Structural Basis of Human p70 Ribosomal S6 Kinase-1 Regulation by Activation Loop Phosphorylation

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The ribosomal S6 kinase family belongs to the AGC3 subfamily of serine–threonine protein kinases. In humans two forms of p70 ribosomal S6 kinases (S6K1 and S6K2) have been reported that are encoded by two different genes (RPS6KB1 and RPS6KB2), respectively (1, 2). RPS6KB1 encodes two isoforms that differ only at the N termini by 23 amino acid residues (2). The longer form of S6K1 contains an N-terminal nuclear localization signal, whereas the shorter isoform of S6K1 predominantly localizes in the cytosol.

Several substrates of p70S6K have been identified including 40 S ribosomal protein S6, insulin substrate (IRS1), preapoptotic protein BAD, eukaryotic initiation factor (eIF4B), eukaryotic elongation factor (eEF2K) and cAMP-response element modulator (CREM) (3). The most studied substrate is the 40 S ribosomal protein S6, a major component of the machinery involved in protein synthesis in mammalian cells, suggesting that p70S6K plays a role in regulating translation.

Several observations suggest a role for p70S6K in cancer (4, 5). For example, upstream regulators of p70S6K are deregulated in multiple types of cancer, and gene and protein overexpression is observed in various cancers (4, 5). In addition, p70S6K is also a downstream kinase of insulin receptor-mediated signaling and is a potential therapeutic target for the management of obesity and diabetes as shown by enhanced metabolic rate and insulin sensitivity in p70S6K knock-out mice (4, 5).

The activation of p70S6K requires multiple phosphorylation events in both the kinase and autoinhibitory domains (Fig. 1). The C-terminal autoinhibitory domain, which is believed to block phosphorylation within the hydrophobic motif and the activation loop, is phosphorylated by upstream kinases such as ERK (6, 7). Other activating phosphorylation events occur at Thr-412 in the hydrophobic motif by mTOR (mammalian target of rapamycin) and at Thr-252 in the activation loop by PDK1 (8, 9). In addition, phosphorylation of Ser-394 in the turn motif is also necessary for full activation (10).

Given the potential of p70S6K as a therapeutic target, we determined crystal structures of both the unphosphorylated and activation-loop phosphorylated p70S6K1 kinase domains bound to staurosporine. The structures represent the first crystal structures of any member of the p70S6K family and provide a platform for the structure-guided design of selective p70S6K inhibitors.

EXPERIMENTAL PROCEDURES

Cloning and Expression—A PCR product encoding residues 75–399 of human p70S6K1 was amplified from full-length p70S6K1 cDNA (Open Biosystems; NM_003161) and ligated into a custom destination vector designed for baculovirus expression.
recombination derived from the transfer vector pVL1392 (Phar-mingen) that adds a N-terminal His₆ tag (11). The generation of recombinant baculovirus using flashBAC (NextGen Sciences) was performed using standard procedures. Viruses harboring the p70S6K1 kinase domain spanning residues 75–399 (hereafter p70S6KD) were used to infect Sf21 insect cells at a multiplicity of infection of 0.07. The cells were grown were resuspended (4.5 ml/g of cells) in lysis buffer (25 mM HEPES, pH 7.0, 350 mM Li₂SO₄, 25 mM imidazole, 10% glycerol, 50 units/g OF benzonase, 2 mM MgCl₂) and lysed with microfluidization. The clarified lysate was loaded on to a 15-ml HisTrap FF crude column (GE Healthcare) and detergent removed with 40 column volumes of 25 mM HEPES, pH 7.0, 350 mM Li₂SO₄, 10% glycerol, 2 mM TCEP, 50 mM imidazole. Protein was eluted with a linear gradient of 0–500 mM imidazole, and the fractions containing p70S6KD were pooled and dialyzed against 25 mM HEPES, pH 7.0, 350 mM Li₂SO₄, 10% glycerol, 1 mM TCEP, 2 mM MgCl₂, 800 units/mg OF λ-phosphatase (New England Biolabs). The His tag was removed with thrombin (Sigma-Aldrich), and the cleaved protein was further purified on a 10-ml HisTrap FF crude column followed by a HiLoad 26/60 Superdex 75 column (GE Healthcare) equilibrated in 25 mM HEPES, pH 7.0, 250 mM Li₂SO₄, 5% glycerol, 2 mM TCEP.

Purification of PDK1 Phosphorylated p70S6KD—Phosphorylation of p70S6KD (0.35 mg/ml) was performed in 1 mM ATP, 5 mM MgCl₂, 25 mM HEPES, pH 7, 10% glycerol, 350 mM Li₂SO₄, and 0.1 mg/ml PDK1 for 3 days at 4 °C. Completion of the phosphorylation reaction was confirmed with mass spectrometry. PDK1 was removed with a HiLoad 16/60 Superdex 75 column (GE Healthcare) equilibrated in 25 mM HEPES, pH 7.0, 250 mM Li₂SO₄, 5% glycerol, 2 mM TCEP.

Kinase Assay—Activity was measured with the QuickScout Screening Assist™ p70S6K FP kit (Carna Biosciences). Reactions contained 100 nm substrate and 25 μM ATP in 20 mM HEPES, pH 7.4, 5 mM MgCl₂, 0.01% Tween 20, and 2 mM dithiothreitol and varying concentrations of unphosphorylated p70S6KD, PDK1-phosphorylated p70S6KD, or activated p70S6K (residues 1–412) with the mutation T412E, purchased from Carna Biosciences (hereafter p70S6KΔCT) in a final volume of 10 μl. Reaction mixtures were incubated at 25 °C for 0, 20, 40, and 60 min and terminated with IMAP™ binding reagent for 2 h (400-fold dilution, Molecular Devices). Fluorescence polarization was measured with PHERAstar (BMG Labtech) using excitation and emission wavelengths of 485 and 520 nm, respectively.

Thermal Shift Assay—Tₘ values were measured with ThermoFluor (Johnson and Johnson). Protein-ligand or protein-buffer solutions (1–4 μl) were dispensed into 384-well polypropylene PCR microplates (ThermoFisher) and overlaid with silicone oil (1.5 μl, Fluka 85411). Protein solutions contained 0.2 mg/ml p70S6KD, 100 mM buffers (sodium acetate (pH 4.6), sodium citrate (pH 5.0 and 5.6), cacodylic acid (pH 6.0 and 6.6), HEPES (pH 7.0 and 7.6), Tris-HCl (pH 8.0 and 8.6), CAPSO (pH 9.0 and 9.6), or CAPS (pH 10.0)) and 250 mM NaCl or Li₂SO₄, 50 μM 1-anilino-8-naphthalenesulfonate, or 0–100 μM adenosine, ATP, AMP-PCP, and staurosporine. The change in 1-anilino-8-naphthalenesulfonate fluorescence was monitored with temperature using an imaging time of 2–30 s. Fluorescent intensities per well were averaged when multiple images were recorded for a given temperature. Reference wells contained protein without inhibitor. Plates were heated from 25 to 90 °C in increments of 1 °C with a 20-s equilibration time.

Analytical Size Exclusion Chromatography—Samples (15 mg/ml) were incubated for 0 or 2 days without ligand or for 5 days with 1 mM staurosporine at 4 °C. After incubation, samples were analyzed with a Superdex 200 5/150 GL column (GE Healthcare) at a flow rate of 0.15 ml/min using an injection volume of 5 μl in buffer containing 25 mM HEPES, pH 7.0, 5% glycerol, 250 mM Li₂SO₄, and 2 mM TCEP with 0 or 10 μM staurosporine.

Dynamic Light Scattering—Dynamic light scattering experiments were performed at 20 °C with a DynaPro dynamic light scattering instrument with a 781.8-nm wavelength laser (Protein Solutions). The protein solution was pretreated by centrifugation at 4 °C and 10,000 × g for 10 min. At least 15 measurements were taken for each sample. Regularization histogram analysis of samples was performed with DYNAMICS Version 5.25.44.

Analytical Ultracentrifugation—Sedimentation-equilibrium experiments were performed with a Beckman XL-I. Data were collected using 12-mm path length 6-sector centerpieces at 280 nm. Samples were dialyzed at 4 °C against the reference buffer (25 mM sodium phosphate, pH 7.5, 250 mM LiSO₄, 5% glycerol, 2 mM TCEP) for 48 h. Data were collected at 20 °C using loading concentrations spanning 3–30 μM and at rotor speeds of 20 and 30 krpm.

Data were analyzed with Origin (Beckman) and fit to ideal single species and monomer-dimer models. Solvent densities of 1.04 g ml⁻¹ and partial molar volumes of 0.743 ml g⁻¹ were calculated as described elsewhere(12). Parameters that were varied during fits to the ideal single-species model were molecular mass and base-line offset. Parameters that were allowed to vary during fits to the self-associating model were the log of the dissociation constant and the base-line offset, with the molecular mass held constant at the known monomeric value (36,882 Da). Monomer-dimer association constants (1/K₁) were converted from values obtained from the fits in absorbance units (Kₐ) to molar values (Kₐ) using Kₐ = Kₐ,abs/ε, where ε is the calculated extinction coefficient of 30,160 M⁻¹ cm⁻¹, and l is the path length.

Crystalization and Structure Determination of Unphosphorylated p70S6KD—Orthorombic crystals were grown by vapor diffusion at 20 °C by mixing equal volumes of protein (15 mg/ml) preincubated with 1 mM staurosporine with 30% (w/v)
polyethylene glycol 3350, 100 mM Bis-Tris, pH 5.5, 200 mM Li$_2$SO$_4$. The monoclinic crystal form was grown by mixing the same protein solution with 22.5% (w/v) polyethylene glycol 3350, 100 mM Bis-Tris, pH 5.5, 200 mM Li$_2$SO$_4$. Crystals were transferred to a cryoprotectant buffer containing the mother liquor and 15–20% (w/v) glycerol and vitrified by plunging into liquid nitrogen.

Diffraction data were collected at the Industrial Macromolecular Crystallography Association beamline 17-ID at the Advanced Photon Source and reduced using HKL2000 (13). The structure was determined by molecular replacement with AMoRe (14) using a homology model based on RSK1 (PDB code 2Z7R) as the search model. The structures were refined with CNSX (15). Model building was performed with Coot (16). Figures were made with PyMOL (DeLano Scientific LLC).

**Crystallization and Structure Determination of Phosphorylated p70S6KD**—Crystals were grown by vapor diffusion at 15 °C by mixing an equal volume of protein solution (15 mg/ml pre-incubated with 1 mM staurosporine) and 3.5 M sodium formate, 100 mM Tris, pH 8.5. Large, single crystals were obtained with macroseeding techniques. Crystals were transferred to a cryoprotectant buffer containing 12.5% (w/v) glycerol and vitrified by plunging into liquid nitrogen.

X-ray diffraction data were collected at beamline 17A at the Photon Factory and reduced with HKL2000 (HKL Research, Inc.). The structure was determined by molecular replacement using Phaser (17) and the unphosphorylated p70S6KD structure as the search model and refined as described above.

**RESULTS**

**Purification of p70S6KD**—The initial purification of p70S6KD in the presence of NaCl (250–500 mM) resulted in aggregation upon concentration. The effects of different buffer components on the thermal stability of p70S6KD were monitored to identify a more suitable buffer. The protein was most stable at pH 7.0 (Fig. 2A), and replacement of NaCl with 250 mM Li$_2$SO$_4$ increased the $T_m$ by 1.5 °C (Fig. 2B). Purification of p70S6KD in 25 mM HEPES, pH 7.0, and 250 mM Li$_2$SO$_4$ significantly reduced aggregation, and the protein could be concentrated to 15 mg/ml in the presence of Li$_2$SO$_4$.

**Kinase Activity**—The purified kinase domain of p70S6KD was a mixture of the phosphorylated and unphosphorylated forms as monitored by mass spectrometry (data not shown). To minimize heterogeneity due to phosphorylation, p70S6KD was dephosphorylated with A-phosphatase. The dephosphorylated protein was found to be catalytically inactive. Some activity could be restored upon phosphorylation of Thr-252 with PDK1 (Fig. 3A). Phosphorylation of Thr-252 was confirmed with Western blotting (Fig. 3B). The apparent activity of PDK1-phosphorylated p70S6KD (residues 75–399) was ~400-fold lower than that of the longer kinase domain (residues 1–412) that contains both phosphorylated Thr-252 and an activating T412E mutation in the hydrophobic motif (Fig. 3A). This observation is in accord with the multiple phosphorylation steps that regulate p70S6K1 (Fig. 1).

**Ligand-dependent Crystallization**—Crystals were not obtained with p70S6KD in the absence of ligands and dynamic light scattering suggested that the apoprotein tended to form higher order oligomers with increasing protein concentration.

**FIGURE 2. Effect of buffer conditions on p70S6KD stability.** A, shown are $T_m$ values of p70S6KD in buffers ranging in pH from 4.6 to 10. B, shown are $T_m$ values in the presence of varying salts (1. 250 mM NaCl, 2. 250 mM Li$_2$SO$_4$, and 3. no salt) at pH 7.0 (25 mM HEPES).

**FIGURE 3. Effects of phosphorylation of p70S6KD activity.** A, shown is activity of unphosphorylated p70S6KD (X), PDK1-phosphorylated p70S6KD (△, 12.5 mM; □, 25 mM), and p70S6KDΔCT (∗, 0.03125 mM; O, 0.0625 mM) on polarized light levels. B, analysis of phosphorylation of p70S6KD, PDK1-activated p70S6KD, and p70S6KDΔCT with Coomassie-stained SDS-PAGE and Western blotting using an anti-Thr(P)-252 antibody (Ab) is shown.
Staurosporine binds at the ATP binding pocket situated at a putative helices and predicted to be involved in a C helix (residues 130–140 of the N terminus and residues 372–399 at the C terminus). Additionally, it is worth noting that staurosporine appears to stabilize a single species with a given molecular mass, which is consistent with the unphosphorylated variant. The N- and C-terminal domains of the protein may be differentially affected by the presence of staurosporine, as suggested by the analytical size exclusion chromatography data.

### Table 1: Structure determination statistics for the unphosphorylated and PDK1-phosphorylated p70S6KD structures

|                | Unphosphorylated | Phosphorylated |
|----------------|------------------|----------------|
|                | Form I           | Form II        |
| Space group    | P2₁             | P2₁,2,2₁       |
| Unit-cell parameters |
| a              | 78.6 Å           | 66.8 Å         |
| b              | 62.9 Å           | 67.4 Å         |
| c              | 87.0 Å           | 98.3 Å         |
| β              | 94.3°            | 94.3°          |
| Resolution (Å) | 2.8 (2.9–3.8)    | 3.4 (3.52–3.40) |
| R-factor (%)   | 21.0 (28.8)      | 26.5 (32.1)    |
| Rfree (%)      | 26.8 (35.2)      | 34.9 (40.4)    |
| No. of solvent molecules | 6              | 38            |
| r.m.s.d. bond length (Å) | 0.009          | 0.011         |
| r.m.s.d. bond angle (°) | 1.50           | 1.38          |

**Ramachandran plot statistics**

| Residues in most favored region (%) | 84.3 | 75.7 | 90.0 |
| Residues in additional allowed region (%) | 13.4 | 21.5 | 9.0 |

Structure of Unphosphorylated p70S6KD—Crystallization screening of p70S6KD in complex with staurosporine yielded two crystal forms grown under similar crystallization conditions belonging to two different space groups (Table 1). Form I diffracted to 2.8 Å and belonged to the monoclinic space group P2₁, with two molecules per asymmetric unit. Form II diffracted to 3.4 Å and belonged to the orthorhombic space group P2₁,2,2₁, with one molecule per asymmetric unit.

The two molecules in the asymmetric unit of form I are similar to each other with a Cα r.m.s.d. (over 228 ordered residues) of 0.63 Å. However, minor conformational differences between the two molecules were observed in flexible regions of the two molecules, particularly around residues 235–243 of the activation loop, and a rotational difference of the helix αD (residues 178–189) and the disordered regions (residues 130–140 in chain A and residues 128–150 in chain B) around the αC helix.

The overall topology of the p70S6KD resembles other AGC kinase folds (Fig. 6A). The N-terminal domain (residues 85–172) is composed largely of β-sheet, and the C-terminal domain (residues 173–371) is predominantly α-helical. Residues 75–84 at the N terminus and residues 372–399 at the C terminus are disordered. In addition, residues 130–140 of the putative helices eB and eC and residues 243–255 of the putative activation loop are also disordered (Fig. 6B).

Staurosporine binds at the ATP binding pocket situated at the hydrophobic cleft between the N- and C-terminal domains (Fig. 6A). One side of the hydrophobic core of the indolocarbazole moiety makes many favorable van der Waals contacts with...
the backbone and side chains of residues from the N-terminal domain of p70S6KD, which includes the backbone and side chains of Leu-97, Val-105, Ala-121, Lys-123, Leu-172, and Tyr-174. The other side of the indolocarbazole moiety makes van der Waals interactions with the side chains of Leu-175, Met-225, Thr-235, and Asp-236. The N1 atom of the staurosporine lactam moiety forms a hydrogen bond (3.1 Å) to the backbone carbonyl oxygen of Glu-173, whereas the O5 atom of the inhibitor accepts a hydrogen bond (2.7 Å) from the amide nitrogen of Leu-175 in the linker region. The tetrahydropyran ring group sits in the ribose-binding pocket surrounded by Gly-98, Gly-100, Glu-179, Glu-222, Asn-223, Met-225, and Asp 236. The N4 atom of staurosporine hydrogen-bonds to Glu-179 of p70S6KD (2.9 Å). The observed van der Waals interactions and characteristic polar contacts are consistent with the apparent high affinity of staurosporine for p70S6K (19).

Dimerization and Domain Swapping in the Unphosphorylated Crystal Structure

—in the monoclinic crystal form I, two molecules in the asymmetric unit form a domain-swapped dimer. Residues 256–268 in chain A and residues 254–268 in chain B of the activation loop intertwine across the dyad axis (Fig. 7). Contributing to the dimer interface are parts of the catalytic loop, P + 1 loop, and helix αEF (Fig. 7). A comparison with the monomeric structure of the orthorhombic crystal form II (Cα r.m.s.d. over 239 ordered residues of 0.46 Å) illustrates that the overall monomeric structure of p70S6KD is preserved in the two crystal forms. The domain swapping in p70S6KD appears to be hinged at residue Gly-269 in the loop region between helices F and EF.

Structure of Phosphorylated p70S6KD—PDK1-phosphorylated p70S6KD exhibits kinase activity (Fig. 3A), consistent with previous reports (8, 9). To understand the structural basis for the differential activity between the PDK1-activated and unphosphorylated p70S6K kinase domains, PDK1-activated p70S6KD was crystallized as a complex with staurosporine. Crystals diffracted to 2.35 Å and belonged to the cubic space group P41212 (Table 1). The phosphorylated domain crystallized as an apparent monomer with one molecule in the asymmetric unit.

The overall folds of the unphosphorylated and phosphorylated p70S6K kinase domains are very similar (Cα r.m.s.d. over 241 ordered residues of 0.71 Å) (Fig. 6C). The binding pose of staurosporine in the ATP binding pocket is preserved between the two structures.
A significant feature of the phosphorylated structure is a hydrogen bond between phosphorylated Thr-252 (Fig. 6D) and Arg-217 in the catalytic loop and between Thr-252 and Arg-267 in the loop between αEF and αF. This interaction leads to local ordering of the activation loop compared with the unphosphorylated structure. Residues 241–250 of the activation loop as well as helices αB and αC (residues 128–141) remain disordered.

**Dimerization in Solution**—Unphosphorylated p70S6KD crystallized as both a monomer and a dimer in distinct crystal forms. In contrast, phosphorylated p70S6KD crystallizes as an apparent monomer. The self-association properties of both the unphosphorylated and phosphorylated kinase domains in solution were analyzed with sedimentation equilibrium over a 10-fold range of concentration. The apparent molecular masses for both the unphosphorylated and PDK1-phosphorylated forms returned by fits to a single-species model were higher than expected for a monomer but less than that of a dimer and increased systematically with increasing total protein concentration. The apparent molecular mass (52 kDa) are consistent with a self-associating species. The data are accounted for by a monomer-dimer fit, reflected in the non-systematic distribution of residuals. Equivalent results were obtained at three loading concentrations and two rotor speeds for the phosphorylated domain and at six loading concentrations and two rotor speeds for the unphosphorylated domain. Phosphorylated p70S6KD exists in solution in a monomer-dimer equilibrium. Results of a fit to sedimentation equilibrium data to single-species and monomer-dimer models are shown. The systematic deviation of the residuals for the single-species fit and the apparent single species are accounted for by a monomer-dimer fit, reflected in the non-systematic distribution of residuals. Equivalent results were obtained at three loading concentrations and two rotor speeds for the phosphorylated domain and at six load- ing concentrations and two rotor speeds for the unphosphorylated domain.

### DISCUSSION

The mTOR-dependent signaling cascade is implicated as a disease-related pathway in many different cancers and in diabetes (4, 5). p70S6K is one of the key component in the TOR pathway and is a potential target for therapeutic intervention. Here we report the first crystal structures of the p70S6K1 kinase domain in both the inactive and partially active phosphorylated activation loop forms. Biophysical analyses of thermal stability and aggregation were essential to obtain high quality p70S6KD protein and crystals.

**Comparison with Unphosphorylated AGC Family Kinases**—The overall structure of unphosphorylated p70S6KD resembles that of the catalytic domain of other AGC kinases. For example, the kinase domain of p70S6KD shares 47% sequence identity with the AGC kinase AKT2. The two structures superimpose with a Ca r.m.s.d. of 1.5 Å (over 236 ordered residues of p70S6KD) for the inactive form (PDB code 1GZK) and 1.2 Å (over 242 ordered residues) for the active form (PDB code 1O6K). The disordered regions of AKT2 and unphosphorylated p70S6KD are also similar. In the inactive form of AKT2, helices αB and αC of the N-terminal domain (residues 189–197) and the activation loop of the C-terminal domain (residues 297–312) are disordered. Likewise, helices αB and αC (130–140) and the activation loop (243–255) of p70S6KD are disordered. These regions are also disordered in structures of the inactive forms of other members of AGC family such as serum- and glucocorticoid-induced protein kinase (20) and RSK1 (21).

In the active conformation of all known AGC kinase structures, the conserved Glu in the helix αC (Glu-143 of p70S6K1) forms a salt bridge with the conserved Lys in the ATP binding pocket (Lys-123 of p70S6K1) that is critical for catalysis. In the structure of unphosphorylated p70S6KD, such an interaction is missing due to disordered Cε and terminal amine of Lys-123. The lack of this salt bridge, the similarity of disordered regions within p70S6K1 with inactive structures, and the observation that the crystallized protein showed no enzymatic activity are all consistent with the observed inactive conformation of the p70S6KD structure.

The lack of the hydrophobic motif region in the p70S6KD construct may also contribute to the inactive conformation of the helix αC. The hydrophobic motif undergoes phosphorylation as part of the activation process that results in stabilization of the catalytically competent conformation of the AGC kinases (22).

The activation loop of protein kinases is the region beginning with the conserved residues DFG and ending with residues (A)PE (23), which corresponds to residues 236–263 in p70S6KD. Part of the activation loop (residues 256–268) of chain A interacts with chain B in the homodimer (Fig. 7). The DFG motif at the beginning of the activation loop is known as the conserved activity related motif. In the active conformation, the Asp residue of the DFG motif points toward the β-phosphate of the bound ATP and coordinates with the Mg2+ cation, and Phe points away from the ATP to permit transfer of the phosphate group. Such a conformation is referred to as DFG-in
conformation. In the inactive p70S6KD structure, DFG assumes the DFG-in conformation. Other kinases in inactive states, such as cyclin-dependent kinase 2 (24), RSK1 (21), and serum- and glucocorticoid-induced protein kinase (20), also exhibit DFG-in conformations. Half of the activation loop of the inactive form (residues 243–255, including unphosphorylated Thr-252) is disordered, which is consistent with the inactive form of the kinase.

Domain Swapping and Dimerization—The unphosphorylated kinase domain crystallizes in both monomeric and dimeric forms, whereas only the monomeric form was observed for the PDK1-phosphorylated domain. Dimerization is mediated by a domain swap involving the activation loop. Domain swapping for the isolated kinase domains of cell cycle checkpoint kinase 2, death-associated protein kinase 3, lymphocyte-oriented kinase, and Ste20-like kinase has been proposed as a possible mechanism of trans-auto phosphorylation at non-consensus sites (25, 26). More recently, domain swapping has been observed for the isolated OSR1 kinase domain, which in contrast to cell cycle checkpoint kinase 2, death-associated protein kinase 3, lymphocyte-oriented kinase, and Ste20-like kinase, does not undergo autophosphorylation (27). Autophosphorylation has not been observed for p70S6KD. Interestingly, the domain orientation of the p70S6KD dimer is more similar to that of the OSR1 dimer than that of cell cycle checkpoint kinase 2, death-associated protein kinase 3, lymphocyte-oriented kinase, and Ste20-like kinase (Table 2). It is also noteworthy that the affinity of the p70S6K dimer is 10-fold greater than that of lymphocyte-oriented kinase and death-associated protein kinase 3 (25). It is possible that domain swapping within kinases may be a general phenomenon for regulating kinase autophosphorylation at non-consensus sites (25, 26). However, domain swapping has been observed to be an artifact in a number of proteins where isolated domains are dimers and yet the full-length protein is a monomeric form (28). The potential biological significance, or otherwise, of the domain-swapped kinase domain within the context of the full-length enzyme awaits further investigation.

Structural Basis for Activation—At least three phosphorylation events appear necessary for activation of p70S6K1 at Thr-252, Ser-394, and Thr-412. The domain used for crystallization does not contain Thr-412. Although the unphosphorylated domain is catalytically inactive, some activity was restored upon phosphorylation of p70S6KD by PDK1 at Thr-252. The crystal structures of the inactive unphosphorylated and partially active PDK1-phosphorylated forms of p70S6KD show the similar structural features of a partially disordered activation loop and αB and αC helices, which is consistent with the relatively low activity of the Thr-252-monophosphorylated form.

Despite the partial disorder of the structural elements that are involved in activation of the kinase, one significant feature of the monophosphorylated p70S6KD is a hydrogen bond between phosphorylated Thr-252 and Arg-217 of the catalytic loop (Fig. 6D). The salt bridge interaction seen in p70S6KD between the catalytic loop arginine and a phosphorylated side chain in the activation segment is conserved among activated states of other known kinases (23). This interaction in PDK1-phosphorylated p70S6KD may represent a transient conformation of the activation loop between fully inactive and active state of the kinase or a partially ordered state that may account for the activity observed with the Thr-252-phosphorylated enzyme that lacks the phosphorylated hydrophobic motif.

The other known structure of an AGC kinase that is phosphorylated in the activation loop is AKT2/PKBα (29). Like p70S6K, activation loop phosphorylation of AKT2/PKBα results in partial activation of the enzyme (10% in the case of AKT2/PKBα). In contrast to p70S6K, the structure of the activation loop of AKT2/PKBα remains completely disordered upon phosphorylation. This result was interpreted at the time to be an artifact of the AKT2/PKB crystallization (29).

Implications for Drug Design—Inactive forms of kinases may offer better selectivity for small-molecule inhibitors than the activated forms due to the highly conserved conformation of the ATP binding pocket in the activated state (30). In contrast, greater conformational variability has been observed for the ATP binding pocket of inactive kinases. The high degree of structural and sequence conservation of the ATP binding pocket presents potential challenges in designing selective active-site kinase inhibitors. For example, the N-terminal kinase domains of the AGC kinase RSK1 is closely related to the kinase domain of p70S6K1 with 55% sequence identity within the p70S6K1 kinase domain. Despite the significant sequence similarity, the cellular functions are different with RSK1 functioning in the ERK mitogen-activated protein kinase pathway (31) and p70S6K1 in the mTOR pathway (4, 5).

Comparison of the structures of p70S6K1 and RSK1 (21), both bound to staurosporine, provides insights into the structural differences that may be exploited in the design of selective inhibitors for p70S6K1. Overall, the structures of RSK1 and unphosphorylated p70S6K1 are very similar (Cα r.m.s.d. over 236 residues of 1.0 Å). Helices αB, αC, and part of activation loop are disordered in both structures. Significant main-chain position differences are observed in residues 115–119 of RSK1 (ILADV) and residues 141–150 (KKAERNILEEVEE) of p70S6K1. This region of p70S6K1 adopts a helical conformation and is observed as a loop in RSK1. In the ATP binding pocket, the DFG motif assumes the DFG-in conformation in RSK1 and p70S6K1. The binding mode of staurosporine (Fig. 9) suggests hydrophobic and hydrogen-bonding interactions are conserved in the two structures. However, two residues in the ATP binding pocket are not conserved; Asp-148 and Leu-194 in RSK1 are spatially equivalent to Glu-179 and Met-225 in p70S6K1. Such small but significant differences have been successfully exploited in designing selective kinase inhibitors (32, 33). The conformational and sequence differences between p70S6K and other kinases together with the proximity of helix

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**TABLE 2**

Comparison of domain-swapped kinases with p70S6KD

| Compared kinase | PDB code | r.m.s.d. | Cα atoms | Difference in the dimer orientation |
|-----------------|----------|---------|----------|-----------------------------------|
| OSR1           | 3DAK     | 3.9     | 201      | 6                                 |
| DAPK3          | 2J90     | 1.3     | 186      | 27                                |
| LOK            | 2J7T     | 2.7     | 210      | 16                                 |
| SLK            | 2J51     | 2.4     | 197      | 26                                 |
| CHK2           | 2CNS     | 2.4     | 205      | 25                                 |

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**Crystal Structures of the p70S6K1 Kinase Domain**
Crystal Structures of the p70S6K1 Kinase Domain

![Comparison of the crystal structures of unphosphorylated p70S6KD and RSK1. Superimposition of the ATP binding pocket of p70S6KD (red) and RSK1 (cyan) is shown. Staurosporine, Glu-179 and Met-225 of p70S6KD are shown in green, and those of RSK1 are shown in blue.

αC to the ATP binding pocket may hold promise for designing p70S6K1 selective inhibitors.

In conclusion, the crystal structures of inactive and partially activated forms of p70S6K1 reported in this paper provide the first structural description of the p70S6K1 kinase domain in both the inactive and partially active forms and offer opportunities for the design of selective p70S6K1 inhibitors.

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