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Domain-Swapping Switch Point in Ste20 Protein Kinase SPAK

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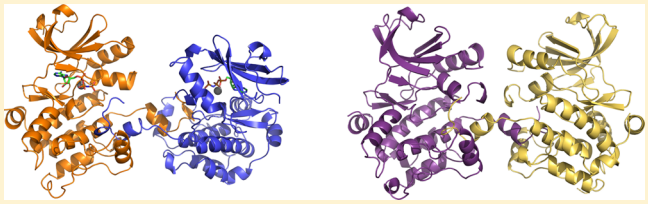
Supporting Information

ABSTRACT: The related protein kinases SPAK and OSR1 regulate ion homeostasis in part by phosphorylating cation cotransporter family members. The structure of the kinase domain of OSR1 was determined in the unphosphorylated inactive form and, like some other Ste20 kinases, exhibited a domain-swapped activation loop. To further probe the role of domain swapping in SPAK and OSR1, we have determined the crystal structures of SPAK 63−403 at 3.1 Å and SPAK 63−390 T243D at 2.5 Å resolution. These structures encompass the kinase domain and different portions of the C-terminal tail, the longer without and the shorter with an activating T243D point mutation. The structure of the T243D protein reveals significant conformational differences relative to unphosphorylated SPAK and OSR1 but also has some features of an inactive kinase. Both structures are domain-swapped dimers. Sequences involved in domain swapping were identified and mutated to create a SPAK monomeric mutant with kinase activity, indicating that monomeric forms are active. The monomeric mutant is activated by WNK1 but has reduced activity toward its substrate NKCC2, suggesting regulatory roles for domain swapping. The structure of partially active SPAK T243D is consistent with a multistage activation process in which phosphorylation induces a SPAK conformation that requires further remodeling to build the active structure.



In animals, ion concentrations and cell volume are controlled to maintain blood pressure, hearing, neurotransmission, and fluid secretion and to preserve cell viability for all other physiological functions.[1−3] The Ste20-related proline-alanine-rich kinase (SPAK; also called PASK and STK39) and its close relative, oxidative stress-responsive kinase 1 (OSR1), directly contribute to the regulation of ion balance through phosphorylation of the cytoplasmic tails of the SLC12 family of Na^+-Cl^-, Na^+-K^+-$2Cl^-$, and K^+-Cl^- ion cotransporters (NCC, NKCC1 and -2, and KCC1−4).[4−8] Reflecting the close connection between salt flux and blood pressure, mutations in the gene encoding SPAK have been linked to increased susceptibility to hypertension.[9] Additionally, kinase-dead SPAK knockin mice are hypotensive and show reduced levels of activation and expression of NKCC2 and NCC in the kidney, whereas OSR1 knockouts die during embryonic development with blood vessel defects.[7,10−12] These results suggest that therapies specifically targeting SPAK or OSR1 have the potential to treat hypertension, which is estimated to cost more than $130 billion annually worldwide.[5,13,14]

SPAK and OSR1 are members of the Ste20 germinal center kinase (GCK) VI subfamily of protein kinases.[6,15] They share a common structural organization with the kinase domain near the N-terminus. The C-terminus contains two conserved domains only found in this subfamily, initially termed PF1 and PF2 (PF stands for PASK and Fray, the *Drosophila* homologue).[16] The PF1 domain is required for kinase activity and may fold with the kinase domain. The PF2 domain, also termed the conserved C-terminal (CCT) domain, is involved in protein−protein interactions and binds a short consensus motif [RFX(V/I)] found in SCL12 family cotransporter substrates, as well as in upstream regulators, the WNK [with no lysine (K)] kinases.[17−21] Structural studies illuminated the basis for this interaction.[22]

Mutations in the upstream WNK1 and WNK4 kinases were shown by positional cloning to be responsible for single-gene forms of inherited hypertension, pseudohypoaldosteronism type II.[23] WNKs are activated by hypotonic, low-Cl^-, and hyperosmotic conditions at least in part because of a direct Cl^- sensing mechanism involving the WNK1 kinase domain.[24] SPAK and OSR1 are activated by all four WNK kinases by phosphorylation at two conserved sites, T243 and S383 (mouse SPAK numbering).[18,20,25] Threonine 243 is located within the activation loop of the kinase domain, and serine 383 is located in the PF1 domain and has been suggested to be part of an autoinhibitory element; however, its precise function remains unclear.[26]

Previous crystal structures of the unphosphorylated OSR1 kinase domain have been determined.[27,28] The structures revealed OSR1 activation loop domain-swapped dimers, where the active sites of both kinases in the dimer are formed with residues from both monomers.[29] The structures of the inactive form of the OSR1 kinase domain left open many questions: What is the activation mechanism of the kinase? How does the

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PF1 domain, known to be important for kinase activity and regulation, fold with the kinase domain? What is the functional significance of dimerization?

To gain insight into these questions, we have determined the structures of SPAK 63−403:ATP and the phosphomimetic mutant SPAK 63−390 T243D:AMP-PNP, which mimics the phosphorylation of the activation loop by WNKs. The structures encompass the kinase domain and part of the C-terminal regulatory region. Both structures reveal domain-swapped dimers with the N-terminal part of the PF1 domain folded onto the backside of the kinase domain. The new structural data offer insights into the function of domain swapping and the function of the PF1 domain in stabilizing the kinase structure.

EXPERIMENTAL PROCEDURES

Cloning. DNA encoding mouse SPAK was amplified from cDNA clone 6843981 (ATCC) and ligated into pHisParallel.[30] Mutants were created using QuikChange (Stratagene).[20]

Protein Expression and Purification. SPAK 63−390 T243D was expressed in Rosetta (DE3) *Escherichia coli* (Novagen). Cells were grown at 37 °C until the OD_{600} reached 0.4−0.6 and then induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside overnight at 16 °C. Cells were lysed in 1 mg/mL lysozyme in 50 mM HEPES (pH 8.0), 0.3 M NaCl, 10% glycerol, and protease inhibitors (1 mM phenylmethanesulfonyl fluoride, 10 mM benzamidine, 0.2 μg/mL leupeptin, and 2 μg/mL aprotinin), on ice for 30 min followed by sonication. Soluble proteins were applied to nickel-nitrilotriacetic acid-agarose (Ni^{2+}-NTA), and SPAK was eluted with 250 mM imidazole. Fractions with SPAK were identified by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS−PAGE) and dialyzed against 50 mM HEPES (pH 7.5) and 50 mM NaCl at 4 °C overnight. The tag was cleaved with TEV protease at a ratio of 50:1 (protein:protease) and removed by rechromatography on Ni^{2+}-NTA agarose. SPAK was further purified on Mono S using a 0.05 to 1 M NaCl gradient in 50 mM HEPES (pH 7.5), 1 mM dithiothreitol (DTT), and 1 mM EDTA and on Superdex75 equilibrated in 50 mM HEPES (pH 7.0), 50 mM NaCl, 1 mM DTT, and 1 mM EDTA. Similar procedures were effective for all SPAK 63−390 mutants evaluated.

SPAK 63−403 was purified similarly. The lysis buffer was as described above except that the level of glycerol was increased to 15% and the buffer included 2.5 mM β-mercaptoethanol. The dialysis buffer contained 20 mM Hepes (pH 7.5), 0.1 M NaCl, and 5% glycerol. Before gel filtration, the protein was further purified on Mono Q eluted with a gradient from 0.1 to 0.5 M NaCl in dialysis buffer also containing 1 mM DTT.

Crystallization. SPAK 63−390 T243D (~20 mg/mL) was preincubated with 5 mM AMP-PNP and 5 mM $MgCl_2$ for 30 min on ice. Crystallization (from Hampton Screening Kit I) was conducted at 20 °C in hanging drops by mixing protein with an equal volume of well solution, 0.2 M $Mg(OAc)_2$, 0.1 M Tris (pH 8.5), 16% PEG3350 (Hampton), 0.01 M Na-$(CH_3)_2AsO_2$ (Hampton), and 0.01 M $CaCl_2$. Blimp-shaped rods 0.4 mm long with a maximal cross section of 0.05 mm were flash-frozen in liquid nitrogen after being sequentially soaked in well solution with an additional 5, 10, and 15% glycerol as a cryoprotectant.

SPAK 63−403 at 15 mg/mL was preincubated with 5 mM ATP and 5 mM $MgCl_2$ for 30 min on ice. Crystallization was conducted at 20 °C in hanging drops by mixing protein with an equal volume of well solution, 100 mM MES (pH 5.5), 150 mM ammonium sulfate, and 18% PEG4000. Rectangular plates (0.2 mm × 0.1 mm) with a maximal cross section of 10−15 μm were flash-frozen in liquid nitrogen after being soaked in cryoprotectant, 20% ethylene glycol, 22% PEG4000, 100 mM MES (pH 5.5), 150 mM ammonium sulfate, 100 mM NaCl, 5 mM $MgCl_2$, 20 mM HEPES (pH 7.5), and 5% glycerol.

Data Collection and Structure Determination. Diffraction data were collected at beamline 19-ID at the Advanced Photon Source (Argonne National Laboratory, Argonne, IL). Data were indexed, integrated, and scaled in HKL2000.[31] The structure of SPAK 63−390 T243D was determined by molecular replacement in PHASER in the CCP4 suite.[32,33] The p21-activated kinase PAK6 [Protein Data Bank (PDB) entry 2c30] provided the best search model. The structure contains two molecules in the asymmetric unit (the solvent content was ~50%, and V_M = 2.51 Å^3/Da). Model building was conducted in Coot, and refinement was performed with REFMAC5 in the CCP4 suite.[34,35] As$(CH_3)_2$ was covalently linked to a subpopulation of SPAK T243D cysteine residues, as a result of the crystallization conditions. TLS (translation/libration/screw) refinement was performed in TLSMD.[36] The structure was checked on the MolProbity web server;[37] figures were made in PyMOL (The PyMOL Molecular Graphics System, Schrödinger, LLC).

The structure of SPAK 63−403 was determined using molecular replacement in PHASER in Phenix.[32,33,38] The refined structure of the SPAK 63−390 T243D chain A N-terminal domain excluding helix αC and the C-terminal domain excluding the activation loop were used as search models. The structure contains two SPAK molecules per asymmetric unit. Manual rigid body docking of the SPAK 63−390 T243D chain A activation loop and helix αC excluded from the molecular replacement search models was followed by iterative rounds of refinement (TLS, rigid body, and individual ADPs) in phenix.refine and model building in Coot.[34,38,39] Secondary structure restraints were used until the final rounds of refinement. Model validation was conducted in phenix.refine, which uses analyses derived, in part, from the MolProbity web server.[37−39] Dimer interface surface areas were calculated by directly inputting the structures into the PISA server (http://http://www.ebi.ac.uk/pdbe/pisa), and using the A to B ranges with symmetry operator *x*,*y*,*z*.[40]

Kinase Assays. SPAK proteins (0.5−1 μg) were added to a 30 μL kinase reaction mixture containing 20 mM Hepes (pH 7.6), 50 μM ATP (10 μCi of [γ-^{32}P]ATP), 10 mM $MgCl_2$, 10 mM β-glycerophosphate, 1 mM DTT, and 1 mM benzamidine, with 5 μg of either GST-PAK1 1−230 or GST-NKCC2 1−175 as a substrate for a 30 min reaction at 30 °C. For the coupled assays using WNK1 preactivated SPAK, SPAK was first phosphorylated by the WNK1 kinase domain (residues 132−483) by incubation of 1 μg of SPAK with 2.5 μg of WNK1 in a 20 μL kinase reaction mixture containing 1 mM DTT, 20 mM HEPES (pH 7.6), 50 μM ATP, 75 mM NaCl, 5% (w/v) glycerol, and 15 mM $MgCl_2$ for 30 min at 25 °C. Then 5 μL of a substrate mixture containing 2 μg of GST-NKCC2 (residues 1−175), 250 μM ATP (10 μCi of [γ-^{32}P]ATP), and 75 mM $MgCl_2$ was added to the reaction mixture and the mixture incubated for 5 min at 25 °C. Samples were analyzed by SDS−PAGE. Gels were stained with Coomassie blue, dried, and exposed to film.

***In Vitro* Binding Assay.** Equimolar amounts of GST, GST-SPAK 63−390, and GST-OSR 1−557 purified from bacteria

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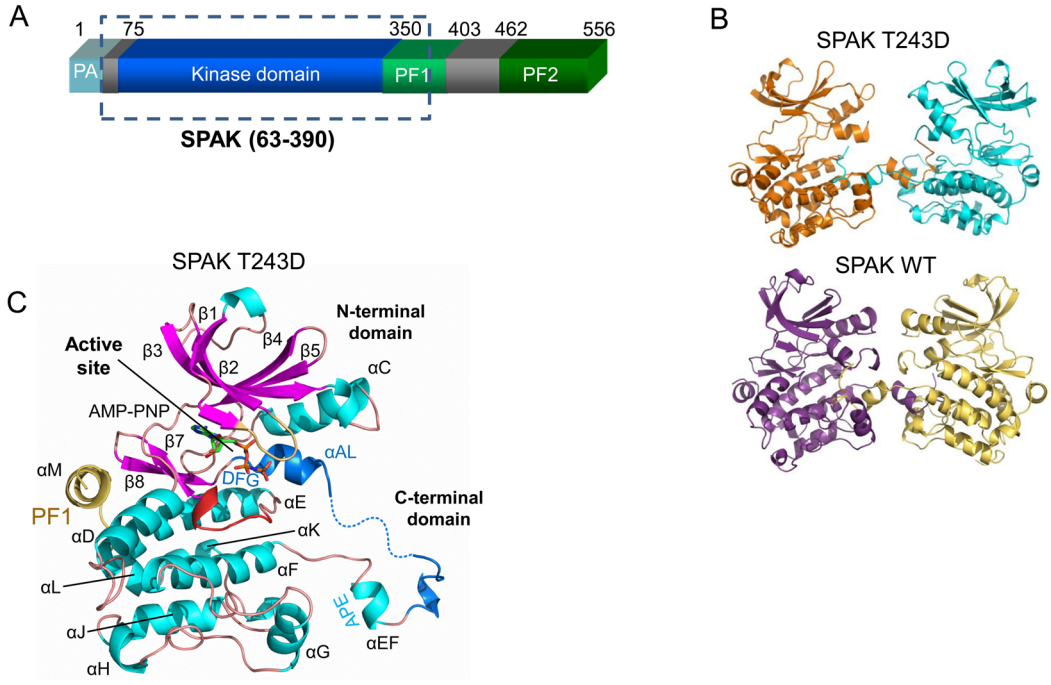

Figure 1. Domain organization and structure of SPAK WT and T243D. (A) Domain organization of mouse SPAK. The kinase domain (residues 75−350) is colored blue, the PF1 domain (residues 350−403) green, and the PF2 domain (residues 462−556) darker green. The crystallized SPAK T243D fragment is boxed. (B) Activation loop domain-swapped dimers of SPAK T243D and SPAK WT. The two SPAK T243D dimer subunits are colored orange and blue, and the two SPAK WT subunits are colored purple and yellow. (C) Ribbon diagram of a SPAK T243D subunit (or monomer) as observed in the domain-swapped dimer: α-helices, cyan; β-strands, magenta; glycine-rich loop, brown; catalytic loop, red; activation loop, blue; PF1 domain, yellow. AMP-PNP is shown as sticks.

were incubated with His_6-SPAK 63−390 also purified from bacteria in buffer containing 50 mM Tris (pH 7.5), 100 mM NaCl, and 5 mM $MgCl_2$ with or without 1 mM ATP or ADP. Samples were washed extensively, separated by SDS−PAGE, and analyzed by immunoblotting using anti-His_6 antibody.

Gel Filtration Chromatography. Gel filtration chromatography of 250 μM SPAK mutants was conducted on a Superdex 75 10/300 GL column in 50 mM HEPES (pH 7.0), 50 mM NaCl, 1 mM DTT, and 1 mM EDTA.

RESULTS

SPAK Crystallization and Structure Determination. Extensive bacterial expression and crystallization screening of the SPAK kinase domain together with both N- and C-terminal extensions led to crystals of SPAK 63−403 and SPAK 63−390 T243D. The constructs crystallized encompass the kinase domain (residues 75−350), an N-terminal extension (residues 63−74), and residues 351−390 or 351−403 from the PF1 homology box (residues 351−403) (Figure 1A).[16,41] SPAK 63−403 will be termed SPAK WT throughout, and SPAK 63−390 T243D will be termed SPAK T243D. The previously reported structure of wild-type OSR1, residues 1−295, used for comparison in this study (PDB entry 3DAK) will be termed OSR1 WT.[28]

SPAK WT and SPAK T243D crystallized under different conditions and in different space groups. SPAK WT crystallized in the presence of ATP, and its structure was determined at 3.1 Å resolution with a final R_{work} of 23.7% and an R_{free} of 26.9%. SPAK T243D crystallized in the presence of AMP-PNP at 2.5 Å resolution with a final R_{work} of 20.6% and an R_{free} of 24.6%. The refined models display reasonable geometry and Ramachandran statistics (Table 1). Two SPAK molecules are in each asymmetric unit in both structures (Figure 1B). The conformations of the two monomers in SPAK WT and SPAK T243D are similar, with average root-mean-square deviations (rmsds) in Cα positions of 0.31 and 0.45 Å, respectively. Complete information about crystallization, data

Table 1. Crystal Data and Refinement Statistics

	SPAK 63−403	SPAK 63−390 T243D
	Crystal Data	
space group	$P2_1$	$P2_12_12_1$
cell dimensions (Å)	a = 72.,4 b = 56.1, c = 100.0	a = 66.4, b = 101.7, c = 104.1
α, β, γ (deg)	90, 108.3, 90	90, 90, 90
resolution (Å)	42.9−3.10 (3.20−3.10)	50−2.45 (2.54−2.45)
no. of observed reflections	38612	308327
no. of unique reflections	13658	27322
completeness	85.6 (44.3)	93 (80.7)
R_{merge} (outer shell)	0.069 (0.49)	0.043 (0.41)
$I/\sigma\langle I\rangle$ (outer shell)	15.1 (2.1)	31.4 (1.98)
	Refinement	
R_{work} (%)	23.6	20.6
R_{free} (%)	26.9	24.6
rmsd for bond lengths (Å)	0.009	0.006
rmsd for bond angles (deg)	1.18	1.03
Ramachandran favored (%)	92.8	96.6
Ramachandran outliers (%)	0.2	0.0
average B factor (Å^2)	76.9	70.6

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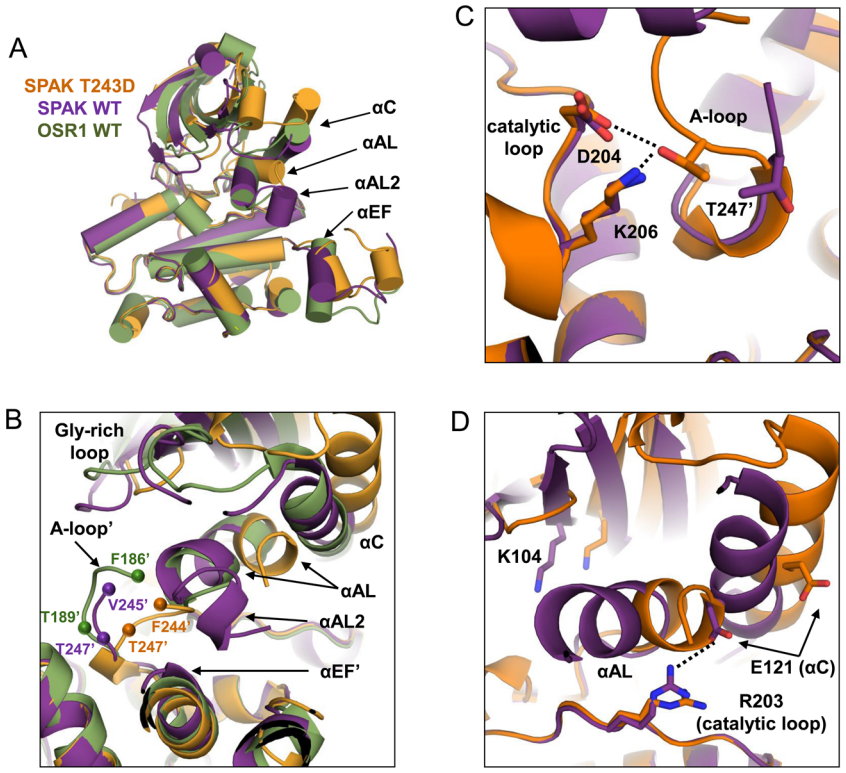

Figure 2. Conformational changes occurring in SPAK T243D compared to SPAK WT and OSR1 WT. (A) Cartoon representation (α-helices represented as cylinders for the sake of clarity) of C-terminal domain alignments of SPAK WT (chain A, purple), SPAK T243D (chain A, orange), and OSR1 WT (chain A, green; PDB entry 3DAK). Structural elements of particular interest are labeled. (B) Dimer interface near the domain-swapped activation loop and SPAK T243/OSR1 T185 WNK phosphorylation site. Labels marked with a prime correspond to the second monomer in the dimer. Spheres represent the first residue observed in the activation loop electron density and the SPAK T247′/OSR1 T189′ catalytic residue. (C) The D-K-T catalytic triad interaction, formed by catalytic loop residues D204 and K206 and domain-swapped activation loop residue T247′, is present in only SPAK T243D. (D) The essential K−E ion pair, between β3 K104 and αC E121, which is a hallmark of active kinases, is not present in SPAK WT or SPAK T243D. An interaction between catalytic loop residue R203 and E121 is present in SPAK WT but not in SPAK T243D.

collection, and refinement can be found in Experimental Procedures.

SPAK Structural Overview. Both SPAK WT and SPAK T243D structures adopt a canonical two-domain kinase fold, with a small N-terminal domain (residues 63−155) connected to a larger C-terminal domain (residues 156−365) through a hinge (Figure 1C). The C-terminal domain resembles that of OSR1 and has canonical kinase helices D−I, as well as small, C-terminal helices J−L. Both structures also possess an extra helix, αM (residues 355−363), that occupies a groove at the back of the kinase close to helix αD and strands β7 and β8. This helix is in a position similar to those of docking motif interactions found in MAPKs, which allosterically regulate sites distal to the docking site.[42−45] The constructs used to determine the structures of OSR1 lacked the C-terminal extension, including residues in αM.[28] An overlay of an individual subunit of both the SPAK WT and OSR1 WT structures reveals a rmsd of 0.99 Å. Apparently, neither the sequence variation between SPAK and OSR1 nor the presence of the PF1 domain in SPAK WT (of which helix αM is visible in the density) gives rise to significant conformational differences between the molecules (Figure 2A). However, conformational differences between the SPAK T243D and SPAK WT structures do exist. A slight (6°) domain rotation is present, with SPAK T243D in the more open configuration. αC and αAL (in the activation loop) show significant differences, again with αAL adopting the more open configuration in SPAK T243D (Figure 2A,B).

The nucleotide positions in SPAK T243D and SPAK WT primarily track with the N-terminal domains and reflect the difference in domain rotation between the two structures (Figure S1). In the open T243D structure, the phosphates are bound to the glycine-rich loop. In the more closed SPAK WT structure, the γ-phosphate maintains contact with K206 in the catalytic loop in the C-terminal domain. Neither position is identical to that in PKA because PKA has a closed domain structure and ATP contacts both domains. The position of the ATP in the SPAK T243D structure is more similar to that in PKA.[59,60]

Both SPAK WT and SPAK T243D have similar disordered regions. In the activation loop, SPAK T243D has only helix αAL, while SPAK WT has two helices, αAL and αAL2. Notably missing from the electron density is the C-terminal part of the PF1 domain (after residue R365 in SPAK T243D). To determine if this C-terminal region of the PF1 box affects activity, we compared wild-type SPAK 63−370 and 63−390, both phosphorylated by WNK1, and found that the longer construct had more activity (Figure S2A). SPAK T243D has 10−20-fold more activity than wild-type SPAK 63−390, but only ∼10% of the activity of SPAK 63−390 phosphorylated by WNK1 (Figure S2B).

SPAK Activation Loop Domain Swapping. The most prominent feature of both the SPAK WT and SPAK T243D structures is that the activation loops are domain-swapped (Figure 1B and Figure S3A,B). To ensure that the dimers observed in the crystal structures were not artifacts of

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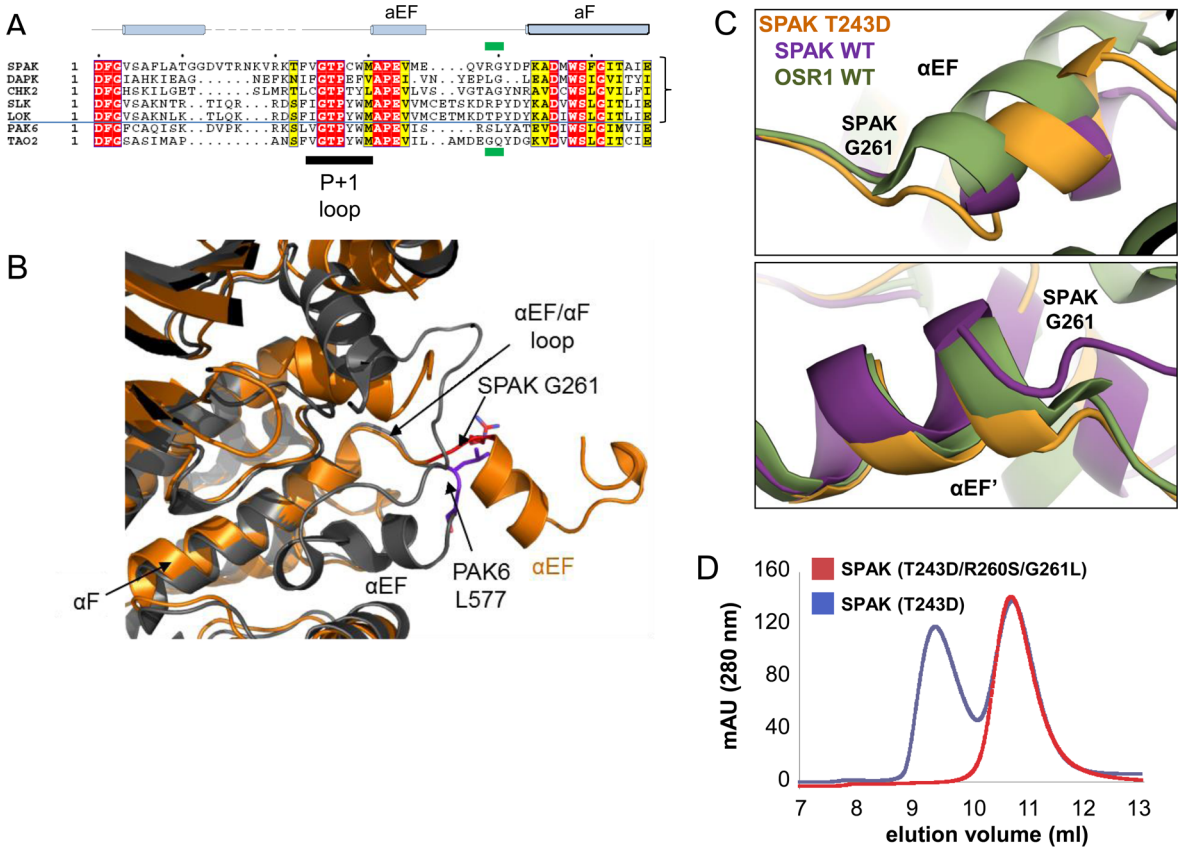

Figure 3. Identification and mutation of the domain-swap hinge. (A) Multiple-sequence alignment of representative sequences of domain-swapped kinases SPAK, DAPK3, CHK2, SLK, and LOK, compared to the nonswapped kinases PAK6 and TAO2. The position corresponding to G261 in SPAK is boxed in green. (B) Superposition of SPAK T243D and the nonswapped kinase PAK6 (PDB entry 2C30) reveals the difference in the αEF/αF loop. SPAK is colored orange and PAK6 gray. The switch points of R260 and G261 in SPAK and S576 and L577 in PAK6 are represented as sticks and colored red and purple, respectively. (C) View near αEF and αEF′ helices using chain A structural alignments of SPAK WT, SPAK T243D, and OSR1 WT dimers. Positional differences begin to occur near G261 and G261′ (G203 in OSR1). (D) Gel filtration profile of SPAK T243D and SPAK (T243D/R260S/G261L).

crystallization, we performed gel filtration experiments. Chromatography on Mono S revealed two peaks. Gel filtration showed that the peaks correspond to monomer and dimer (Figure S3C). Rechromatography of proteins from each peak gave rise to both populations (data not shown), indicating SPAK exists in a monomer−dimer equilibrium in solution. In addition, ATP favors dimerization (Figure S3D).

Activation loop domain swapping encompasses residues G233−G261: G233−G246 of the activation loop and P+1 specificity pocket (binds the P+1 site of the substrate), T247 in the active site of the other monomer, helix αE/F (E254−V259), and the following loop leading to helix αF (Figure S4A). In SPAK T243D, T247 is hydrogen-bonded to two invariant catalytic residues, D204 and K206, from the opposite monomer (Figure 2C and Figure S4B). The local structure in this area is similar to that of active canonical non-domain-swapped protein kinases, such as PKA (Figure S4B).

The dimer interfaces in SPAK T243D and SPAK WT bury 1906 and 2003 Å^2 of the dimer surface areas, respectively, or approximately 5% of the total surface areas of the dimers as calculated by the PISA server, and are almost entirely hydrophobic.[40] Part of the activation loops in both SPAK WT and T243D are disordered; in SPAK T243D, these residues encompass T231−D243 in the activation loop of one monomer and D234−D243 in the activation loop of the other monomer (Figure 2B). Similar residues are disordered in SPAK WT, with the exception of the presence of an additional helix αAL2 at the N-terminus of the activation loop. Notably, densities for T243 and the phosphomimetic mutation, T243D, are not present in either structure (Figure 2B).

At the quaternary level, the dimeric arrangements of SPAK WT and SPAK T243D differ significantly. Both form 2-fold symmetric dimers. However, these dimers are not equivalent because of differences in the orientation of the dimer axes (Figure S5A). This difference in rotation correlates with tighter dimer packing in SPAK WT, including close contact between the αAL2 helices, which are ordered in only SPAK WT (Figure S5C). In SPAK T243D, the two monomers are farther apart by a surprising 5 Å, although the function of this enhanced separation is unclear. Thus, these conformational differences are found not only in specific kinase substructures but also in the overall organization of the two monomers. These differences might be responsible for alteration of the relative orientation and position of the N- and C-terminal domains, or activation loop accessibility, which are generally associated with kinase activation.[57,58]

Comparison of the SPAK Structures Suggests a Multistage Activation Mechanism. SPAK WT and SPAK T243D monomers superimpose with a rmsd of ∼1.7 Å. The superposition improves considerably using the C-terminal domain alone, with a rmsd of 0.31 Å. The large discrepancy in rmsd is primarily due to the relative positions of the N- and

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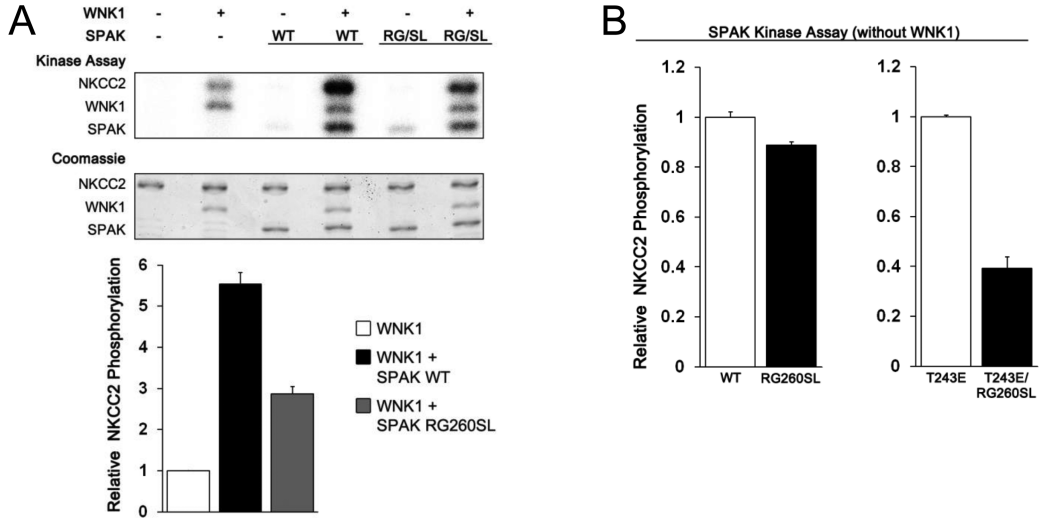

Figure 4. Effect of the R260S/G261L mutation on SPAK activity. (A) Coupled protein kinase assay. SPAK 63−403 WT and the R260S/G261L mutant were preincubated with ATP-$MgCl_2$ for 30 min with or without WNK1, after which [γ-^{32}P]ATP was added for radiolabeling, and GST-NKCC2 1−175 was added as a substrate. (B) Kinase assays comparing activity of SPAK 63−403 with and without the R260S/G261L mutation. T243E is an activating phosphomimetic mutation in the SPAK activation loop. All results in panels A and B are reported as relative activities, and as the mean and standard deviation of triplicate measurements quantified by phosphoimaging.

C-terminal domains, with SPAK T243D in the more open configuration, as discussed above (Figure 2A). Other specific differences occur in the glycine-rich loop, helix αC, and at both ends of the activation loop. The glycine-rich loop of SPAK T243D falls closer to the ATP binding pocket than it does in both SPAK WT and OSR1 WT and adopts a closed conformation on the first two β-strands. The C-termini of the activation loops adopt different conformations in the SPAK WT and T243D structures (Figure 2B). The SPAK T243D configuration is similar to that of active kinases, as discussed above, with a well-formed P+1 pocket, and T247 from the opposing monomer making standard active site contacts with D204 and K206 in the catalytic loop (Figure 2C and Figures S4B). In contrast, SPAK WT and OSR1 WT have poorly formed P+1 pockets. In SPAK T243D, the N-terminus of helix αC is shifted ∼5 Å relative to SPAK WT. As noted previously in the report on OSR1 WT, SPAK WT R203 from the catalytic loop makes an electrostatic interaction with E121 in αC.[28] This interaction is lost in SPAK T243D, potentially as the result of the movement of helix αAL (Figure 2D). However, SPAK T243D still lacks the K104−E121 ion pair, which is a hallmark of active kinases (Figure 2D and Figures S7A,B). Thus, SPAK T243D appears to be more active near the P+1 pocket and T247 but is still in an inactive configuration, because it lacks the K−E ion pair.

Another difference between the SPAK WT and SPAK T243D structures lies in the orientation of the N-termini of the activation loops, and in particular the αAL helix. αAL helices occur in diverse classes of kinases, e.g., EGF receptor,[51] and are known to be inhibitory by sterically blocking the inward movement of αC to form the K−E ion pair. Recent work has shown that deletion of the αAL helix in OSR1 leads to enhanced kinase activity.[52] Interestingly, the positions of SPAK WT and OSR1 WT αAL helices are incompatible with the position of the SPAK T243D activation loop (Figure 2B). We compared the position of αAL using both N- and C-terminal domain structural alignments and found that the movement of αAL was partially independent of the gross movements of the N- and C-terminal domains (Figure S8A,B). Several hydrophobic interactions between αAL and αC are lost in the SPAK T243D structure. In addition, αAL partially unfolds in SPAK T243D, and both αAL and αC exhibit higher *B* factors (Figure S8C−E).

Recent work has shown that the small activating protein MO25, also known as Cab39 (calcium binding protein 39), potently activates SPAK and OSR1 even in the presence of phosphomimetic mutation of the activation loop.[46] Structures of MO25 in complex with the related MST kinases reveal that this occurs through stabilization of the K−E ion pair. Therefore, MO25 binding may be required for SPAK and OSR1 to attain fully active conformations.[46−50,53]

Residues Permissive for Activation Loop Swapping. The exact N-terminal switch point for the swapping is within the segment of disordered residues D234−T243. The C-terminus of the swapped region ends in the loop that connects helices αEF (short helix following activation loop) and αF (Figure 1C and Figure S4A). Several other protein kinases exhibit activation loop domain swapping. Ste20-like kinase (SLK), lymphocyte-originated kinase (LOK, also known as STK10), and OSR1, in the Ste group, and checkpoint kinase 2 (CHK2) and death-associated protein kinase 3 (DAPK3), in the CaMK group, show domain swapping.[28,54−56] In each case, the αE/F helices are intact but bound to the opposite subunit.

A structure-based multiple-sequence alignment of SPAK and other kinases in the domain-swapped segment shows that all the domain-swapped kinases contain either a glycine or a proline at the position corresponding to G261 in SPAK, while the nonswapped kinases (for example, PAK6 and TAO2) have leucine and glutamine instead (Figure 3A). Proline is frequently found in the hinge loops of domain-swapped proteins.[29] Comparison of domain-swapped and nonswapped kinase structures reveals a major conformational difference in the αEF/αF loop in the backbone conformation between residues R260 and G261 in SPAK and S576 and L577 in PAK6 (Figure 3B). In particular, G261, in SPAK T243D, adopts a left-handed conformation with a φ of 83° and a ψ of 175°. Additionally, via alignment of chain A of the SPAK WT, OSR1 WT, and SPAK T243D dimer structures, the positional differences begin to

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occur around SPAK G261, or OSR1 G203 (Figure 3C). Thus, we suspected that G261 is part of the domain-swap hinge point.[29]

To investigate the role of G261 in activation loop swapping, we mutated R260 and G261 of SPAK to S and L, respectively, as in PAK6. The Superdex75 gel filtration profile of SPAK 63−390 (T243D/R260S/G261L) suggests that it is primarily a monomer in solution, in contrast to SPAK T243D (Figure 3D). Thus, apparently, R260 and G261 are part of the domain-swap hinge.

We then addressed the question of whether the monomer is active by comparing the activity of SPAK WT and the dimerization-blocked mutant. The activity profile was studied in the context of constructs encompassing the entire PF1 domain (SPAK 63−403 and SPAK 63−403 R260S/G261L). In an *in vitro* kinase assay, the SPAKs were first preactivated by phosphorylation by WNK1 for 30 min, and both were phosphorylated similarly (Figure S9). The SPAK 63−403 R260S/G261L mutant had ∼40% of the activity of SPAK WT toward the substrate GST-NKCC2 1−175 (Figure 4A). Data that show SPAK exhibits a small amount of autophosphorylation are also presented, and WNK1 exhibits some phosphorylation activity toward GST-NKCC2 1−175 in the absence of SPAK. Similarly, SPAK T243E/R260S/G261L had ∼40% of the activity of SPAK T243E (Figure 4B). However, the dimerization blocking mutation has no effect on the basal activity of SPAK (Figure 4B). Taken together, we believe these results suggest that the monomeric form of SPAK can be activated by WNK1, and it has kinase activity.

DISCUSSION

Domain swapping of activation loops is not uncommon among protein kinases. SLK, lymphocyte-originated kinase (LOK, also known as STK10), and OSR1, in the STE group, and checkpoint kinase 2 (CHK2) and death-associated protein kinase (DAPK3), in the CAMK group, are examples of domain-swapped enzymes.[28,55,56] Several structures of domain-swapped kinases, some phosphorylated, have been reported. These structures vary considerably in conformation, with some having hallmarks of an active kinase, such as the essential K−E ion pair interaction between helix αC and strand β3, and others appearing to be inactive. These results show that domain swapping is not a reflection of the state of phosphorylation or activity of the kinase.

Here we report the domain-swapped structures of SPAK WT and SPAK T243D, which mimics the WNK activating phosphorylation site in the SPAK activation loop. SPAK WT adopts an inactive configuration very similar to the previously reported structure of OSR1 WT.[28] SPAK T243D, in contrast, has some features reminiscent of active kinases. Thus, we hypothesize that the structures presented here reflect two different activity states in a multistage activation process: activation loop phosphorylation, mimicked here with the T243D mutation, induces a partially active conformation that can be further activated. Possible interactions that could lead to a fully active structure are with substrates or with known activator protein MO25 (Cab39). In support of the multistage activation concept, SPAK and OSR1 harboring the phosphomimetic mutation in the activation loop are still activated by nearly 100-fold in the presence of MO25.[46] We also note that the SPAK T243D active site is much broader than those of SPAK WT and OSR1 WT because of both N-terminal domain rotation and outward movement of helix αAL. The more open SPAK T243D active site could potentially be more accessible to substrate, but the precise function is unclear from this study.

By comparing structures and sequences of domain-swapped and nonswapped kinases, we identified a switch point for domain swapping. By mutating switch point residues to ones incompatible with domain swapping, we were able to generate a monomeric mutant form of SPAK and show that it can be phosphorylated by WNK1 and retains activity, albeit lower than that of the wild-type kinase. The dimerization blocking mutation should prove to be valuable in future studies aimed at understanding the role of SPAK and OSR1 domain swapping.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00593.

Figure captions of the nine supporting figures (PDF)
Nine supporting figures (PDF)

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Notes

The authors declare no competing financial interest.
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ABBREVIATIONS

SPAK, Ste20-related proline-alanine-rich kinase; WNK, with no lysine (K); OSR1, oxidative stress-responsive kinase; NKCC, Na^+-K^+-$2Cl^-$ cotransporter; DTT, dithiothreitol; SDS−PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate; PKA, cyclic AMP-dependent protein kinase; SLK, Ste20-like kinase; LOK, lymphocyte-originated kinase; CHK2, checkpoint kinase 2; DAPK3, death-associated protein kinase; PAK6, p21-

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activated protein kinase 6; TAO2, thousand-and-one protein kinase 2.

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