Auto-activation mechanism of the *Mycobacterium tuberculosis* PknB receptor Ser/Thr kinase

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Many Ser/Thr protein kinases are activated by autophosphorylation, but the mechanism of this process has not been defined. We determined the crystal structure of a mutant of the Ser/Thr kinase domain (KD) of the mycobacterial sensor kinase PknB in complex with an ATP competitive inhibitor and discovered features consistent with an activation complex. The complex formed an asymmetric dimer, with the G helix and the ordered activation loop of one KD in contact with the G helix of the other. The activation loop of this putative 'substrate' KD was disordered, with the ends positioned at the entrance to the partner KD active site. Single amino-acid substitutions in the G-helix interface reduced activation-loop phosphorylation, and multiple replacements abolished KD phosphorylation and kinase activation. Phosphorylation of an inactive mutant KD was reduced by G-helix substitutions in both active and inactive KDs, as predicted by the idea that the asymmetric dimer mimics a trans-autophosphorylation complex. These results support a model in which a structurally and functionally asymmetric, 'front-to-front' association mediates autophosphorylation of PknB and homologous kinases.

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Introduction

Tight regulation of Ser/Thr and Tyr protein kinases is required to control phospho-signaling pathways. The exquisite control of kinase activity is mediated by the convergence of multiple allosteric mechanisms that combine to establish kinase domain (KD) conformations (Huse and Kuriyan, 2002). These diverse activation signals include not only the binding of regulatory subunits but also the phosphorylation of the KD. In the Src family kinases, for example, the inactive form is maintained by a coupled network of interactions set up by phosphorylation of Tyr527, intramolecular binding of this pTyr residue by the Src SH2 domain, release of partner

proteins by the Src SH3 domain, association of the SH3 domain with an extended linker that engages the KD and dephosphorylation of a characteristic motif called the activation loop. In contrast, activation-loop phosphorylation in Src and many homologous kinases promotes the assembly of a platform on the edge of the kinase catalytic site that engages the regulatory C helix in an active conformation and mediates binding of protein substrates. In some kinases, this phosphorylation also relieves a steric blockade of the ATP-binding site mediated by the unphosphorylated activation loop (Huse and Kuriyan, 2002; Biondi and Nebreda, 2003). Despite the central importance of activation-loop phosphorylation in kinase activation, the recognition mechanisms that mediate this reaction are not well defined.

Here, we investigate the autophosphorylation mechanism of *Mycobacterium tuberculosis* (*Mtb*) PknB, one of 11 *Mtb* Ser/Thr protein kinases (STPKs) that are structurally and mechanistically similar to eukaryotic homologues (Av-Gay and Everett, 2000; Greenstein *et al.*, 2005). The primary sequences of the mycobacterial STPKs show up to 30% identity with those of human family members. The three-dimensional structures of the KDs of *Mtb* PknB, PknE and PknG resemble those of human kinases, with conserved sequence motifs having functionally analogous roles (Ortiz-Lombardia *et al.*, 2003; Young *et al.*, 2003; Gay *et al.*, 2006; Scherr *et al.*, 2007). By a mechanism with striking parallels to the activation of human double-stranded RNA-dependent protein kinase (PKR; Dar *et al.*, 2005; Dey *et al.*, 2005), the dephosphorylated *Mtb* PknD KD is activated by dimerization through an allosteric, 'back-to-back' interface in the N-terminal lobe (Greenstein *et al.*, 2007a). This apparently ancient allosteric interface shows high conservation among orthologous bacterial STPKs and promotes KD autophosphorylation, which activates the KDs for transphosphorylation of heterologous substrate proteins (Young *et al.*, 2003; Greenstein *et al.*, 2007a, b).

Similar to many eukaryotic homologues, the mycobacterial KDs are activated by autophosphorylation of specific residues in the 'activation loop', a conserved segment bordered in the amino-acid sequence by the DFG and APE tripeptides. The KDs from PknB, PknD, PknE and PknF, for example, show a conserved pattern of autophosphorylation on the activation loop and several other sites (Boitel *et al.*, 2003; Young *et al.*, 2003; Duran *et al.*, 2005). Dephosphorylation of the KDs invariably causes a large reduction in kinase activity *in vitro*. Single Ala replacements for Thr171 and Thr173 in the PknB activation loop reduced kinase activity on the non-cognate substrate myelin basic protein (MyBP) by 15- and 20-fold, respectively, and the double mutant was 300-fold less active (Boitel *et al.*, 2003). Phosphorylation of Thr171 and Thr173 was confirmed in PknB overexpressed in *Mtb*, suggesting that these sites are autophosphorylated *in vivo* (Kang *et al.*, 2005). Overexpression of the full-length PknD kinase in *Mtb* led simultaneously to the accumulation of phosphorylated PknD and to increased phosphorylation of cellular

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proteins (Greenstein *et al*, 2007a). Addition of the PknD inhibitor SP600125 reduced both autophosphorylation and transphosphorylation, supporting the idea that autophosphorylation activates the kinase *in vivo*. These studies established that the activation loops of several mycobacterial KDs are autophosphorylated similar to those of numerous eukaryotic kinases, and these phosphorylation reactions activate the bacterial STPKs *in vitro* and *in vivo*.

Here, we investigate the mechanisms of inhibitor recognition and autophosphorylation of *Mtb* STPKs. Because these kinases afford validated targets for new anti-infectives (Wehenkel *et al*, 2006), we sought to define the basis for *Mtb* PknD recognition by the eukaryotic protein kinase inhibitor, KT5720. To overcome our failure to cocrystallize the KD–inhibitor complex, we mutated the ATP-binding site of the Leu33Asp variant of PknB, which crystallizes readily, to closely resemble the active site of PknD. Here, we show that this ‘PknD surrogate’ acquired enhanced affinity for KT5720 and staurosporine and yielded cocrystals with KT5720 suitable for structural analysis. In addition to revealing the mechanism of KT5720 selectivity, this structure showed unanticipated features that suggested clues about the mechanism of KD autophosphorylation. The two PknD surrogate KDs formed an unusual offset dimer in which the activation loop of only one monomer was ordered, whereas that of the other monomer was disordered and positioned to extend into the active site of the other KD. Asymmetric contacts between G-helix residues in both KDs formed most of the dimer interface. Supporting the idea that this structure represents an autophosphorylation ES complex, activation-loop autophosphorylation occurred in an intermolecular reaction and mutations in helix G altered the efficiency of autophosphorylation. We used a new strategy to map the phosphorylation states of the activation loops of the PknB G-helix mutants, and the results suggested that autophosphorylation may occur in a preferred order. These results support a model in which two distinct interfaces mediate PknB activation and suggest that asymmetric G-helix recognition has a key function in the activation of STPKs in prokaryotes and eukaryotes.

Results

Structure of PknD surrogate bound to KT5720 revealed an asymmetric interface

To identify selective inhibitors of *Mtb* STPKs, we screened a variety of commercially available inhibitors of eukaryotic kinases. Staurosporine and the staurosporine analogue, KT5720, inhibited the purified KD of *Mtb* PknD with IC_{50} values in the mid-nanomolar range (Figure 1). Because efforts to crystallize the complex of KT5720 with the PknD KD failed, we made mutations in the PknB KD to mimic the ATP-binding site of PknD and to promote crystallization. With two mutations, Met145Leu and Met155Val, we converted the sequence of the PknB ATP-binding site to that of PknD (Figure 1A). Importantly, this PknD surrogate KD phosphorylated the non-cognate substrate MyBP with activity comparable to the wild-type (WT) PknB KD (data not shown) and gained the ability to bind KT5720 and staurosporine with IC_{50} values below 1 μ M (Figure 1A). To aid in crystallizing the KD–inhibitor complex, we also introduced the Leu33Asp mutation in the ‘back-to-back’ dimer interface

that mediates activation of the unphosphorylated KD. This single mutation reduces the strength of the back-to-back interface, preserves significant autophosphorylation activity and promotes crystallization of the PknB KD (T Noelle Lombana *et al*, unpublished results).

As expected, the back-to-back dimer found in three crystal forms of the WT PknB KD (Ortiz-Lombardia *et al*, 2003; Young *et al*, 2003; Wehenkel *et al*, 2006) was not formed in the crystals of the KT5720 complex of the Leu33Asp PknD surrogate (Table I). Instead, the surrogate KD crystallized as an unusual asymmetric dimer (Figure 1B). The monomers adopted similar conformations (backbone root mean square deviation (r.m.s.d.) = 0.43 Å), and each monomer bound KT5720 in the ATP-binding site. KT5720 bound in a position similar to that seen earlier in the complexes of staurosporine or its analogues with numerous eukaryotic protein kinases (Supplementary Figure 1) (Lawrie *et al*, 1997). The structure revealed that the Met145Leu and Met155Val mutations in the PknB ATP-binding site created space to accommodate the inhibitor (Figure 1C).

Unlike previous structures of the PknB KD (Ortiz-Lombardia *et al*, 2003; Young *et al*, 2003; Gay *et al*, 2006; Wehenkel *et al*, 2006), the activation loop of monomer B was largely visible in the electron density map (Figure 1D). The phosphorylated Thr171 made intramolecular contacts to the CD loop and intermolecular contacts to the opposing EF loop. In contrast, the activation loop of monomer A was disordered. Unexpectedly, monomers A and B were not related to each other by a simple two-fold rotation axis. Instead, a rotation of 132° and a translation of 4.4 Å were required to optimally superimpose the two KDs. This offset resulted from the zipper-like interdigitation of residues in helix G of each monomer. The offset placed the ends of the disordered activation loop of monomer A into the entrance to the kinase active site of monomer B.

This asymmetric dimer interface buried a total of 1492 Å², sequestering a number of hydrophobic residues from solvent (Figure 1E; Supplementary Figure 2). Val222, Ala225, Tyr226 and Val229 in the G helix and other residues, including Leu183 and Arg189, at the margins of the activation loop made the principal intermolecular contacts. The relative translation of the monomers placed identical residues in distinct environments. For instance, Val222 in monomer B makes contacts with Ala225 and Val229 in monomer A, whereas Val222 in monomer A makes contacts with Ala225, Ala180 and Leu183 in monomer B. Additional interactions included a van der Waals contact between Arg189 (B) and Tyr226 (A), a salt bridge between Asp219 (A) and Arg161 (B), and a possible cation– π interaction between Arg230 (A) and Tyr226 (B) (Figure 1F). Asymmetric backbone interactions also occur throughout the interface. Most contact residues are well conserved in PknB orthologues from 90 species (Figure 1G), supporting the hypothesis that the observed interactions are not simply the adventitious consequence of crystallization, but instead may mediate important functions.

Substitutions in PknB G-helix dimer alter autophosphorylation and kinase activation

To explore the function of the helix G interface, mutations were introduced into the WT PknB KD, and the effects on the rates of auto-activation and transphosphorylation were analysed. In these experiments, the purified KDs were first

Figure 1 Complex of PknD surrogate KD with KT5720. (A) Autoradiograms showing MyBP phosphorylation catalysed by the KDs (100 nM) of PknD, PknB and the Met145Leu/Met155Val double mutant of PknB. In this double mutant 'PknD surrogate', all the ATP contact residues except Tyr94 (bottom) matched those in PknD. Each set of three lanes shows the reactions without inhibitor and in the presence of 10 μ M staurosporine and 10 μ M KT5720, respectively. The double substitution to PknB allows KT5720 to bind at 10 μ M. (B) Ribbon diagram showing the KT5720-PknD surrogate KD structure. Monomers A (yellow) and B (blue) form an asymmetric dimer stabilized by contacts of the G helices (highlighted in transparent surface) and the activation loop (red) of monomer B. (C) Superposition of the active site of the KT5720-PknD surrogate KD (blue) with wild-type PknB (grey). The Met145Leu and Met155Val substitutions derived from PknD create room in the active site for the inhibitor. (D) Superposition of the two independent PknD surrogate monomers. The two monomers adopt similar overall structures (0.43 Å r.m.s.d.). The activation loop (residues 161–179) of monomer A was disordered, but this segment (red surface) except for residues 174–177 could be placed in the electron density in monomer B. (E) View of G-helix interface. The dimer is not two-fold symmetric, putting identical residues in the monomers in distinct environments. Van der Waals contacts include Val222 (B, blue)-Val229 (A, yellow), Ala225 (A)-Val222 (B), Leu183 (B)-Val222 (A) (not shown). (F) Interactions in the G-helix interface. Several backbone interactions occur between the EF loop (monomer A) and the activation loop (monomer B). Arg161 and Asp219 form an ion pair, and Leu183 also contacts the opposing G helix. The only phosphorylated residue with clear density, pThr171 (B), is exposed and contacts Thr217 (A) in the EF loop. (G) Sequence alignment of 90 PknB orthologues mapped onto surface of PknB. Conserved regions (red >95% identical, blue least conserved) include the ATP site, the putative protein-substrate-binding site, the N-lobe interface (not shown) and the G helix.

Table I Data collection and refinement statistics

	PknB L33D/M145L/ M155V-KT5720	PknB L33D/V222D- ADP
<i>Data collection</i>		
Space group	F4 ₃ 2	P 2 ₁ 2 ₁ 2 ₁
<i>Unit cell dimensions</i>		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	297.56, 297.56, 297.56	38.98, 51.54, 152.54
Resolution (Å) ^a	50–2.8 (2.9–2.8)	50–1.8 (1.86–1.8)
<i>R</i> _{merge}	9.5 (84.9)	5.6 (36.0)
<i>I</i> / <i>σI</i>	29.7/0.6 (2.7/0.6)	136.1/2.8 (6.4/1.7)
Completeness (%)	99.2 (99.9)	99.3 (93.4)
Redundancy	32.3 (32.7)	8.0 (6.4)
<i>Refinement</i>		
Resolution (Å)	39.8–2.80	37.8–1.80
Unique reflections	26 637	27 635
<i>R</i> _{work} / <i>R</i> _{free}	21.8/26.8	20.3/22.8
Atoms	4267	2326
Protein	4147	2111
Ligand/ion	90	31
Water	30	184
B-factors (overall)	25.5	27.4
<i>r.m.s.d.</i>		
Bond lengths (Å)	0.010	0.012
Bond angles (deg)	1.41	1.46
Backbone dihedral angles preferred/allowed (%)	91.0/9.0	93.5/6.5

^aValues in parentheses describe the highest resolution shell.

dephosphorylated using the *Mtb* Ser/Thr phosphatase, PstP, and autophosphorylation was measured using γ -³²P-ATP. Single amino-acid replacements in the hydrophobic G-helix interface reduced the rate of autophosphorylation *in vitro* (Figure 2A). Double substitutions such as Val222Asp/Val229Asp and Ala225Glu/Val229Asp further reduced the autophosphorylation rate. In contrast, replacement of Ala225 in helix G with Leu increased the autophosphorylation of the KD, supporting the idea that this site is buried during the autophosphorylation reaction.

To assess the autophosphorylation activities of the KDs in a cellular context, electrospray ionization mass spectrometry (ESI-MS) was used to define the populations of phosphates on the recombinant KDs purified from *Escherichia coli*. *E. coli* was used as a host organism because of the ease of analysis and the absence of any other STPKs capable of phosphorylating the KD variants. Single mutations such as Val222Asp and Val229Asp in the G-helix interface reduced the number of phosphates on the PknB KD (Table II). Moreover, these reductions in autophosphorylation were associated with decreased *in vitro* activity against MyBP (Figure 2B). Double or triple substitutions in helix G successively reduced phosphorylation of the KD during expression of the protein. These hypophosphorylated KD variants showed minimal autophosphorylation activity and, as anticipated from the deficit of activating phosphoryl groups, also had negligible activity on MyBP *in vitro*. The Ala225Glu/Val229Asp PknB KD, for example, was predominantly unphosphorylated and the Leu183Asp/Val222Asp/Val229Asp KD was 90–95% unphosphorylated. Ala225Glu/Val229Asp phosphorylated MyBP 30-fold more slowly than the WT KD. The interface triple mutant, Leu183Asp/Val222Asp/Val229Asp, showed negligible autophosphorylation in *E. coli* and *in vitro* kinase activity

on MyBP. Paralleling the results of the *in vitro* activation of the dephosphorylated KDs, however, the Ala225Leu variant was hyperphosphorylated in *E. coli* (4–7 phosphates versus 3–6 phosphates for WT PknB; Table II). The hyperphosphorylated Ala225Leu variant was more active than the WT KD in MyBP transphosphorylation (Figure 2). These results suggested that PknB autophosphorylation is mediated by the G-helix interface.

Interestingly, mutations in the G-helix interface synergized with the Leu33Asp mutation in the previously identified N-lobe interface that mediates activation of the unphosphorylated kinase (Young *et al*, 2003; Greenstein *et al*, 2007a). PknB orthologues in over 90 bacterial species contain Leu33 in this ‘back-to-back’ interface. When we combined the Leu33Asp mutation with the moderately attenuating Val222Asp mutation in the G-helix interface, the double mutant KD showed negligible autophosphorylation and MyBP transphosphorylation *in vitro* (Figure 2). Moreover, this double mutant expressed in *E. coli* lacked phosphoryl groups (Table II). This synergy revealed that both helix G in the C-lobe and the back-to-back, allosteric interface containing Leu33 in the N-lobe contribute to the activation process.

Site-specific activation-loop phosphorylation

To define the products of PknB autophosphorylation, tandem mass spectrometry (MS/MS) was used to map the phosphorylation sites on the activation loops of the variant KDs. Because traditional MS/MS results in neutral losses of phosphates, mapping the phosphorylated residues is problematic, especially for the activation loop of PknB, where up to four phosphates are present on a tryptic peptide of 28 residues (Boitel *et al*, 2003; Young *et al*, 2003; Duran *et al*, 2005). An alternative strategy has been reported in which the phosphorylated residues are chemically modified to a lysine mimetic to enable tryptic or LysC digestion at the modified site (Knight *et al*, 2003). We used a variation of this bottom-up method in which the PknB KD was digested with trypsin and the peptides were chemically modified on pSer and pThr residues. As the lysine mimetic is stable under typical ESI/MS/MS conditions, the positions of the modifications were deduced unambiguously. This top-down approach has advantages over digesting the modified sites, as each phosphorylated residue is mapped within a single fragment that may contain additional phospho-residues. This chemical context provides additional information about the potential coupling of phosphoryl modifications.

As reported earlier, 2–4 modifications were observed in the activation loop of WT PknB 1–308 (Figure 3) (Boitel *et al*, 2003; Young *et al*, 2003; Duran *et al*, 2005). The earlier reported major phosphorylation sites, Thr171 and Thr173, were identified, and two minor sites were found at Ser169 and Thr179. The partial phosphorylation of Thr179 has not been reported earlier. Consistent with earlier data (Boitel *et al*, 2003), we found that the Ser169Ala mutant produced minimal effects on autophosphorylation and MyBP phosphorylation (data not shown).

To explore the site-specific effects of G-helix mutations, we mapped the phosphorylation sites on the PknB KD variants purified from *E. coli* (Figure 4) and measured the activity of the KD variants *in vitro*. Unexpectedly, each G-helix mutation not only reduced the autophosphorylation activity (Figure 2A) but also preferentially reduced phosphorylation

Figure 2 Helix G substitutions reduced autophosphorylation of the PknB KD. **(A)** Autoradiogram and plot showing autophosphorylation activities of dephosphorylated PknB KD variants. The Val222Asp, Ala225Glu, Ala225Leu and Val229Asp substitutions (cyan) were made in the G helix. The acidic substitutions of residues 222, 225 and 229 individually reduced autophosphorylation 3.7-, 4.3- and 4.5-fold after 15 min, respectively (values are tabulated in Supplementary Figure 3). The Ala225Leu PknB KD showed 2.4-fold increased autophosphorylation activity at 15 min compared with the wild-type KD. The Leu33Asp substitution (orange) in the conserved regulatory N-lobe interface on the opposite side of the KD and the Val222Asp G-helix substitution showed synergistic effects. **(B)** Relative activities of the PknB 1–308 KD variant populations purified from *E. coli*. Activities were determined by measuring *in vitro* phosphorylation of MyBP. The relative transphosphorylation activities of each variant correlated well with the degree of intrinsic autophosphorylation (Table II) and the autophosphorylation activity observed *in vitro*.

of specific sites. The activation loop of the Val229Asp variant, for example, was phosphorylated nearly exclusively at Thr171 and Thr173, with fewer modifications at other sites in the activation loop. In comparison to WT PknB 1–308, less triply modified activation loop was observed by LC/MS, and no tetra-phosphopeptide was detected (Supplementary Figure 4). Helix-G double mutants, such as Ala225Glu/Val229Asp, which showed reduced autophosphorylation and MyBP phosphorylation activities (Figure 2), were partially phosphorylated at only one site, Thr171. Interestingly, for variants where only one modification was detected, Thr171 was exclusively modified. When two modifications were observed in the activation loop, Thr171 and Thr173 were modified exclusively. In the case of three or more activation-loop phosphorylations, Thr179 and/or Ser169 were the additional sites of modification. For the WT KD, Ser169 was the preferred third phosphorylation site detected. For Val229Asp PknB, Thr179 was the predominant third modification site.

Structure of the PknB Leu33Asp/Val222Asp variant bound to ADP

To test whether substitutions in the G-helix interface inhibit PknB activation directly by weakening the asymmetric KD association or indirectly through long-range conformational changes, we determined the 1.8-Å resolution crystal structure of the unphosphorylated Val222Asp/Leu33Asp PknB KD bound to ADP. This inactive, double-mutant protein crystallized in a distinct habit in which neither the G-helix nor the N-lobe surface containing Asp33 made extensive intermolecular contacts (Figure 5). The Val222Asp/Leu33Asp PknB KD was completely unphosphorylated when purified from *E. coli* (Table II), and typical of an unphosphorylated KD, it had negligible phosphotransfer activity on MyBP (Figure 2B). Nonetheless, the unphosphorylated KD bound ADP at the concentration (3 mM) used in the crystallization. The structure of the double interface mutant PknB KD resembled that of the PknB Leu33Asp variant bound to ATP γ S (backbone r.m.s.d. = 0.31 Å; T Noelle Lombana, Nathaniel Echols,

unpublished results). No electron density was observed for the activation loop in either structure. The lack of a conformational perturbation associated with the Val222Asp mutation suggested that changes in the G helix exert an effect not by altering the kinase conformation, but rather by directly inhibiting helix-G interactions.

Table II G-helix substitutions alter the extent of PknB KD phosphorylation

PknB KD	Phosphates (major species)
Wild type	3–6 (5)
Val222Asp	2–5 (4)
Ala225Leu	4–7 (5, 6)
Val229Asp	2–5 (3, 4)
Val222Asp/Val229Asp	0–2 (1)
Ala225Glu	2–4 (3)
Ala225Glu/Val229Asp	0–2 (0)
Leu183Asp/Val222Asp/Val229Asp ^a	0–1 (0)
Leu33Asp	2–3 (3)
Leu33Asp/Val222Asp	0

^aPredominant species (90–95%) was unphosphorylated.

Phosphorylation states were determined from the mass spectra of intact PknB variants expressed in *E. coli*. The predominant species in the LC-MS spectrum are shown in parentheses. Of the mutants tested, rates of autophosphorylation and activity on MyBP correlate with the number of phosphates present on each PknB variant. The mutations were made in the G-helix except for Leu33Asp, which disrupts the conserved N-lobe dimer interface. Up to four phosphates were found in the wild-type PknB activation loop, and two phosphates have been mapped to the C-terminal region of the kinase domain (Boitel *et al*, 2003; Young *et al*, 2003; Duran *et al*, 2005).

Helix G mediates intermolecular phosphorylation of PknB

The hypothesis that the KT5720–KD dimer represents an autophosphorylation complex predicts that PknB activates through an intermolecular interaction involving the G helix of both the substrate and enzymatic KDs. PknB has been observed earlier to autophosphorylate *in trans* (Kang *et al*, 2005). We confirmed this result by measuring phosphorylation of the inactive Asp138Asn PknB KD by the WT protein. Because Asp138 functions as the catalytic base in the phosphotransfer reaction, the Asp138Asn mutation inactivates the enzyme. WT (1–308) and Asp138Asn (1–279) PknB KD variants of different lengths were used to distinguish the phosphorylated products using SDS–PAGE. In a phosphoryl transfer assay, the inactive Asp138Asn variant was only phosphorylated in the presence of the WT KD, indicating that the modification(s) occurred in an intermolecular reaction (Figure 6). To test whether this reaction was mediated by helix G, the Val229Asp replacement was engineered into the inactive PknB substrate. The mutation inhibited phosphorylation by the WT KD 6.3 (± 1.1)-fold at 1 μ M substrate KD. The same trend also was observed when the Val229Asp replacement was made in the active PknB KD (10-fold reduction in activity). Combining the Val229Asp mutation in both the active PknB KD and the inactive substrate KD produced a larger reduction (~ 50 -fold) in substrate phosphorylation (Figure 6). Similarly, the Ala225Glu and Leu183Asp substitutions in the G-helix interface of the substrate KD reduced phosphorylation by the WT PknB KD by 43 (± 3.5)- and 8.4 (± 0.9)-fold, respectively (Supplementary Figure 5). These

Figure 3 Tandem mass spectrum (MS/MS) showing modifications of the 28-residue activation-loop peptide. (A) Mass spectrum showing ions formed by electrospray ionization. The inset shows detail for the $(M + 2H)^{2+}$ ions of the activation-loop fragment of PknB (residues 162–189) with two, three and four modifications. The asterisks denote ions that are 18 Da lower in mass, presumably due to losses of water molecules. (B) Quadrupole isolation mass spectrum of the $(M + 2H)^{2+}$ ion with three modifications. (C) MS/MS spectrum of the $(M + 2H)^{2+}$ ion with three modifications showing fragment ions formed by collisionally activated dissociation.

Figure 4 MS/MS spectra and cleavage maps of the $(M + 2H)^{2+}$ ions of the activation-loop fragment of PknB (residues 162–189) with (A) two, (B) three and (C) four modifications. Chemical modification increases the mass of each phospho-residue by 60 Da. The asterisks in the spectra denote residual precursor ion and the arrowheads in the cleavage maps denote modification sites (bold). The MS/MS spectrum of the ion with three modifications (B) indicates a mixture of isoforms with $\sim 80\%$ having the third modification on Ser169 and $\sim 20\%$ on Thr179.

results demonstrated directly that mutations in the helix-G interfaces in both the active and inactive KDs inhibited autophosphorylation *in trans*.

Analogous experiments were performed to investigate the contribution by the N-lobe interface to intermolecular autophosphorylation. The Leu33Asp mutation reduces autophosphorylation (Figure 2), reflecting the role of the back-to-back, N-lobe interface in activating the KD (Greenstein *et al*, 2007a). When the Leu33Asp mutation was engineered into the inactive Asp138Asn substrate, however, efficient phosphorylation by active WT PknB was observed (Figure 6B). This phosphorylation was comparable to the level observed for the substrate containing the Asp138Asn mutation alone. These results suggest that the active, dimeric KD can efficiently phosphorylate a monomeric KD. Thus, N-lobe dimerization is important for activating the unphosphorylated KD, but is not required in the substrate KD.

Discussion

Earlier studies using synthetic peptides identified the motif Thr-Gln-X-hydrophobic-hydrophobic as favouring phosphorylation by PknB and supported the idea that the Thr-Gln-Thr-Ala-Ala sequence in the PknB activation loop functions as a

local determinant of autophosphorylation (Kang *et al*, 2005). In contrast, here we identified distinct structural recognition interfaces that mediate autophosphorylation of the WT PknB KD. The structure of the KT5720 complex of the PknD surrogate KD suggested not only that the larger volume of the PknD ATP-binding site (compared with that of PknB) mediates inhibitor selectivity but also revealed an unprecedented asymmetric dimer involving offset G-helix contacts. Although the inhibitor occupied the nucleotide-binding site in both monomers, this asymmetric dimer stabilized in the crystals displayed several features expected in an authentic kinase ES or EP complex.

Characteristic of the active conformation of the KD (Huse and Kuriyan, 2002), the activation loop of one monomer was largely ordered in the dimer interface. The conformation of this loop resembled that seen in activated PKA (Madhusudan *et al*, 2002) and other active eukaryotic protein kinases (Huse and Kuriyan, 2002). In contrast, the other KD monomer resembled a protein substrate. In particular, a segment of 16 residues in the activation loop was disordered with the ends positioned at the mouth of the active site of the 'enzymatic' monomer (Figure 1B). A disordered phosphorylation target site was also identified earlier in the protein substrate in the complex of the eIF2 α bound to the cognate kinase, PKR (Dar

Figure 5 The Val222Asp substitution causes minimal changes in the PknB KD structure. Leu33Asp PknB KD bound to ATP γ S (light pink) superimposed on the Leu33Asp/Val222Asp PknB KD bound to ADP (dark blue). Spheres show the locations of Leu33Asp in the N-lobe and Val222 in the G-helix.

et al, 2005). Thus, the specific asymmetry of the KT5720–KD complex reconciles the apparently contradictory requirements for the activation loop to fold in an active kinase and to unfold to position the multiple target Thr (and Ser) residues in the partner kinase active site.

The asymmetry of the PknB KD dimer is consistent with distinct roles for the two kinase molecules. In this sense, the PknB KD structure resolves a paradox apparent in studies of p21-activated kinase-2 (PAK2) autophosphorylation inspired by the crystal structure of the activated homologue, PAK1 (Pirrucello *et al*, 2006). Similar to the PknD surrogate KD complex, the PAK1 KD formed a front-to-front dimer mediated primarily by hydrophobic contacts of the G (and E) helices. Unlike the PknD surrogate KD, however, the PAK1 KDs were related by a crystallographic two-fold rotation axis that places the G helices in register, and the activation loops of both molecules were ordered and oriented away from the active site of the partner KD. Even though this presumptive autophosphorylation complex required structural remodeling to place the phosphorylation site of one PAK1 monomer in the partner active site, amino-acid substitutions in the G-helix interface reduced intermolecular autophosphorylation in the homologous KD of PAK2 (Pirrucello *et al*, 2006). Transient unfolding of one activation loop was proposed to resolve this paradox. Alternatively, if PAK2 formed

Figure 6 Intermolecular PknB autophosphorylation involves G-helix contacts. Autoradiograms showing intermolecular phosphorylation of inactive Asp138Asn PknB (1–279) KD substrates by active PknB (1–308) KD variants. Corresponding SDS–PAGE gels are shown as loading controls. **(A)** The Val229Asp mutation in helix G of both the active kinase (lanes 6–8) and inactive substrate KD (lanes 5 and 8) reduced trans-autophosphorylation of the Asp138Asn KD substrate. **(B)** The Leu33Asp mutation reduced intermolecular autophosphorylation (by 60%) when engineered into the active kinase domain. However, PknB (1–308) efficiently phosphorylated both the PknB (1–279) Asp138Asn KD substrate with and without the Leu33Asp mutation.

an asymmetric complex in solution akin to the offset PknD surrogate dimer, the target activation loop would be positioned to access the partner kinase active site and G-helix substitutions would reduce autophosphorylation. Whether the functional PAK2 interaction is equivalent to or distinct from that of the PknB KD, analogous G-helix substitutions in the two KDs cause qualitatively similar reductions in autophosphorylation. Such functional conservation in distant homologues predicts that the G helix may mediate activation-loop autophosphorylation in prokaryotic and eukaryotic STPK family members.

Recently, symmetric dimer interfaces distinct from the E- and G-helix contacts of the PAK KDs were observed in the structures of checkpoint kinase 2 (CHK2), STE20-like kinase (SLK), lymphocyte-originated kinase (LOK) and death-associated protein kinase 3 (DAPK3) (Oliver *et al*, 2006; Pike *et al*, 2008). Each of these KDs forms homodimers detectable in solution, and they crystallize as dimers with swapped activation loops projecting into the active site of the neighbouring monomer (Oliver *et al*, 2006; Pike *et al*, 2008). N-lobe contacts through the C helix occur in some of these dimers, making them fundamentally different from the associations seen for either PAK1 or PknB. Similar to the PAK1 dimer, however, the activation-loop exchange complexes are generally two-fold symmetric, and structural fluctuations would be required to reposition the target residue(s) for phosphoryl transfer by the catalytic machinery of the partner KD. In contrast, one activation loop (monomer A) in the PknB KD pair is poised to access the opposing active site and the disorder of this activation-loop segment recapitulates the mechanism by which the Ser51 phosphorylation site of eIF2 α accesses the cognate kinase, PKR (Dar *et al*, 2005).

Mutational analysis of the WT PknB KD supported the idea that the G helix mediates KD autophosphorylation. Substitutions of conserved, external residues in every turn of the G helix reduced autophosphorylation, and multiple G-helix changes eliminated autophosphorylation (Figure 2 and Table II). Several lines of evidence suggest that these reductions were due to direct disruption of the kinase interactions rather than indirect effects of conformational changes in the active KD. First, mutations in the G helix of an inactive substrate KD reduced intermolecular autophosphorylation by the WT PknB KD (Figure 6, Supplementary Figure 5). Second, the Ala225Leu substitution in the G helix increased autophosphorylation, whereas the Ala225Glu substitution slowed activation (Figure 2). This pattern is consistent with burial of residue 225 on the edge of the G-helix interface. Third, the Val222Asp mutation in the PknB G helix caused only small structural changes in the Leu33Asp variant and directly eliminated G-helix contacts in the crystals (Figure 5). Fourth, the Val229Asp substitution in the G helix of the active KD further reduced transphosphorylation of the inactive Val229Asp/Asp138Asn substrate (Figure 6). These results support the idea that the G helix mediates PknB interactions on both sides of the autophosphorylation interface, as seen in the asymmetric KT5720–PknD-surrogate structure.

To analyse autophosphorylation in a cellular context that lacks other STPKs, we mapped the activation-loop phosphates in the mutant KDs purified from *E. coli*. A chemical modification strategy (Knight *et al*, 2003) was adapted to define multiply phosphorylated species. The G-helix variants revealed cumulative effects that followed a particularly

Table III MS/MS measurements of the chemically modified PknB activation loop

Variant	Number of modifications	Location of modification
WT	2	Thr171, Thr173
WT	3	Thr171, Thr173, Thr179 or Ser169 ^a
WT	4	Thr171, Thr173, Thr179, Ser169
Val229Asp	2	Thr171, Thr173
Val229Asp	3	Thr171, Thr173, Thr179 ^a or Ser169
Ala225Glu/ Val229Asp	1	Thr171
Val222Asp/ Val229Asp	1	Thr171

^aPredominant site of third modification.

Substitutions in the G helix decreased phosphorylation of the activation loop at preferential sites.

simple pattern. Single mutations in the G helix reduced total autophosphorylation, and combining these substitutions caused larger reductions (Table II). One double and one triple G-helix mutant were >90% unphosphorylated when purified from *E. coli*. The Thr-Gln-Thr motif was retained in the activation loop of all of these G-helix variants, indicating that this local sequence motif in the activation loop is insufficient by itself to mediate KD autophosphorylation. Instead, the G-helix variants showed that this structural interface can influence directly or indirectly all autophosphorylation of the PknB KD.

The four activation-loop phosphorylation sites showed different sensitivities to G-helix mutations (Table III). Mutations first eliminated Ser169 and Thr179 phosphorylation, followed by Thr173. Thr171 was the last activation-loop phosphorylation site to be eliminated by G-helix substitutions (Table III). These effects were recapitulated in the pattern of phosphorylation of the WT KD, in which Thr171 and Thr173 were fully phosphorylated, whereas Ser169 and Thr179 were partially phosphorylated. Our results suggest that sites in the activation loop may be phosphorylated in a specific order. In the simplest interpretation of the results, the autophosphorylation sequence is Thr171 followed by Thr173 followed by the other two activation-loop sites.

Only the Thr171 and Thr173 phosphorylations have been reported to occur *in vivo* (Kang *et al*, 2005) and to mediate activation *in vitro* (Boitel *et al*, 2003). Thus, the fraction of the population doubly phosphorylated on Thr171 and Thr173 may control specific activity. However, PknB variants with single G-helix replacements were doubly phosphorylated on Thr171 and Thr173 regardless of whether they were more (Ala225Leu) or less (Val222Asp, Ala225Glu and Val229Asp) active in phosphorylating MyBP than the WT KD. Moreover, the hypophosphorylated G-helix mutants were less active on MyBP than the WT PknB KD, and the Ala225Leu mutant is more phosphorylated and more active. Thus, it is possible that additional phosphorylation sites may contribute to full kinase activity. Skepticism is needed, however, in considering functional roles for the minor phosphorylation sites in the activation loop. The Ser169Ala mutation does not alter activity (Boitel *et al*, 2003), and Thr179 is analogous to Thr201 in PKA, which has a key function in positioning the catalytic loop in the active conformation of the kinase (Pirrucello *et al*, 2006). Alternatively, the parallel effects of G-helix mutations on KD and MyBP phosphorylation could

Figure 7 Model of the PknB intermolecular autophosphorylation complex. Active, wild-type PknB KD dimer (grey) superimposed on the KT5720–PknD surrogate complex (monomers A and B represented by yellow and blue, respectively). This ‘two-on-one’ model is consistent with mutational effects and the structural organization of the N-lobe and G-helix (C-lobe) interfaces. The wild-type N-lobe dimer superimposes on each monomer in the asymmetric C-lobe dimer with an average r.m.s.d. of 1.59 Å with no overlapping contacts. For the putative substrate monomer (yellow), the disordered activation loop is modelled (grey surface). The ordered activation loop of the putative active monomer (blue) is highlighted in red. A KD monomer is phosphorylated efficiently, but the substrate KD may also be phosphorylated as a dimer. Because the monomeric KD is not directly activated by an extracellular cue and the phosphorylated monomer is active, phosphorylation can amplify the signal.

be explained if this structural interface mediated direct substrate interactions or controlled KD specific activity in auto- and trans-phosphorylation reactions. Additional studies are needed to distinguish these models.

The synergistic effects of mutations in the G helix and the back of the N-lobe also indicate that these distinct interfaces mediate PknB autophosphorylation. The Leu33Asp mutation in the N-lobe dimer interface and the Val229Asp mutation in the G helix individually reduced PknB KD autophosphorylation from 3–6 phosphates on the WT KD to an average of 3–4 phosphates, respectively (Table II). In contrast, the combination of these two substitutions, which are located >48 Å apart in the PknB KD structure, eliminated autophosphorylation (Table II). Interestingly, when the same changes were made only in the inactive KD substrates, the Val229Asp substitution was inhibitory, whereas the Leu33Asp replacement was not. These distinct effects suggest that, although the N-lobe interactions are essential to activate the unphosphorylated KD (Greenstein *et al*, 2007a), the G-helix contacts mediate recognition of monomeric and dimeric KD substrates. Consistent with this model, the superposition of monomers from the WT and PknD surrogate KD structures produces no steric clashes (Figure 7). Because the phosphorylated kinase can be fully active as a monomer (Greenstein *et al*, 2007a), phosphorylation of the monomer activation loop provides a mechanism to amplify the activating signal.

The PknD surrogate structure, however, does not readily explain how reported phosphorylation sites (Thr294, Ser295 and Thr309) outside the activation loop can access the catalytic site (Boitel *et al*, 2003; Young *et al*, 2003; Duran *et al*, 2005). Global relative reorientation of the KDs would seem to be necessary for these sites in the juxtamembrane segment C-terminal to the KD to access the kinase active site.

Considering the many combinations of phosphorylation states (Boitel *et al*, 2003; Young *et al*, 2003; Duran *et al*, 2005), the sequential autophosphorylation we observed in PknB may afford a mechanism to regulate the activities of prokaryotic STPKs. Our studies implicate two interfaces with different roles in this process—the conserved N-lobe interface orients the catalytic sites in the dimer away from each other and activates the unphosphorylated KDs by an allosteric mechanism (Greenstein *et al*, 2007a), whereas the G-helix interface mediates intermolecular activation-loop autophosphorylation.

Materials and methods

Protein expression and purification

PknB (1–308) with a thrombin-cleavable, N-terminal His₆ tag was expressed and purified as described (Young *et al*, 2003). KD variants were made by site-directed mutagenesis using the QuikChange method (Stratagene). WT and mutant KDs were purified using Ni-NTA chromatography (GE Healthcare) using 25 mM Tris pH 8.0, 300 mM NaCl, 1 mM TCEP, 10% glycerol, 10 mM imidazole as wash buffer and proteins were eluted in wash buffer containing 300 mM imidazole. To remove the His tag, 10 U thrombin (Sigma) was added, and the reaction was dialysed against 25 mM Tris pH 8.0, 200 mM NaCl, 1 mM TCEP, 5% glycerol, 10 mM imidazole and 1 mM CaCl₂. This solution was passed over a Ni-NTA affinity column, and the flow-through was dialysed against 25 mM Tris pH 7.5, 125 mM NaCl, 1 mM TCEP and 5% glycerol. Glycerol was added to 40%, and the samples were flash frozen in liquid N₂ and stored at –80°C for 5–15 days before use in kinase activity assays. For crystallization, proteins were further purified on a MonoQ column (GE Healthcare) eluted using a NaCl gradient. Proteins were concentrated to >10 mg/ml and dialysed against 20 mM Tris pH 7.5, 75 mM NaCl and 1 mM TCEP.

Crystallization and structure determination

KT5720:PknD-surrogate. The inhibitor complex of PknB Leu33Asp/Met145Leu/Met155Val KD was crystallized using the vapour diffusion method. A PknD surrogate solution (10 mg/ml) containing 400 μM KT5720 (Calbiochem) was mixed with an equal volume of 100 mM Tris pH 8.5, 25% PEG 3350 and 200 mM ammonium sulphate and equilibrated against precipitant at 4°C. Crystals that formed in 1–3 weeks were harvested by transferring to mother liquor containing 37% PEG 3350 and flash frozen in liquid N₂. X-ray data were collected at the Lawrence Berkeley National Laboratory Advanced Light Source Beamline 8.3.1 at 100°K and 11111 eV and indexed using ELVES (Holton and Alber, 2004). Data were processed and scaled using HKL2000 (Otwinowski and Minor, 1997). Molecular replacement was carried out using Phaser with the structure of the Leu33Asp PknB KD bound to ATP_γS as the search model (Potterton *et al*, 2003; Storoni *et al*, 2004). Two molecules were located in the asymmetric unit. Models were modified using Coot and refined with REFMAC (Murshudov *et al*, 1997; Emsley and Cowtan, 2004). Structures were superimposed using SSM-based methods (Krissinel and Henrick, 2004). Coordinates for KT5720 were generated using the PRODRG server (Schüttelkopf and van Aalten, 2004). The quality of the models was evaluated using PROCHECK (Laskowski *et al*, 1993). Structures were displayed using PYMOL (DeLano, 2002). Solvent-accessible surface area was calculated using Arealmol (Potterton *et al*, 2003). Surface conservation mapping was performed using sequence alignments generated by ClustalW (Thompson *et al*, 1994).

PknB Leu33Asp/Val222Asp:ADP. The Leu33Asp/Val222Asp PknB KD was expressed and purified as described earlier for WT PknB 1–308 (Young *et al*, 2003). Crystals were grown from 20% PEG 3000, 3 mM ADP, 0.2 M NaF and 100 mM Tris pH 8.5. Crystals were flash frozen in 30% PEG 3000, 0.2 M NaF, 100 mM Tris pH 8.5 and 5% glycerol. Data collection, phasing and refinement were carried out as described above for the PknD surrogate structure.

Kinase activity assays

Autophosphorylation assays were carried out using completely dephosphorylated, purified WT and mutant PknB KDs. The KDs were dephosphorylated using a 1:10 ratio of PstP phosphatase (with a non-cleavable N-term His₆ tag) to PknB KD overnight at 25°C in 20 mM Tris pH 7.5, 25 mM NaCl, 0.5 mM TCEP and 1 mM MnCl₂. PstP and PknB were separated using Ni-NTA chromatography and dephosphorylation of PknB was verified using SDS-PAGE (Invitrogen) and intact MS. Kinase assays were carried out at 25°C in 20 mM Tris pH 7.5, 25 mM NaCl, 0.1 mM MgCl₂, 2 mM MnCl₂, 0.5 mM TCEP using 2 µM dephosphorylated KD. Reactions were initiated by adding 50 µM ATP containing γ-³²P-ATP (0.2 µCi/µl). Reactions were quenched using SDS-PAGE loading buffer containing 20 mM EDTA.

Transphosphorylation assays were performed in the same buffer by incubating active PknB KD (500 nM) with 1 mg/ml MyBP or active PknB (1 µM) with the Asp138Asn PknB 1–279 KD (1 µM) in 100 µM ATP with γ-³²P-ATP added (0.2 µCi/µl). KD concentrations were determined by measuring the absorbance at 280 nm (Perkin Elmer) and verified by SDS-PAGE analysis. Reaction products were quenched at 5 min. For both auto- and transphosphorylation assays, products were separated using SDS-PAGE and blotted onto a PVDF membrane (Perkin Elmer). Dried membranes were exposed to a Storage Phospho Screen (Molecular Dynamics), imaged using a Molecular Dynamics Typhoon scanner and analysed quantitatively using Imagequant software. Values reported are from triplicate sets of data.

Chemical modification of phosphopeptides and mass spectrometry

PknB phosphopeptides were prepared by tryptic digestion of purified PknB (25 mM Tris, pH 8.5) followed by enrichment of phosphopeptides through Fe-IMAC chromatography using Chelating Sepharose Fastflow (GE Healthcare) charged with FeCl₃ (Knight *et al*, 2003). Phosphopeptides were eluted in 100 mM Na₂HPO₄ and desalted using Spec C18 desalting tips (Varian). Chemical modification of pThr and pSer residues using 2-aminoethanethiol was performed as described (Knight *et al*, 2003).

Mass spectra of chemically modified PknB peptides were acquired using a quadrupole time-of-flight (Q-TOF) mass spectrometer equipped with a Z-spray source (Q-TOF PremierTM; Waters). Ions were formed by nano-ESI from emitters made from borosilicate capillary tubes (1.0 mm o.d./0.78 mm i.d.; Sutter Instruments) pulled to a tip with an inner diameter of ~5 µm. The flow rates were ~50–200 nl/min. For MS/MS experiments, the quadrupole

resolution was adjusted to encompass the ion of interest and the accelerating voltage into the argon-filled collision cell was 60 V. External mass calibration was performed using sodium formate immediately prior to measuring samples. Mass spectra were processed using MassLynx software (v4.1; Waters). Scaling of mass spectral ions and assignment of fragment ions were performed as described earlier (Roepstorff and Fohlman, 1984; Marshall *et al*, 2002).

The Stanford University Mass Spectrometry Laboratory performed intact mass analysis (LC/MS) of purified PknB KDs. The LC-MS system (Waters) consisted of an Alliance 2795 HPLC with inline 2487 UV detector and Micromass ZQ single quadrupole mass spectrometer. Protein samples were separated on a 2.1x 40 mm C8 Targa Sprite column (Higgins Analytical) at 300 µl/min, using a linear gradient of 2–95% B; solvent A was 0.1% formic acid in H₂O, solvent B was 0.1% formic acid in acetonitrile. UV response was monitored at 254 and 280 nm. MS data were collected up to 2000 m/z and raw spectra were deconvoluted using the MaxEnt1 algorithm.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Accession codes

Coordinates and structure factors were deposited in the Protein Data Bank under accession codes 3F69 (Leu33Asp/Met145Leu/Met155Val PknB-KT5720 complex) and 3F61 (Leu33Asp/Val222Asp PknB-ADP complex).

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Competing interests statement

The authors declare that they have no competing financial interests.

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