Crystal structures of S6K1 provide insights into the regulation mechanism of S6K1 by the hydrophobic motif

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The activity of S6K1 (p70 ribosomal protein subunit 6 kinase 1) is stimulated by phosphorylation of Thr389 in the hydrophobic motif by mTORC1 (mammalian target of rapamycin complex 1) and phosphorylation of Thr229 in the activation loop by PDK1 (phosphoinositide-dependent kinase 1); however, the order of the two events is still ambiguous. In the present paper we report six crystal structures of the S6K1 kinase domain alone or plus the hydrophobic motif in various forms, in complex with a highly specific inhibitor. The structural data, together with the biochemical data, reveal in vivo phosphorylation of Thr389 in the absence of Thr229 phosphorylation and demonstrate the importance of two conserved residues, Gln460 and Arg571, in the establishment of a hydrogen-bonding network between the N-lobe (N-terminal lobe) and the hydrophobic motif. Phosphorylation of Thr389 or introduction of a corresponding negatively charged group leads to reinforcement of the network and stabilization of helix αC. Furthermore, comparisons of S6K1 with other AGC (protein kinase A/protein kinase G/protein kinase C) family kinases suggest that the structural and sequence differences in the hydrophobic motif and helix αC account for their divergence in PDK1 dependency. Taken together, the results of the present study indicate that phosphorylation of the hydrophobic motif in S6K1 is independent of, and probably precedes and promotes, phosphorylation of the activation loop.

Key words: activation loop, hydrophobic motif, mammalian target of rapamycin (mTOR) signalling, phosphorylation, ribosomal protein subunit 6 kinase 1 (S6K1), zinc finger.

INTRODUCTION

S6K1 (p70 ribosomal protein subunit 6 kinase 1), belonging to the AGC [PKA (protein kinase A)/PKG (protein kinase G)/PKC (protein kinase C)] family of kinases is an important downstream serine/threonine kinase of the mTOR (mammalian target of rapamycin) signalling pathway, and its phosphorylation state is an indicator of the mTOR activity [1,2]. In mammalian cells, S6K1 is widely expressed and the S6K1a isoform is more sensitive to rapamycin (mTORC1) signalling than the S6K1b isoform [3].

Like many other AGC family kinases, S6K1 contains a hydrophobic motif which is C-terminal to the kinase domain, and to achieve full activity it requires phosphorylation at multiple serine/threonine sites through a series of phosphorylation events. Two phosphorylation events occur first, including phosphorylation of Thr389 by the activated mTORC1 [17,18], and phosphorylation of Ser411, Ser417, Ser421 and Ser424 in the activation loop [19]. Ser380 and Thr389 of S6K1 are autophosphorylation sites which are important for full activity [20]. The importance and variety of S6K1 functions, aberration of the mTOR/S6K1 signalling pathway has been correlated with various diseases, including cancers, diabetes, obesity and aging [11]. For example, hyperactivation of the mTOR/S6K1 cascade is a hallmark of cancer cells [12,13]. It was also reported that the S6K1−/− mouse could antagonize obesity induced by aging and a rich nutrition diet [8]. Therefore S6K1 is a potential therapeutic target and currently a number of small-molecule inhibitors targeting S6K1 are undergoing clinical trials [14].

Like many other AGC family kinases, S6K1 contains a hydrophobic motif which is C-terminal to the kinase domain, and to achieve full activity it requires phosphorylation at multiple serine/threonine sites through a series of phosphorylation events. Two phosphorylation events occur first, including phosphorylation of Ser380, Ser417, Ser421 and Ser424 in the intrinsically disordered AID (autoinhibitory domain) at the C-terminus to relieve its inhibitory effect [15] and phosphorylation of Ser371 in the turn motif bridging the kinase domain and the hydrophobic motif [16]. To be fully activated, S6K1 also needs phosphorylation of Thr389 in the hydrophobic motif by the activated mTORC1 (mTOR complex 1) [17,18], and
phosphorylation of Thr\(^{229}\) in the activation loop by PDK1 (phosphoinositide-dependent kinase 1) which is also a member of the AGC kinase family [3]. However, the order of the latter two events is still ambiguous. The conventional model proposes that mTORC1 phosphorylates Thr\(^{389}\) of the hydrophobic motif first and the phosphorylated hydrophobic motif provides a docking site for PDK1, which subsequently phosphorylates Thr\(^{229}\) of the activation loop [19]. However, an alternative model argues that phosphorylation of Thr\(^{229}\) occurs before phosphorylation of Thr\(^{389}\) [20] as PDK1 is able to phosphorylate Thr\(^{229}\) of S6K1 when the hydrophobic motif is absent or unphosphorylated [3,21].

Previously, the first crystal structures of the S6K1 kinase domain with either unphosphorylated or phosphorylated Thr\(^{229}\) of the activation loop were reported, providing valuable insights into the regulation of S6K1 by phosphorylation of Thr\(^{229}\) in the activation loop [21]. However, the structural information is limited due to the lack of the hydrophobic motif and the disordering of helices αB and αC, and the regulation mechanism of S6K1 activity by the hydrophobic motif remains elusive. In the present paper we report six crystal structures of the S6K1 α2 kinase domain alone or connected with the hydrophobic motif in various forms. The structural data, together with the biochemical data, reveal unique structural features of S6K1 and provide new insights into the regulation mechanism of S6K1 activity.

**MATERIALS AND METHODS**

**Cloning, protein expression and purification**

The full-length S6K1 α2 gene was amplified from a human brain cDNA library using PCR and cloned into the pET28a vector (Novagen). The gene fragments encoding the kinase domain (encompassing residues 52–379; termed S6K1-KD) and the kinase domain plus the hydrophobic motif (encompassing residues 52–394; termed S6K1-HM\(^{WT}\)) were subcloned into the pFastHTB vector (Invitrogen) respectively. The plasmids were transfected into High-Five insect cells. The viruses extracted and transfected into insect cells were harvested and resuspended with lysis buffer [50 mM Hepes (pH 7.0), 250 mM lithium sulfate, 10% (w/v) glycerol, 2 mM magnesium chloride and 1 mM PMSF] supplemented with protease inhibitor cocktail (Roche). Cells were lysed with a hydraulic chamber and the supernatant was incubated with pre-equilibrated Ni-NTA (Ni\(^{2+}\)-nitrilotriacetate) sepharose resin (Qiagen) for 1.5 h. The resin was loaded on to a column and eluted in turn with lysis buffer alone and lysis buffer supplemented with 40 mM imidazole. The target protein was eluted with lysis buffer supplemented with 200 mM imidazole and dialysed to buffer A containing 20 mM Hepes (pH 7.0), 250 mM lithium sulfate and 10% (w/v) glycerol. The N-terminal His\(^{1}\) tag was removed by TEV (tobacco etch virus) digestion overnight. The protein was treated with the λ-protein phosphatase to remove non-specific phosphorylation at 16°C, and then reloaded on to an Ni-NTA column to remove the undigested protein and the phosphatase. The protein was treated with the λ-protein phosphatase, phosphorylation of Thr\(^{389}\) was detected in the S6K1-HM\(^{WT}\) structure and confirmed using Western blotting with an antibody specific for phosphorylated Thr\(^{389}\) of S6K1 (Cell Signaling Technology). The purified protein was pooled and concentrated to 10 mg/ml.

The S6K1-HM\(^{WT}\), S6K1-HM\(^{389A}\), S6K1-HM\(^{121A}\), S6K1-HM\(^{217A}\), S6K1-HM\(^{217A/H222A}\) and S6K1-HM\(^{217A/H222A/H228A}\) mutants were constructed with the QuikChange\textregistered site-directed mutagenesis kit (Stratagene), and the S6K1-HM\(^{P2F}\) mutant was generated with the Blunt-PCR kit (TaKaRa). All of the S6K1-HM mutants were expressed and purified using the same procedures as for S6K1-HM\(^{WT}\).

**Crystallization, data collection and structure determination**

To obtain the protein–inhibitor complex, the target protein was incubated with PF-4708671 on ice for 30 min at a molar ratio of 1:4. Crystallization was performed using the hanging-drop vapour-diffusion method, and all crystals were grown in drops containing 1 μl of the protein solution and 1 μl of the crystallization solution. For S6K1-KD, the crystallization solution contained 0.1 M Hepes (pH 7.5) and 2.5 M sodium formate. For S6K1-HM\(^{WT}\), the crystallization solution contained 0.1 M Hepes (pH 6.8), 0.01 M magnesium chloride and 2.2 M ammonium sulfate. Crystals of S6K1-HM\(^{389A}\) were grown in the crystallization solution containing 0.1 M sodium cacodylate (pH 6.5) and 1.4 M sodium citrate, and those of S6K1-HM\(^{P2F}\) in the crystallization solution containing 0.1 M Mes (pH 6.2) and 2.0 M ammonium sulfate. Form I and form II crystals of S6K1-HM\(^{389A}\) were grown in the crystallization solution containing 0.1 M Bis-Tris propane (pH 7.0) and 1.6 M ammonium citrate, and that containing 0.05 M Hepes (pH 7.2), 0.05 M magnesium chloride and 2 M lithium sulfate respectively. Diffraction data were collected at beamline BL-17U at the SSSRF (Shanghai Synchrotron Radiation Facility) and processed with HKL2000 [22].

The S6K1-KD structure was solved with the molecular replacement method implemented in the program suite CCP4 using the S6K1-pKD structure (PDB code 3A62) as the search model, which then served as the search model for the S6K1-HM structures. Structure refinement was carried out with Phenix [23] and REFMAC5 [24] following standard protocols. Model building was performed manually with COOT [25]. Throughout the refinement, 5% of randomly chosen reflections were set aside for free R-factor monitoring. The final stereochemical qualities of the structure models were checked with Molprobity [26]. All graphics were drawn using PyMOL (http://www.pymol.org).

**Phosphorylation and in vitro kinase activity assay of the S6K1-HM proteins**

The wild-type or mutant S6K1-HM protein was incubated with TEV to remove the His\(^{1}\) tag, and then incubated with 1.5 μM His-tagged PDK1 kinase domain in the presence of 5 mM magnesium chloride and 2 mM ATP for 48 h. After the reaction, PDK1 was removed with an Ni-NTA column. The phosphorylated S6K1 protein was further purified by gel filtration using a Superdex G200 16/60 column (GE Healthcare) equilibrated in 20 mM Hepes (pH 7.0), 250 mM lithium sulfate, 5% (w/v) glycerol and 2 mM TCEP [tris-(2-carboxyethyl)phosphine]. The protein was concentrated to 10 mg/ml and stored at −80°C.

The in vitro kinase activity assay was performed with an ADP-Glo\textsuperscript{TM} kit (Promega) following standard procedures. Briefly, a 20 μl reaction system contained 1 mM ATP, 10 mM magnesium chloride, 1 mM S6 peptide (with the sequence RRRLSSLA, corresponding to residues 231–239 of S6) and 25 ng/μl S6K1-HM protein in a buffer consisting of 50 mM Hepes (pH 7.5), 150 mM sodium chloride and 1 mM TCEP. The reaction was performed at room temperature (25°C) for 30 min and terminated by the addition of the ADP-Glo\textsuperscript{TM} reagent. After incubation for 40 min with the ADP-Glo\textsuperscript{TM} reagent, the ADP-Glo\textsuperscript{TM} Max Detection
Cell parameters bilobal architecture (Figure 1), and the ATP-binding cleft formed during the crystallization.

For crystallization of the S6K1 proteins, a new inhibitor with high specificity for S6K1, namely PF-4708671 [28], was supplemented.

In all of the structures, the kinase domain took a typical bilobal architecture (Figure 1), and the ATP-binding cleft formed between the N- and C-lobes (N-terminal and C-terminal lobes respectively) was occupied by the inhibitor via both hydrophilic and hydrophobic interactions. In some of the structures, a Zn$^{2+}$ ion was detected to bind at the activation loop (see below).

Although the overall structures of S6K1 are similar to the structure of the PKB$^{S472D}$ (protein kinase B)–p[NH$^\gamma$]ppA (adenosine 5'-[β,γ-imido]triphosphate)–GSK3β (glycogen synthase kinase 3β) complex [29] with RMSDs of approximately 1.2 Å (1 Å = 0.1 nm) over 276 Cα atoms and the previously reported structure of S6K1 containing phosphorylated Thr$^{389}$ (S6K1-pKD) [21] with RMSDs of less than 0.5 Å over 248 Cα atoms, significant differences were observed in the hydrophobic motif, the activation loop, helix αC and the active site (discussed below).

### Results and Discussion

#### Overall structures

To study the regulation mechanism of S6K1 by the hydrophobic motif, we determined the structures of the S6K1 α2 kinase domain alone (S6K1-KD, residues 52–379) and the S6K1 α2 fragment containing the kinase domain and the hydrophobic motif (S6K1-HM, residues 52–394) in various forms including the wild-type form (S6K1-HMW) or the mutant forms carrying the T389E or T389A mutation at the phosphorylation site of the hydrophobic motif (S6K1-HMT389E and S6K1-HMT389A) (Table 1). The structure of a chimaeric S6K1 protein (S6K1-HMPS) was also determined in which residues 380–394 of the hydrophobic motif of S6K1 was substituted with the PIF sequence (EEQEMFRDFEYIADW) [27], a mimic of the hydrophobic motif of PKC-related kinase 2. For crystallization of the S6K1 proteins, a new inhibitor with high specificity for S6K1, namely PF-4708671 [28], was supplemented during the crystallization.

Complete electron density (Supplementary Figure S1 at http://www.biochemj.org/bj/454/bj4540039add.htm). The hydrophobic motif is not observed to bind in a hydrophobic groove across a top surface of the N-lobe (Figures 1B and 2A) with evident electron density (Supplementary Figure S1 at http://www.biochemj.org/bj/454/bj4540039add.htm).

### Table 1 Summary of diffraction data and structure refinement statistics

Values in parentheses are for the highest-resolution shell. HM, hydrophobic motif.

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| Ramachandran plot (%)
| Allowed | 100 | 100 | 100 | 100 | 100 | 100 |
| Most favoured | 97.2 | 90.6 | 94.9 | 91.6 | 92.7 | 91.2 |
| Allowed | 100 | 100 | 100 | 100 | 100 | 100 |

* | Ramachandran plot (%)
| Allowed | 100 | 100 | 100 | 100 | 100 | 100 |
| Most favoured | 97.2 | 90.6 | 94.9 | 91.6 | 92.7 | 91.2 |

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Values in parentheses are for the highest-resolution shell. HM, hydrophobic motif.

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reagent was added and the luminescence was detected with a plate-reading luminometer (PerkinElmer).

### Results and Discussion

#### Overall structures

To study the regulation mechanism of S6K1 by the hydrophobic motif, we determined the structures of the S6K1 α2 kinase domain alone (S6K1-KD, residues 52–379) and the S6K1 α2 fragment containing the kinase domain and the hydrophobic motif (S6K1-HM, residues 52–394) in various forms including the wild-type form (S6K1-HMW) or the mutant forms carrying the T389E or T389A mutation at the phosphorylation site of the hydrophobic motif (S6K1-HMT389E and S6K1-HMT389A) (Table 1). The structure of a chimaeric S6K1 protein (S6K1-HMPS) was also determined in which residues 380–394 of the hydrophobic motif of S6K1 was substituted with the PIF sequence (EEQEMFRDFEYIADW) [27], a mimic of the hydrophobic motif of PKC-related kinase 2. For crystallization of the S6K1 proteins, a new inhibitor with high specificity for S6K1, namely PF-4708671 [28], was supplemented during the crystallization.

In all of the structures, the kinase domain took a typical bilobal architecture (Figure 1), and the ATP-binding cleft formed between the N- and C-lobes (N-terminal and C-terminal lobes respectively) was occupied by the inhibitor via both hydrophilic and hydrophobic interactions. In some of the structures, a Zn$^{2+}$ ion was detected to bind at the activation loop (see below).

Although the overall structures of S6K1 are similar to the structure of the PKB$^{S472D}$ (protein kinase B)–p[NH$^\gamma$]ppA (adenosine 5’-[β,γ-imido]triphosphate)–GSK3β (glycogen synthase kinase 3β) complex [29] with RMSDs of approximately 1.2 Å (1 Å = 0.1 nm) over 276 Cα atoms and the previously reported structure of S6K1 containing phosphorylated Thr$^{389}$ (S6K1-pKD) [21] with RMSDs of less than 0.5 Å over 248 Cα atoms, significant differences were observed in the hydrophobic motif, the activation loop, helix αC and the active site (discussed below).

#### Phosphorylation of Thr$^{389}$ of the hydrophobic motif in the S6K1-HMW structure

In the structure of S6K1-HMW, the hydrophobic motif was observed to bind in a hydrophobic groove across a top surface of the N-lobe (Figures 1B and 2A) with evident electron density (Supplementary Figure S1 at http://www.biochemj.org/bj/454/bj4540039add.htm). The hydrophobic motif is not involved in crystal packing in this structure and the other hydrophobic-motif-containing structures, suggesting that the
stabilization of the motif should be due to its interactions with other structural elements intramolecularly (discussed below). Intriguingly, although the protein was treated with the λ-protein phosphatase before crystallization, phosphorylation of Thr^{389} of the hydrophobic motif was revealed during refinement of the crystal structure (Supplementary Figure S2 at http://www.biochemj.org/bj/454/bj4540039add.htm), and further confirmed using Western blotting analysis with an antibody specifically targeting phosphorylated Thr^{389} of S6K1-HMWT (Figure 2C). Taken together, these results demonstrate that in vivo phosphorylation of the hydrophobic motif in the S6K1-HMWT protein does not require phosphorylation of the activation loop.

Our observation is in line with previous findings. Thr^{389} phosphorylation of S6K1 overexpressed in mammalian cells has been detected in the full-length S6K1 α1 and the Δ2-46ΔCT truncate of S6K1 α1 corresponding to residues 24–398 of S6K1 α2, which most resembles our S6K1-HMWT protein [30]. Moreover, in the full-length T229A mutant S6K1, Thr^{389} phosphorylation could also be readily detected [30]. In addition, in in vivo studies with PDK1^{155E/155E} knock-in cells, it was shown that although Thr^{229} of the activation loop of S6K1 is unphosphorylated, phosphorylation of Thr^{389} of the hydrophobic motif is constitutive and can be enhanced with the treatment of IGF-1 (insulin-like growth factor 1) or okadaic acid, but suppressed with rapamycin [31]. Taken together, these results support the notion that the in vivo phosphorylation of the hydrophobic motif of S6K1 by mTORC1 is independent of the phosphorylation of the activation loop by PDK1. Therefore the S6K1-HMWT structure containing the phosphorylated Thr^{389} might provide a snapshot of S6K1 during its activation process.

**Figure 1** Overall structures of S6K1

(A) Front view of the overall structure of S6K1-KD. The kinase domain assumes a typical bilobal structure containing the N-lobe (cyan) and the C-lobe (yellow). The P-loop of the N-lobe is coloured in light blue, and the activation loop of the C-lobe is coloured in red. The inhibitor PF-4708671 is bound at the ATP-binding cleft at the interface of the N- and C-lobes and is shown with a grey ball-and-stick model. A Zn^{2+} ion is bound at the activation loop and is shown with a grey sphere. (B) Back view of the overall structure of S6K1-HMWT. The hydrophobic motif (HM) is bound in the hydrophobic groove of the N-lobe formed by helices αB and αC and the β3–β5 sheet. The activation loop and the turn motif (TM) connecting the kinase domain and the hydrophobic motif are partially disordered, and the missing residues are indicated with broken lines.

**Interactions between the hydrophobic motif and the N-lobe and the divergence among the AGC family kinases**

As shown in Figure 2(A), the hydrophobic motif is bound in and makes extensive hydrophobic interactions with a hydrophobic groove at the top of the N-lobe formed by helices αB and αC and strands β3–β5. The phenyl rings of the invariant Phe^{385} and Phe^{388} of the hydrophobic motif pack against the side chains of Leu^{106} on strand β3, Ile^{107} on helix αB and Leu^{145} on strand β5. In addition, the phenol ring of the invariant Tyr^{140} packs against the conserved Leu^{135} on strand β4. Similar hydrophobic interactions have been observed in the structures of PKA (PDB code 1ATP) and PKB (PDB code 1O6L) [29]. With the phosphorylation of Thr^{389}, a hydrophilic network is also formed, which plays an important role in the stabilization of the hydrophobic motif (Figure 2D). Specifically, the side chain of Gln^{140} on strand β4 forms hydrogen bonds with the main-chain carbonyl of Phe^{388} and the phosphoryl group of pThr^{389}. The phosphoryl group of pThr^{389} is also hydrogen-bonded to the main-chain amide of Phe^{388}, whereas the main-chain carbonyl of Phe^{388} forms hydrogen bonds with the side chain of Arg^{121} on helix αC.

To examine the functional roles of the hydrophobic motif and its phosphorylation at Thr^{389}, the structures of S6K1-HM in various mutant forms were determined. As shown in Figure 2(E), most of the interactions between the hydrophobic motif and the N-lobe observed in the S6K1-HMWT structure were retained in the S6K1-HM^{T389E} structure in which the T389E mutation mimics the phosphorylation of Thr^{389}, except that the side chain of Glu^{108} formed only one hydrogen bond with Gln^{140} while its main-chain carbonyl formed an extra hydrogen bond with the side chain of Arg^{121}. Owing to the difficulties in obtaining the unphosphorylated S6K1-HM, mutation of Thr^{389} to alanine was performed and two forms of the S6K1-HM^{T389A} structures were solved. In the form I S6K1-HM^{T389A} structure, as expected, the hydrogen bonds mediated by the phosphoryl group of pThr^{389} were lost (Figure 2F). Intriguingly, in the form II S6K1-HM^{T389A} structure, a strong electron density was detected at the phosphorylation site of Thr^{389}, which was interpreted as an SO_{4}^{2−} ion as the crystals were grown in a crystallization solution containing a high concentration (2 M) of lithium sulfate, yielding a reasonable average B-factor of 58.1 Å^{2}. The SO_{4}^{2−} ion occupies a position similar to the phosphoryl group of pThr^{389} and interacts with the side chain of Gln^{145} and the main-chain amide of Phe^{388}, and additionally forms a hydrogen bond with the main-chain amide of Ala^{389} (Figure 2G and Supplementary Figure S1). In both structures of S6K1-HM^{T389A}, the hydrophobic motif retains a similar conformation as in S6K1-HM^{WT} and S6K1-HM^{T389E}. In the structure of S6K1-HM^{PIF}, the PIF segment assumes a conformation resembling that of the hydrophobic motif in the other S6K1-HM structures and makes similar interactions with the N-lobe (Figure 2H). Detailed analysis and comparison of
Figure 2  Hydrophobic motif in the S6K1 structures

(A) Hydrophobic interactions of the hydrophobic motif (in yellow) with the N-lobe of the kinase domain (in light blue) in the S6K1-HMWT structure. The electrostatic potential surface of the hydrophobic groove of the N-lobe is shown with positive charges in blue, negative charges in red and neutral in white. The side chains of the residues of the hydrophobic motif are shown with stick models in yellow and those of the N-lobe in light blue. (B) Phosphorylation states of Thr389 of the hydrophobic motif in S6K1-HMWT and S6K1-HMT389A. The phosphorylation states of Thr389 of the freshly prepared proteins without treatment of the λ-protein phosphatase were examined by Western blot analysis using an antibody specifically targeting the phosphorylated Thr389 of S6K1. (C) Phosphorylation states of Thr229 of the activation loop in S6K1-HMWT with or without the treatment of PDK1. The phosphorylation states of Thr229 of the freshly prepared protein without the treatment of the λ-protein phosphatase were examined by Western blot analysis using an antibody specifically targeting the phosphorylated Thr229 of S6K1 before and after incubation with PDK1. (D–H) Hydrophilic interactions of the hydrophobic motif with the N-lobe in the structures of (D) S6K1-HMWT, (E) S6K1-HMWM, (F) form II S6K1-HMT389A, (G) form I S6K1-HMT389A and (H) S6K1-HMT389A respectively. The interacting residues Gln140 in strand β4 and Arg121 on helix αC are shown with stick models, and the hydrogen bonds are indicated with broken lines. The colour-coding is the same as in Figure 1(B). (I) Hydrophilic interactions of the hydrophobic motif with the N-lobe in the PKB(pS473)-p(N148pA-GSK3β) structure (PDB code 1O6L). PKB is coloured in grey, and the hydrophobic motif in green. The residues involved are shown with stick models, and the hydrogen bonds are indicated with broken lines. HM, hydrophobic motif.
these hydrophilic interactions in different S6K1-HM structures demonstrate that Gln\(^{40}\) on strand \(\beta_4\) and Arg\(^{121}\) on helix \(\alpha C\) play critical roles in the establishment of the hydrogen-bonding network between the N-lobe and the hydrophobic motif. In particular, in the absence of a negatively charged group conjugated close to Thr\(^{49}\), Arg\(^{121}\) plays a major role in the hydrophilic interactions (Figure 2F). In agreement with the structural data, mutation of Arg\(^{121}\) to alanine almost completely abolished the in vitro kinase activity of S6K1-HM (Supplementary Figure S3 at http://www.biochemj.org/bj/454/bj4540039add.htm).

It was proposed that the phospho-serine/threonine in the hydrophobic motif of the substrate kinases of PDK1 provides a binding site for the PIF-binding pocket of PDK1 [32,33]. Both in vitro and in vivo studies have shown that the PIF-binding pocket of PDK1 is required for the activation of S6K1, RSK (ribosomal S6 kinase) and SGK1 (serum- and glucocorticoid-induced protein kinase 1), but not PKB [31,33]. Intriguingly, the interactions between the hydrophobic motif and the N-lobe in the S6K1-HM structures are similar to those in the PKC\(\theta\) [34] and PKC\(\beta\)II [35] structures, except the orientations of the corresponding phosphoryl groups (downwards in S6K1 compared with upwards in PKCs), but display substantial differences from those in the PKB\(\beta^{\theta}\)–p[NH\(\\alpha\)ppA–GSK3\(\beta\) and PKB\(\beta^{\phi}\) structures [29] (Figure 2I). In the PKB structures, Ser\(^{201}\) occupies a position equivalent to that of Arg\(^{121}\) in S6K1 and forms a hydrogen bond with the main-chain carbonyl of Phe\(^{206}\) (equivalent to Phe\(^{388}\) in S6K1) and the immediately following Arg\(^{202}\) points its side chain upwards to form two hydrogen bonds with the side chain of Gln\(^{204}\) (Figure 2I), whereas in the S6K1 structures, the residue structurally equivalent to Gln\(^{204}\) of PKB is Gly\(^{378}\), which is unable to make such interactions due to the lack of a side chain (Figures 2D–2H). Given that the residues corresponding to Arg\(^{121}\) and Gly\(^{378}\) of S6K1 are conserved in the other substrate kinases of PKD1, including RSK and SGK1 [33], the results not only demonstrate the structural differences between S6K1 and PKB in helix \(\alpha C\) and the phosphorylation site of the hydrophobic motif, but also indicate that the divergence in the two sites confers specificity on the activation processes of the AGC family kinases.

It was proposed that, for PKB, phosphorylation of the hydrophobic motif stabilizes helix \(\alpha C\) which is critical for the concerted restructuring of the activation loop and the kinase bilobal structure [29,36]. Structural analyses of our S6K1 structures show that helix \(\alpha C\) is not involved in crystal packing in the S6K1-KD and S6K1-HM\(^{\phi}\) structures which belong to space group \(P_4_2_2_1\) and \(P_6_2\) respectively; however, it does participate in crystal packing in the other four S6K1-HM structures which all belong to space group \(P_3_2_1\), with Asn\(^{122}\) of helix \(\alpha C\) forming a hydrogen bond with Glu\(^{62}\) of helix \(\alpha D\) of a symmetry-related S6K1. As helix \(\alpha C\) is well defined in all of the structures, its stabilization is unlikely to be induced by the crystal packing. Phosphorylation of the hydrophobic motif has been shown to mediate the full activation of S6K1 [37] and, similarly, in the present study, helix \(\alpha C\) in the S6K1-HM\(^{\phi}\) structure has a lower average B-factor and displays more evident electron density than in the S6K1-KD structure (Table 1 and Supplementary Figure S4 at http://www.biochemj.org/bj/454/bj4540039add.htm), which could be partially accounted for by the interactions of the hydrophobic motif with helix \(\alpha C\), particularly with Arg\(^{121}\).

Inactive conformation of the active site and a zinc-finger-like motif of the activation loop

The active site in the S6K1 structures was analysed by comparing with the PKB\(^{\beta^{\theta}}\)–p[NH\(\\alpha\)ppA–GSK3\(\beta\) structure, given that the residues of PKB involved in the substrate recognition and binding and the catalysis are conserved in S6K1 [29,37]. As in the PKB\(^{\beta^{\theta}}\)–p[NH\(\\alpha\)ppA–GSK3\(\beta\) structure, in the S6K1 structures the conserved Phe\(^{31}\) of the DFG motif on the activation loop makes hydrophobic interactions with Ile\(^{21}\) and Leu\(^{24}\) at the C-terminus of helix \(\alpha C\), which is a signature of activated kinases; and concurrently, a salt bridge between Glu\(^{220}\) on helix \(\alpha C\) and Lys\(^{300}\) on strand \(\beta 3\) is formed, which is also a hallmark of activated kinases [38,39].

Although the DFG motif and helix \(\alpha C\) display features of activated kinases, further structural comparison indicates that the activation loop in the S6K1 structures used in the present study assumes an inactive conformation. In the activated kinases as represented by the PKB\(^{\beta^{\phi}}\)–p[NH\(\\alpha\)ppA–GSK3\(\beta\) structure, the activation loop stretches outwards with the N-terminus forming one short anti-parallel \(\beta\) sheet with the catalytic loop and the C-terminus forming another with the P+1 and P+2 residues of the substrate, which are called two anchor points [40,41]. Superimposition of the S6K1-KD and S6K1-HM\(^{\phi}\) structures in which the activation loop is completely defined on to the PKB\(^{\beta^{\phi}}\)–p[NH\(\\alpha\)ppA–GSK3\(\beta\) structure shows that residues 227–230 of the activation loop of S6K1 might impose steric hindrance on substrate binding, making it impossible to form the second anchor point (Figure 3A). In addition, although the activation loop is partially disordered in the other S6K1 structures, the conformation of the ordered regions is very similar to that of the corresponding segment in the S6K1-KD and S6K1-HM\(^{\phi}\) structures (Supplementary Figure S5 at http://www.biochemj.org/bj/454/bj4540039add.htm). Furthermore, in the previously reported pS6K1-KD structure [21], although Thr\(^{209}\) is phosphorylated and stabilized by Arg\(^{217}\) and Arg\(^{225}\), the activation loop also takes a similar conformation as in the S6K1-KD and S6K1-HM\(^{\phi}\) structures. Therefore the activation loop in all of the S6K1 structures reported so far assumes an inactive conformation.

Intriguingly, in the initial \(F_o − F_c\) map of S6K1-KD there was a strong positive electron density in the space surrounded by residues Cys\(^{317}\), His\(^{322}\), His\(^{328}\) and Cys\(^{331}\) of the activation loop. Anomalous diffraction data were collected to a 2.1 Å resolution at the zinc k-edge wavelength of 1.2826 Å, and the phased anomalous difference Fourier map revealed a strong electron density at the same site, indicating the binding of a Zn\(^{2+}\) ion (Table 1 and Figure 3B). In the S6K1-HM\(^{\phi}\), S6K1-HM\(^{\psi}\) and form II S6K1-HM\(^{310\alpha}\) structures, a Zn\(^{2+}\) ion is found to bind at the equivalent site co-ordinated by the same ligand residues. In the previously reported pS6K1-KD structure [21], although part of the activation loop, including His\(^{224}\), is disordered, a metal ion was also detected at a similar site and was interpreted as an Mn\(^{2+}\) ion, whereas a water molecule occupies the position corresponding to that of His\(^{224}\). Therefore formation of a zinc-finger-like motif and binding of a metal ion, most probably a Zn\(^{2+}\) ion, at the activation loop seem to be a common feature of the S6K1 structures irrespective of the bound inhibitor, the presence and phosphorylation state of the hydrophobic motif, or the phosphorylation state of the activation loop. Since no Zn\(^{2+}\) was supplemented in the protein or crystallization solution, the bound Zn\(^{2+}\) should be co-purified with the protein from the expression, suggesting that the zinc-finger-like structure in the activation loop was naturally formed in vitro. As the conformation of the zinc-finger-like motif with a bound Zn\(^{2+}\) appeared to cause steric hindrance with the substrate binding (Figure 3A), the formation of the zinc-finger-like structure and the accompanied Zn\(^{2+}\) binding might be associated with the inactive conformation of the activation loop. Sequence alignment showed that the residues forming the zinc-finger-like motif are highly conserved.
Structures of S6K1

Figure 3  Inactive conformations of the activation loop and the associated Zn\(^{2+}\) binding in the S6K1 structures

(A) Structural comparison of the S6K1-HMPIF and PKB\(^{S473D}–p[NH]ppA–GSK3\(\beta\) structures (PDB code 1O6L). The colour-coding of the S6K1-HMPIF and PKB\(^{S473D}–p[NH]ppA–GSK3\(\beta\) structures is the same as in Figures 2(H) and 2(I) respectively, and the activation loop and the bound GSK3\(\beta\) peptide in the PKB structure are coloured in green and blue respectively. (B) Binding of a Zn\(^{2+}\) ion by a zinc-finger-like motif of the activation loop. The anomalous difference Fourier map (blue mesh, contoured at 6\(\sigma\)) indicates that the metal ion bound at the activation loop is a Zn\(^{2+}\) ion. Cys\(^{217}\), His\(^{222}\) and His\(^{228}\) of the activation loop form a zinc-finger-like motif to co-ordinate the Zn\(^{2+}\) ion. The side chains of these residues are shown with stick models. The interactions between the residues and the Zn\(^{2+}\) ion are indicated with broken lines. (C and D) Sequence alignments of the activation loop of S6K1 (C) from different species and (D) with those of the other AGC family kinases. The positions corresponding to Cys\(^{217}\), His\(^{222}\) and His\(^{228}\) of S6K1 are marked with stars.

In all of our structures, the S6K1-specific inhibitor PF-4708671 is bound at the ATP-binding cleft with evident electron density. As shown in Figure 4(A), the 1′ amine of the imidazole ring is hydrogen-bonded to the side chain of Asp\(^{113}\), and the 3′ amine of the ethylpyrimidin moiety forms one hydrogen bond each with the main-chain carbonyl of Glu\(^{150}\) and the main-chain amide of Leu\(^{152}\). Also, two fluorine atoms of the trifluoromethyl moiety form two halogen bonds with the main-chain amide of Gly\(^{150}\). In addition to the hydrophilic interactions, the hydrophobic interactions between the ethyl of the ethylpyrimidin moiety and the side chain of Val\(^{182}\) may also contribute to the binding of PF-4708671 by S6K1. In the structures of S6K1-KD and S6K1-pKD bound with staurosporine, helix \(\alphaC\) is disordered [21], whereas in the S6K1 structures bound with PF-4708671, helix \(\alphaC\) becomes ordered, suggesting that the binding of PF-4708671 might play some role in the stabilization of helix \(\alphaC\). Comparison of our S6K1-KD structure with the previously reported S6K1-KD structure showed that in the presence of PF-4708671, the P-loop moved upwards with the Gly\(^{78}\) C\(^\alpha\) atom being displaced by 6.5 Å, accompanied by the formation of a local hydrogen-bonding network among the side chains of Tyr\(^{79}\) on the P-loop, Asp\(^{113}\) on helix \(\alphaC\) and His\(^{228}\) on the activation loop (Figure 4B). Although helix \(\alphaC\) is ordered

Inhibitor binding

It has long been appreciated that kinases play vital roles in many biological processes and their dysfunctions are related to various diseases, making them important targets for drug development [42]. Kinase inhibitors have been used in the treatment of various cancers, among which the BCR-Abl inhibitor imatinib is the most famous one [43]. For the AGC family kinases, dozens of inhibitors are currently undergoing clinical trials [37].
in our S6K1-KD structure, it displays a high degree of flexibility as the average B-factor of helix αC is approximately 44% higher than that of the protein (Table 1). In the other S6K1 structures, the presence of the hydrophobic motif further stabilizes helix αC as the average B-factor of helix αC is lower than that of the protein (Table 1). Therefore there seems to be interplay among the bound inhibitor, helix αC, the hydrophobic motif and the activation loop. It was reported previously that occupation of the ATP-binding pocket by inhibitors induces hyperphosphorylation of PKB [44] and priming phosphorylations of PKC [45]. Similarly, treatment of HEK (human embryonic kidney)-293 cells with PF-4708671 rapidly enhances phosphorylation of S6K1 at both Thr229 and Thr389 [28]. The conformational changes associated with the inhibitor binding, particularly the ordering of helix αC, and the interplay between the hydrophobic motif and the activation loop mediated by helix αC might contribute to the conformational regulation that enhances S6K1 phosphorylation by PF-4708671.

**Implications for S6K1 activation**

Full activation of S6K1 requires multiple phosphorylation events. It is widely accepted that phosphorylations of the AID and the turn motif occur first. However, the order of the following phosphorylations of the activation loop and the hydrophobic motif is still in debate. In a recent report on the study of a S6K1 truncate with deletion of the AID (S6K1ΔAID, residues 1–398), Keshwani et al. [46] claim that phosphorylation of Thr229 in the activation loop by PDK1 is a pre-requisite for phosphorylation of Thr389 in the hydrophobic motif by mTORC1. We noticed that a band was present in the Western blotting results of that paper [46] corresponding to phosphorylated Thr389 in the S6K1ΔAID protein before the treatment of mTORC1 and PDK1 (lanes 1 and 2 of Figure 4A in Keshwani et al. [46]). In addition, the kinase activity assay system was complicated with immunoprecipitated mTORC1 complex, and a critical control (treatment of PDK1 only) was missing. In the present study, the S6K1-HMWT protein produced by insect cells is strongly phosphorylated at Thr389 of the hydrophobic motif in the absence of Thr229 phosphorylation (Figures 1 and 2), indicating that phosphorylation of Thr229 is not essential for the phosphorylation of Thr389. As discussed above, our structural and biochemical data are in agreement with the previous in vivo studies which indicate that Thr389 phosphorylation is independent of Thr229 phosphorylation [30,31].

It was proposed that the phospho-serine/threonine residues of the hydrophobic motif in AGC family kinases may provide a docking site for PDK1 binding, which further promotes the phosphorylation of the activation loop [33]. The paradox arises from the reports that PDK1 is able to phosphorylate S6K1-KD [21] and the T389A S6K1ACT mutant [3] in which the hydrophobic motif is absent or the phosphorylation site is mutated. In the present study we also show that most of the interactions between the hydrophobic motif and the N-lobe are retained in the S6K1-HMWT structure (Figure 2D). However, although it is likely that the hydrophobic motif of the mutant might compensate for some of the activity of the wild-type kinase, there are no in vivo data available so far to support the phosphorylation of Thr229 in the full-length wild-type S6K1 without the phosphorylation of the hydrophobic motif. Moreover, although at a low level, the phosphorylation of Thr389 is detected in serum-deprived cells and, upon stimulation, the level of Thr389 phosphorylation increases rapidly at a rate comparable with that of AID phosphorylation [30]. Additionally, the effect of PF-4708671 on the rapid enhancement of S6K1 phosphorylations at Thr389 and Thr229 is dependent on the mTORC1 activity [28]. Taken together all of these results suggest that Thr389 phosphorylation might be a pre-requisite for Thr229 phosphorylation. Furthermore, the structural divergence of the hydrophobic motif in S6K1 and PKB might provide an explanation for the divergence in their dependence on PDK1 for activation (Figure 2).

Taken together, our structural and biochemical data are in agreement with the previous in vitro and in vivo studies [19,28,30,31,33] and demonstrate that the phosphorylation of the hydrophobic motif of S6K1 is independent of and probably precedes the phosphorylation of the activation loop. In particular, the hydrophobic motif, especially when phosphorylated, stabilizes helix αC and provides a docking site for PDK1, which might promote phosphorylation of the activation loop.

**AUTHOR CONTRIBUTION**

Jianchuan Wang carried out the structural and functional studies. Chen Zhong participated in the data analyses and discussion, and drafted the paper. Fang Wang and Fangfang Qu participated in the functional study. Jianping Ding conceived the study, participated in the experimental design, data analyses and discussion, and wrote the paper.

**ACKNOWLEDGEMENTS**

We thank the staff members at the Shanghai Synchrotron Radiation Facility (SSRF) of China for technical support in diffraction data collection and other members of our group for helpful discussion.
REFERENCES


SUPPLEMENTARY ONLINE DATA

Crystal structures of S6K1 provide insights into the regulation mechanism of S6K1 by the hydrophobic motif

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Figure S1 Stereo views of a simulated annealing omit Fo − Fc map (magenta, 1.5σ contour level) and a composite omit 2Fo − Fc map (blue, 1.0σ contour level) of the hydrophobic motif in the structures of (A) S6K1-HMWT, (B) S6K1-HMT389E, and (C) form I and (D) form II S6K1-HMT389A

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The crystal structures of S6K1-KD, S6K1-HMWT, S6K1-HMT389E, form I S6K1-HMT389A, form II S6K1-HMT389A and S6K1-HMWF, and that of S6K1-KD determined using the zinc anomalous diffraction data have been deposited with the RCSB PDB under accession codes 4L3J, 4L46, 4L45, 4L43, 4L44, 4L42 and 4L3L respectively.

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**Figure S2** Phosphorylation of Thr\(^{389}\) of the hydrophobic motif in the structure of S6K1-HM\(^{WT}\)

(A) Initial \(F_o - F_c\) (green, 4 \(\sigma\) contour level) and \(2F_o - F_c\) maps (blue, 1.0 \(\sigma\) contour level) indicate that the side chain of Thr\(^{389}\) is modified. (B) \(2F_o - F_c\) map (blue, 1.0 \(\sigma\) contour level) indicates that the phosphate group of the modified Thr\(^{389}\) fits the electron density very well.

**Figure S3** *In vitro* kinase activities of the S6K1-HM mutants


**Figure S4** Stabilization of helix \(\alpha\)C by the hydrophobic motif

\(2F_o - F_c\) maps of helix \(\alpha\)C (1.0 \(\sigma\) contour level) in the structures of (A) S6K1-KD and (B) S6K1-HM\(^{WT}\).

**Figure S5** Comparison of the conformations of the activation loop in the structures of S6K1-KD (red), S6K1-HM\(^{WT}\) (green), S6K1-HM\(^{TM\alpha\beta}\) (yellow), form I S6K1-HM\(^{TM\alpha\beta}\) (cyan), form II S6K1-HM\(^{TM\alpha\beta}\) (purple) and S6K1-HM\(^{PF}\) (magenta)

For positional reference, the Zn\(^{2+}\) ion bound by the zinc-finger-like motif of the activation loop in the S6K1-KD structure is shown with a grey sphere.