, and volume regulation. What was unexpected was finding that mutations in suOSR1 kinase, previously demonstrated to confer constitutive activity to mOSR1 [9], resulted in an inactive sea urchin kinase (Fig. 3). This interesting observation led us to hypothesize that either the mutated catalytic or mutated regulatory domain of suOSR1 was preventing kinase activation and stimulation of NKCC1. Therefore, we chose to create sea urchin/mouse OSR1 chimeras to 'map' regions of the suOSR1 (T197E, S338A) mutant that prevented mouse cotransporter stimulation. Our first set of chimeras yielded an interesting surprise: the mutated catalytic domain of suOSR1 (T197E) when combined with the mutated regulatory domain of mOSR1 (S325A) was constitutively-active and capable of stimulating mouse NKCC1. There was also nothing intrinsically wrong with the mutated regulatory domain of suOSR1 (S338A), as it also acquired an active conformation when spliced with the mutated catalytic domain of mOSR1 (T185E). Based on these results, we next hypothesized that inactivation of the suOSR1 (T197E; S338A) mutant was a result of a conformational interaction between portions of the catalytic and regulatory domains. Our chimera with only the N-terminal portion of the regulatory domain (PF1 region) of suOSR1 supports this hypothesis as it prevented kinase stimulation of the mouse cotransporter (see Fig. 7). One possible explanation for the inactivity of this mutant chimera is that the position and/or structure of the suOSR1 PF1 domain affected the spatial rearrangement of the catalytic domain, specifically the phosphorylated T-loop upon sea urchin kinase activation. Another possibility is that residues unique to the suOSR1 PF1 domain lock the interaction between the catalytic and PF1 domains and mutation of S338 into an alanine is not sufficient enough to allow the suOSR1 (T197E, S338A) mutant kinase to achieve an active conformation. On the catalytic side, we identified three putative regions, highlighted in Fig. 9A, which may be involved in the prevention of acquiring an active conformation. The first corresponds to the end of the N-lobe and beginning of the C-lobe (sheet β5 and helix αD); the second region surrounds the Mg^{2+} binding site (sheet β8 and helix αAL); and the third involves residues around helix αG. As indicated in the surface models (see Fig. 5C & 6C), these regions have exposed residues that could possibly interact with residues in the PF1 region. Interestingly, when we mutated additional residues in the PF1 domain of the suOSR1 (T197E, S338A) mutant, we still did not observe stimulation of the mouse cotransporter.

A truly unusual behavior was the variability observed when we co-injected oocytes with mNKCC1 cRNA, the suOSR1 (T197E, S338A) mutant cRNA, and mWNK4 cRNA. Although the oocyte expression system has inherent variability, never in the past had we seen such

striking differences in tracer flux per oocyte between and within experiments. In some cases, we observed: (1) a dominant negative effect of the mutant kinase on cotransporter activity; (2) no difference as a result of kinase co-expression; and (3) full activation of mNKCC1. Because it appeared to be an all or nothing situation, we deduced that some other factor was affecting the outcome of our experiment. Based on recent publications showing that Fray and Cab39 (or MO25) in *Drosophila* operate together or in the same pathway in promoting proper asymmetric divisions during development [16] and that mouse Cab39 regulates mouse SPAK/OSR1 function [15], we hypothesized that perhaps an endogenous Cab39 in the frog oocyte was the variable factor in our tracer flux experiments. Therefore, we cloned and co-injected combinations of mCab39, the suOSR1 (T197E, S338A) mutant, and mWNK4 in oocytes expressing mNKCC1 and found that now the cotransporter exhibited full activation. Even more surprising was the observation that mouse Cab39 rescued suOSR1 (T197E, S338A) kinase activity and fully stimulated the mouse cotransporter, irrespective of mWNK4, in a manner similar to constitutively-active mOSR1 in the absence of mouse Cab39. Based on these results we decided to revisit the need for modification of the PF1 residue in mSPAK (S383) or mOSR1 (S325) along with the T-loop threonine in order to render the kinase constitutively-active [9], and indeed, co-expression of mCab39 with mSPAK (T243E) now resulted in full stimulation of the cotransporter. It is important to note that the necessity of this double mutation was only observed *in vivo* using *Xenopus laevis* oocytes. We, along with others, have used *in vitro* kinase assays to demonstrate that the T-loop threonine was sufficient to render the mouse kinase active [17-19].

In the present study, we now have an explanation for the discrepancy between the *in vivo* and *in vitro* experiments. We propose that activation of mOSR1/mSPAK is a multi-step process involving: (1) phosphorylation of the PF1 serine by upstream kinases; (2) a conformational change in the interaction between the catalytic and regulatory domain; (3) phosphorylation of the T-loop threonine by upstream kinases; (4) dimerization of OSR1/SPAK; and possibly (5) transphosphorylation of secondary T-loop residues resulting in an activated OSR1/SPAK (Fig. 10 A-F). Whether or not the open conformation facilitates dimer formation is unknown. We also propose that mCab39, with or without primary T-loop threonine phosphorylation, stabilizes wild-type OSR1 in an open conformation. This hypothesis suggests an alternative mode of OSR1 activation which might involve another as-yet-unidentified kinase that only phosphorylates the T-loop threonine. Therefore, caution should be taken in interpreting data using phospho-specific antibodies directed against either residue, as their phosphorylation may be part of distinct signaling cascades. Previous studies have demonstrated differential phosphorylation of several threonine residues in the amino-terminus of mNKCC1 which resulted in cotransporter activation, suggesting distinct signaling cascades with different kinases [8, 20-21]. Recently, it was shown that in the presence of mCab39, mSPAK phosphorylated all these residues [15], indicating the intriguing possibility that instead of distinct signaling cascades targeting the cotransporter through different kinases, they may all converge onto a single kinase (i.e. SPAK/OSR1). As a result, which cotransporter threonine residues are phosphorylated might depend on whether the distinct signaling cascades converging on SPAK/OSR1 lead to phosphorylation of the T-loop threonine alone, or the T-loop threonine plus the PF1 domain serine residue. This might also explain why while transport stimulation is only seen when phosphorylation increases from its basal level, it does not always follow that an increase in phosphorylation results in transport activation [22]. Thus, phospho-specific antibodies may provide important information about cotransporter phosphorylation and the integrity of cell signaling pathways but they may not necessarily be reliable for reporting cotransporter stimulation. By affecting the PF1 domain and serving as a scaffold, mCab39 might facilitate the formation of a dimer, which allows the transphosphorylation of an additional T-loop threonine (T189) contained within the swapped domain of mOSR1 [6]. We previously demonstrated that phosphorylation of this additional threonine was essential for mouse kinase activation and mouse cotransporter stimulation [17, 21]. Note that although mCab39 might facilitate dimer formation, its inability to activate wild-type mouse or sea urchin OSR1 (see Fig. 8) indicates that OSR1 activation is not simply due to autophosphorylation of the first T-loop threonine. Furthermore, the serine residue (338) in the PF1 domain is located only 11 residues upstream of the putative binding motif (Trp-Glu-Trp) for mCab39 [11, 15, 23]. The proximity of this motif and the key serine in the PF1 domain suggests that similar to the LKB1-STRAD-Cab39 heterotrimeric complex [24], binding of mCab39 with the suOSR1 (T197E, S338A) mutant opens and stabilizes the sea urchin kinase in an active conformation.

In summary, the non-functional nature of the suOSR1 (T197E, S338A) mutant has provided some new

functional insights into the activation mechanism of Ste20-related kinases. Furthermore, the result of co-expressing mCab39 with the T-loop threonine mutant of mSPAK (T243E) has possibly unveiled an explanation for the previously observed differential phosphorylation of NKCC1. Finally, crystallographic efforts thus far have been unable to resolve the complete crystal structure of hOSR1, citing the flexibility of the PF1 region. Given the fact that several mouse/sea urchin chimeric constructs created for this study appear to be 'locked' in an inactive conformation, perhaps they may be used to resolve the crystal structure of inactive OSR1. Alternatively, as the presence of mCab39 apparently stabilizes suOSR1 in an open and active conformation, perhaps the OSR1 structure could be resolved by co-crystallizing the mCab39-suOSR1 complex. Finally, our study suggests the possibility of multiple avenues to SPAK/OSR1 activation through an intricate interaction with upstream proteins (e.g. Cab39, WNK4). Because of their direct interaction with multiple cotransporters (i.e. NKCC1/2 and NCC [25]), the Ste20 kinases and their regulators are likely to play important roles in modulating salt transport and ion homeostasis [26-29].

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