To define how extracellular signals activate bacterial receptor Ser/Thr protein kinases, we characterized the regulatory functions of a weak dimer interface identified in the *Mycobacterium tuberculosis* PknB and PknE receptor kinases. Sequence comparisons revealed that the analogous interface is conserved in PknD orthologs from diverse bacterial species. To analyze the roles of dimerization, we constructed PknD KD fusions stimulated phosphorylation of unphosphorylated PknD. The phosphorylated PknD KD fusions stimulated phosphorylation activity. Mutations in the dimer interface reduced this activation, limited autophosphorylation, and altered substrate specificity. In contrast, an inactive catalytic site mutant retained the ability to stimulate the wild-type KD by dimerization. These results support the idea that dimer formation allosterically activates unphosphorylated PknD. The phosphorylated PknD KD was fully active even in the absence of dimerization, suggesting that phosphorylation provides an additional regulatory mechanism. The conservation of analogous dimers in diverse prokaryotic and eukaryotic Ser/Thr protein kinases implies that this mechanism of protein kinase regulation is ancient and broadly distributed.

Tight regulation of Ser/Thr protein kinases (STPKs) is essential for signaling in all three kingdoms of life. In keeping with the importance of phospho-signaling pathways, STPKs are subject to multiple mechanisms of allosteric regulation (1). Diverse regulatory surfaces of kinase domains (KDs) can bind additional proteins (as in the cyclin-dependent kinases and cAMP-dependent protein kinase) or other domains of the kinase itself (such as the Src homology 2 and linker domains of Src). These interactions often affect the assembly of the catalytic site by positioning a conserved, substrate-binding element called the C helix. In addition, phosphorylation of a conserved motif called the activation loop often relieves a steric blockade of the active site and promotes assembly of a substrate-binding platform (1). Although many regulatory interactions have been characterized in eukaryotic protein kinases, the mechanisms by which environmental signals regulate prokaryotic STPKs have yet to be elucidated.

Prokaryotic STPKs occur in numerous pathogens and organisms with complex developmental pathways (2). Like their eukaryotic homologs, the bacterial STPKs adopt a characteristic two-domain fold with the ATP-binding site located between the N- and C-terminal lobes (3–5). Unexpectedly, the catalytic domain of the receptor STPK PknB from *Mycobacterium tuberculosis* formed an unstable, back-to-back dimer through an interface in the N-terminal lobe (Fig. 1A) (3–5). Residues in the PknB dimer interface were found to be strictly conserved in orthologs from dozens of bacterial genera, supporting the proposal that KD dimerization plays an important functional role (5). Because the dimer interface included contacts near the C terminus of the C helix, it was proposed that dimerization regulates PknB (5). This idea was reinforced by the structure of the *M. tuberculosis* PknD extracellular domain, which includes a rigid β-propeller motif flexibly tethered to the single, predicted transmembrane helix (6). This arrangement was consistent with a model in which dimerization or localization of the extracellular domain, rather than a conformational change transmitted across the membrane, serves to regulate the intracellular kinase domains (2, 5, 6).

Further support of a regulatory role for dimerization was provided by the crystal structure of the *M. tuberculosis* PknE KD, which crystallized as a back-to-back dimer similar to that formed by the PknB KD (Fig. 1A), despite the absence of sequence homology in the interfacial residues (3). Sequence analysis of the 11 *M. tuberculosis* STPKs indicated that the predicted dimer interface residues are more similar in PknB, -A, and -L; PknD, -E, and -H; and PknF, -I, and -J, but these three clades are distinct from each other (Fig. 1B) (3). This conservation within three paralogous branches of *M. tuberculosis* STPKs supported the importance of the back-to-back pairing interface. Consistent with the proposed regulatory role in PknB and PknE, formation of a structurally similar dimer was found to activate the human dsRNA-dependent protein kinase R (PKR; Fig. 1A) KD in response to double-stranded RNA binding to the ligand-binding domain (7, 8).
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A

B

PKnE 1 MDGAESREHTQFPGYPRLRRLGVRDMQEDVYAEQTVEERLVALKMSETLSSDVPFTRMGREATAGLQEPH 75
PKnH 1 MDSEASRVSMEDGYPHKLRLGVRDMQEDVYAEHTKVKEWTAVKLMAETSKDPFVRMREREAIAGLQEPH 75
PKnD 1 MDAVP-QVSQGPFGYPLLRLGVRDMQEDVYAEETLFRHRVVALKLISQPSDNVFRARMOREADGLLQEPH 74
PKnB 1 . . . . MTPSHELSDRYELGIEI LGEGMGEVHLADRLRHRDVAHKLVRADLARPSFYLERBFREAGANAALNHPA 70
PKnE 76 VVPPEEIDEIDG . . . . QLQVYDMLRINTQVLAAMLRQG6PPRPRAAIVRQGSAALDAHAAGATVRDVQENIL 146
PKnH 76 VVPPEEIDEIDG . . . . QMFLERMLVGETDLDSVLFRGPLTTPRATAIQTQIASSALDAHAADVMSRVDQENIL 146
PKnD 75 VVPPEEIDEIDG . . . . OFFVREMMDGQTSMLRKLQYQGPLTPRARAVIQQIALAAALAAANGTVHVRQENIL 145
PKnB 71 VVPPEEIDEIDG . . . . OQVQ(GTKL)DEIDG . . . QLQVYDMLRINTQVLAAMLRQG6PPRPRAAIVRQGSAALDAHAAGATVRDVQENIL 145
PKnE 147 VSADFARYLVDFRIASATDDEK . . . . LTQLGNTVSTLYMYMAPERSHATYRADITYALTCVLECLT9SPYQDQ 216
PKnH 147 ITRODFAYLVDFRIASATDDEK . . . . LTQLGNTAVTQWKMAPERFSDEVYTRADIYALACVHCEC1GAPPYRA 216
PKnD 146 VTAADFARYLVDFRIASATDDEK . . . . LTQLGNTAVTQWKMAPERFSDEVYTRADIYALACVHCEC1GAPPYRA 216
PKnB 146 VSADFARYLVDFRIASATDDEK . . . . LTQLGNTAVTQWKMAPERFSDEVYTRADIYALACVHCEC1GAPPYRA 216
PKnE 220 LVMDAHINGAIPTPVSPTRPG . . . . LPVAFAVIAIRGMAKNEPDRYVTGDLASAAHAALATAQDRATDIL 296
PKnH 220 AGLVSSIMQPIPOAVSRPG . . . . LPKAFDVAIRGMAKNEPDRYVAAGDLALAAHAEALSDPDQADL 296
PKnD 219 V dripping G 221 PSVAYKHREDPIPPSARHEG . . . . LSADLVAYVKTALKALPKNREYQTAAMRADLVRVHERGEPPEAPKVL 296
PKnB

C

ATP binding site

Y81

H79

N-lobe

C-lobe

D138

180°
Here we provide experimental evidence that dimerization activates *M. tuberculosis* PknD. To test the functional roles of dimerization, we used rapamycin to bring together FKBP (FK506-binding protein) and FRAP (FK506-binding protein-rapamycin-associated protein) fusions to the PknD KD. Dimerization strongly activated the unphosphorylated PknD KD, particularly when tested using a cognate in vivo substrate. In contrast, dimerization caused little or no further activation of the active, autophosphorylated KD fusions. The predicted intersubunit contact residues were found to be conserved in orthologs of *M. tuberculosis* PknD. Mutation of conserved residues in the predicted dimer interface reduced activation, inhibited autophosphorylation, and altered substrate specificity. Remarkably, association with a catalytically inactive mutant containing a normal dimer interface activated the unphosphorylated wild-type (WT) KD fusion. These data support the conclusion that back-to-back dimerization allosterically regulates diverse protein kinases in prokaryotes and eukaryotes.

**EXPERIMENTAL PROCEDURES**

**Sequence Comparisons**—Ser/Thr kinase sequences were extracted from the NCBI RefSeq data base (9) using BLAST searches (10) against PknB, PknE, and representative eukaryotic kinases. Domain assignments were made with hmmpfam using the PFAM data base (11). Sequences containing PASTA or NHL domains were collected, and the kinase domains were aligned using MUSCLE (12). ConSurf (13) was used to visualize the sequence conservation mapped onto the structures of PknB or PknE.

**DNA Constructs**—The pET-24b vector (Novagen) was adapted by inserting 2 bases between the SacI and Sall restriction sites to facilitate the insertion of consecutive in-frame open reading frames. DNA fragments encoding FKBP and the minimal functional domain of FRAP (the FRB domain) (14) were cloned from a vector generously provided by Michael Schelle (University of California, Berkeley) with a 5′-flanking HindIII site and a 3′-flanking XhoI site. Amplified FKBP or FRAP DNA was digested with HindIII and XhoI and ligated into similarly flanking XhoI site. Amplified FKBP or FRAP DNA was digested with HindIII and XhoI and ligated into similarly digested vector DNA to create the vectors pAG-FK and pAG-FR, respectively.

In *M. tuberculosis* PknD, the KD (~290 amino acids) is followed by an 88-amino acid flexible linker (also called the juxtamembrane domain), a 20-amino acid transmembrane helix, a 10-amino acid tether, and a 262-amino acid β-propeller domain. A fragment corresponding to the first 378 amino acids of PknD was cloned with flanking 5′ Ndel and 3′ BamHI sites from *M. tuberculosis* genomic DNA as described (5). The double-digested, amplified PknD fragment was ligated into pAG-FK or pAG-FR to create PknD-FK or PknD-FR, respectively.

**Expression and Purification**—Plasmids were transformed into BL21(DE3) Codon Plus cells (Stratagene) as described (5). Cells were grown at 37 °C to an absorbance (A <sub>600</sub>) of 1.8 in Terrific Broth (Research Products International), moved to 18 °C for 10 min, induced using 300 μM isopropyl 1-thio-β-d-galactopyranoside, and grown for an additional 4 h. The cells were harvested by centrifugation and resuspended in 100 mM Tris-HCl (pH 7.8), 0.3 M NaCl, 10% (v/v) glycerol, and 0.5 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP). Cells were lysed by sonication on ice, and the lysate was clarified by centrifugation. The supernatant was loaded onto a 5-ml chelating Sepharose HP column (Amersham Biosciences) equilibrated with 0.1 M NiSO<sub>4</sub> and eluted in lysis buffer with 300 mM imidazole. After dialysis into 50 mM HEPES (pH 7.65), 150 mM NaCl, and 0.5 mM TCEP, each protein was further purified by gel-exclusion chromatography using a HiLoad 26/60 Superdex 75 column (Amersham Biosciences).

To dephosphorylate the KDs, the His<sub>6</sub> tag was first removed from PknD by incubation with thrombin (10 units per mg of PknD) in 50 mM HEPES (pH 7.65), 150 mM NaCl, 2.5 mM CaCl<sub>2</sub>, and 0.5 mM TCEP. A mixture of 20:1 (w/w) PknD/His<sub>6</sub>PstP was incubated overnight at 4 °C with 5 mM MnCl<sub>2</sub> (15) and separated on chelating Sepharose HP (Amersham Biosciences) equilibrated with 0.1 M NiSO<sub>4</sub>. The flow-through and first wash fractions were concentrated using VivaSpin 2-ml concentrators (VivaScience), and the absence of contaminating His<sub>6</sub>PstP was confirmed by SDS-PAGE of an overloaded sample.

**Kinase Assays**—Reactions were performed in 150 mM NaCl, 50 mM HEPES (pH 7.65), 0.5 mM TCEP, and 2 mM MnCl<sub>2</sub>. Each KD fusion protein was added to a final concentration of 40 nM (80 nm total kinase). Myelin basic protein (MyBP) and purified Rv0516c (containing N-terminal His<sub>6</sub> and maltose-binding protein tags) (16) were assayed at 27 and 16 μM, respectively. Excess rapamycin (final concentration of 10 μM) in ethanol or an equivalent volume of ethanol was added to each reaction. This mixture was incubated for 1 h at 4 °C, and the reaction was initiated by adding 1 μl of [γ<sup>32</sup>P]ATP (250 nCi/μl; ICN) in 2 mM cold ATP (Sigma). The final reaction volume was 20 μl.

MyBP reactions were quenched with 5 μl of 5× SDS-PAGE loading dye. Rv0516c reactions were first quenched by adding EDTA (50 mM) and tobacco etch virus protease (24 μM). After 30 min of protease digestion to separate the His-MBP tag from Rv0516c, 5 μl of 5× SDS-PAGE loading dye was added. All reactions were separated by SDS-PAGE on 4–12% NuPage NOVEX BisTris gels (Invitrogen). The gels were dried, and radioactivity was quantified with a Typhoon 8600 PhorImager (Amersham Biosciences). The autophosphorylation and MyBP reactions were monitored at 10, 20, and 30 min, whereas Rv0516c phosphorylation reactions were monitored at 15, 30, and 60 min to increase the amount of radioactive product. All assays were repeated at least three times. The effects of dimerization were quantified using ImageQuant™ (GE Healthcare).
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assuming a linear activation model by calculating the average ratio of the signals obtained at each time point in the presence and absence of rapamycin.

Kinase Phosphorylation Analysis—Each PknD variant was expressed and purified as described above for the WT protein. The protein was dialyzed into 50 mM NaCl, 20 mM Tris (pH 7.8), 0.5 mM TCEP, and 10% glycerol (v/v), loaded onto a 5-ml HiTrap Q-Sepharose ion-exchange column (Amersham Biosciences) in the same buffer, and eluted with a 0.05–1 M NaCl gradient in 150 ml. The protein was concentrated to ~2 mg/ml.

The mass of the intact protein was determined by electrospray ionization-trap mass spectrometry. The Y81A mutant was further analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry to determine the average mass.

RESULTS

The identification of the anti-σ-factor antagonist homolog Rv0516c as an in vivo substrate of PknD (16) afforded an opportunity to explore the activity and specificity of an M. tuberculosis STPK using a functional, cognate substrate. Phosphorylation of Rv0516c Thr-2 blocks binding to another σ-factor regulator (Rv2638) and markedly alters transcription of σ-F-dependent genes in vivo (16). Unlike other reported M. tuberculosis STPK substrates (2, 17–22), Rv0516c lacks a Forkhead-associated domain and is phosphorylated efficiently at a single site.

PknD Conservation—To explore the potential role of dimerization in regulating M. tuberculosis PknD, we first assessed the conservation of predicted dimer-interface residues in the KDs of PknD orthologs. The PknD KD is 39 and 65% identical, respectively, to the KDs of PknB and PknE, but the structure and capability for dimerization of the PknD KD tethered to the membrane are not known. PFAM domain assignments for all available prokaryotic kinase sequences in the NCBI RefSeq data base identified 10 potential PknD orthologs with shared domain architecture. These receptor kinases contained a KD linked through a single transmembrane helix to extracellular NHL repeats predicted to form a β-propeller. The 10 orthologous KDs showed 34–82% sequence identity with M. tuberculosis PknD. Mapping the level of sequence conservation onto the crystal structure of the KD of M. tuberculosis PknE revealed that the predicted dimer interface of PknD is highly conserved in orthologs (Fig. 1C). In concert with the conservation of the dimer interface in PknB orthologs (5), this analysis suggested that dimerization is conserved in certain prokaryotic receptor STPKs.

Activity of PknD Dimers on Three Substrates—To test the role of the observed dimer interface in PknD regulation, we measured the effects of dimerization on kinase activity. Because the isolated M. tuberculosis KDs are monomeric at concentrations used in biochemical assays (3),3 we fused the PknD KD to proteins that form heterodimers in the presence of the macrolide antibiotic rapamycin. The fusions contained the first 378 amino acids of PknD, encompassing the kinase domain (~290 amino acids) and the juxtamembrane linker (88 amino acids). This intracellular segment of PknD was joined to the FKBP (gi 30585002) or the minimal binding domain (FRB domain) of the FRAP (gi 19924298). FKBP binds rapamycin with subnanomolar affinity, and FRAP subsequently binds the FKBP-rapamycin complex (14). This binding brings together the attached PknD KDs with physical constraints similar to those that would be imposed by dimerization of the extracellular domain of the full-length receptor (Fig. 2A). Rapamycin-induced dimerization raises the effective concentration of the KDs and favors formation of KD dimers.

Assays using a 1:1 mixture of purified PknD KD fusions that were phosphorylated during production in Escherichia coli showed that rapamycin induced little or no increase in kinase activity (Fig. 2B). Activity was monitored on three substrates as follows: the KD itself; a noncognate substrate with multiple phosphorylation sites, myelin basic protein (MyBP); and the in vivo PknD substrate, Rv0516c. Rapamycin-induced dimerization of the phosphorylated PknD KD weakly enhanced (1.7 ± 0.4-fold) phosphorylation of MyBP, and it had little or no observable effect on activity toward Rv0516c or the PknD KD itself (Fig. 2B).

An important caveat in interpreting these assays is the robust autophosphorylation of this KD during expression in E. coli. At least 14 autophosphorylation sites, including three sites in the activation loop, were detected in the PknD KD and juxtamembrane-linker regions (23). Because activation-loop phosphorylation is critical for activating homologous kinases, including PknB (1, 5, 23, 24), the autophosphorylation of the KD may be sufficient to maximally stimulate the enzyme. Moreover, the lack of an increase in autophosphorylation upon dimerization may reflect the presence of nonradioactive phosphoryl groups on the majority of phosphorylation sites in the WT KD. To test the idea that PknD autophosphorylation may bypass the effects of dimerization, we measured the effects of dimerization on the activation of the unphosphorylated PknD KD. The KD fusions to FKBP and FRAP were treated with the M. tuberculosis Ser/Thr phosphatase, PstP, to remove all the phosphoryl groups on the purified proteins (15, 23). The dephosphorylation reactions went to completion, as monitored by SDS-PAGE (data not shown). Kinase assays were repeated after separating PstP from the dephosphorylated KD fusion proteins. As expected, dephosphorylation reduced the activity of the PknD KD on MyBP up to 9.3-fold (Fig. 2C). Because the phosphorylated KD accumulates from the initiation of the assay, the observed activity may result from autophosphorylated KD. This observation, however, established an upper limit for the intrinsic activity of the unphosphorylated PknD KD. Rapamycin-induced dimerization of the unphosphorylated KD stimulated autophosphorylation 4.2 ± 0.3-fold and increased MyBP phosphorylation 2.6 ± 0.3-fold (Fig. 2D). Using the cognate M. tuberculosis substrate protein, Rv0516c, however, a larger increase in activity (6.8 ± 1.3-fold) was observed when the KD was dimerized with rapamycin. In contrast, rapamycin had no effect on the measured activity of each fusion protein in isolation (Fig. 2E). Only when the FKBP and FRAP fusions were mixed did rapamycin induce dimerization and activation. These results suggested that dimerization strongly stimulates phosphorylation of specific, cognate substrates and has smaller

3 T. N. Lombana and T. Alber, unpublished results.
Dimer Interface Mutants Influence Activity and Specificity

Dimer Interface Mutants Influence Activity and Specificity—To pinpoint the role of back-to-back N-lobe contacts in kinase activation, we mutated two predicted dimer-interface residues to alanine and replicated the kinase assays. His-79 forms direct contacts across the dimer interface in the homologous PknE structure, and Tyr-81 is conserved in the predicted dimer interface of all PknD orthologs (Fig. 3A). Mass spectrometry revealed that autophosphorylation of the protein isolated from E. coli was reduced by the Y81A substitution and abolished by H79A (Fig. 3B and Table 1). Because autophosphorylation activates PknB and other protein kinases (1, 5, 23, 24), the different numbers of phosphoryl groups on the WT PknD KD and dimer-interface mutants complicated direct comparisons of the specific activities of these variants.

We used two approaches to overcome this problem. First, we measured the impact of rapamycin-induced dimerization on the activities of KD fusions as isolated from E. coli (before treatment with PstP). Kinase assays were performed using the phosphorylated WT, H79A, and Y81A KD fusions purified from E. coli (Fig. 3C). The H79A mutant was inactive under all conditions in which WT kinase activity was observed (data not shown). The Y81A mutant phosphorylated the KD itself, MyBP, and Rv0516c (Fig. 3C). Like the WT protein (Fig. 2B), rapamycin-induced dimerization did not alter the activity of the autophosphorylated Y81A KD.

In addition, we tested the activity of each mutant kinase after dephosphorylation. The H79A mutant was consistently inactive in all conditions tested (data not shown). Neither auto-phosphorylation nor trans-phosphorylation of MyBP by the Y81A mutant was observed in the presence or absence of rapamycin (Fig. 3D). Rv0516c phosphorylation, in contrast, was stimulated by rapamycin-induced dimerization (Fig. 3D). These results suggested that dimerization may preferentially activate the Y81A KD to phosphorylate cognate substrates independent of KD autophosphorylation.

Allosteric Activation of WT PknD by a Catalytically Inactive Mutant—To assess whether allosteric effects transmitted through the dimer interface are sufficient to activate PknD, we produced a defined heterodimer of dephosphorylated WT

FIGURE 2. Unphosphorylated PknD is activated by dimerization. A, rapamycin-induced association of PknD KDS. The PknD KD and juxtamembrane domain (JMD), encompassing amino acids 1–378, were fused to the minimal functional domain of either the FKBP or FRAP. Rapamycin stabilizes the FKBP-FRAP heterodimer and raises the effective concentration of the kinase domains, favoring KD dimerization. B, rapamycin (Rap)-induced dimerization had little or no effect on the activity of phosphorylated WT PknD kinase domains. Multiple site autophosphorylation and trans-phosphorylation of the noncognate substrate, myelin basic protein (MyBP), dimerization had little or no effect on the activity of phosphorylated WT PknD kinase domains. Multiple site autophosphorylation and trans-phosphorylation of the noncognate substrate, myelin basic protein (MyBP), was monitored at 10, 20, and 30 min, whereas trans-phosphorylation of the singly phosphorylated, functional substrate, Rv0516c, was monitored at 15, 30, and 60 min. Phosphorylation was initiated by adding radiolabeled [γ-32P]ATP and excess unlabeled ATP. The PknD kinase domains were heterogeneously autophosphorylated at up to 14 sites during production in E. coli (23). C, dephosphorylation reduced PknD activity. Phosphorylated PknD KD purified from E. coli and PknD KD dephosphorylated using the PstP phosphatase were used in parallel assays of MyBP phosphorylation, which were analyzed on a single autoradiogram. Phosphorylation (right) increased the apparent specific activity of the KD up to 9.3-fold. Because the dephosphorylated kinase was phosphorylated during the assay, this experiment establishes a lower limit for the activation caused by phosphorylation. D, rapamycin-induced dimerization increased activity of dephosphorylated PknD. The PknD KD fusions were dephosphorylated using the PstP phosphatase and tested for auto- and trans-phosphorylation activity. Band intensities cannot be compared directly with those in the other panels because dephosphorylation reduced the specific activity of the KD (C) and the autoradiogram is displayed to illustrate the effects of dimerization. Dimerization resulted in a 6.8 ± 1.3-fold increase in the activity toward the cognate substrate, Rv0516c, and caused smaller increases in autophosphorylation of the KD and phosphorylation of MyBP. E, rapamycin did not affect the Rv0516c phosphorylation by the isolated, dephosphorylated FKBP or FRAP KD fusions. The 30-min time points are shown. In contrast to the mixture of dephospho-FKBP and FRAP KD fusion (D), rapamycin does not induce dimerization of the separate KD fusions.
PknD with an active-site mutant that renders the kinase catalytically inactive. This D138N KD mutant was 2600-fold less active than WT (16), but the protein was soluble and folded in solution (data not shown). When the purified FKBP fusion of the D138N mutant (which contained no phosphoryl groups) was combined with the dephosphorylated FRAP fusion of the WT kinase, rapamycin-induced dimerization activated the WT KD in a manner analogous to the dephosphorylated WT KD homodimer (Fig. 4A). Autophosphorylation and trans-phosphorylation of MyBP increased 3.3 \pm 1.1-fold and 3.7 \pm 2.0-fold, respectively, in response to rapamycin-induced dimerization. Similar to the unphosphorylated WT KD, dimerization caused the largest increase (5.9 \pm 0.9-fold) in the trans-phosphorylation of Rv0516c. In contrast, a mixture of the FKBP fusions (which are not brought together by rapamycin) of dephosphorylated WT and D138N variant KDs showed no stimulation by rapamycin (Fig. 4B). These results showed that a mutant lacking kinase activity nonetheless activated the WT KD by dimerization.

**DISCUSSION**

Taken together with sequence comparisons (3, 5) (Fig. 1B) and structural studies showing analogous dimers of PknB and PknE KD (3–5) (Fig. 1A), these biochemical data support the idea that dimerization through the conserved, back-to-back interface controls PknD activity and specificity. Phosphorylation of the cognate substrate, Rv0516c, by the unphosphorylated KD showed the largest stimulation by dimer formation. The His-79 and Tyr-81 mutations, respectively, abolished or inhibited the activation of the PknD KD, suggesting that activation requires formation of a structurally specific dimer. These mutations define the location of an allosteric activation surface in PknD, which presumably also includes the other predicted dimer-interface residues conserved in PknD orthologs (Fig. 1C). The failure of rapamycin-induced dimerization to stimulate autophosphorylation and MyBP phosphorylation in the H79A and Y81A mutants (Fig. 3) indicates that activation of the WT KD results from the formation of specific intersubunit contacts, not just the proximity of monomers in the dimer. The back-to-back arrangement of the molecules in the homologous dimers, which holds the active sites of the subunits away from each other (Fig. 1A), bolsters the idea that dimerization allosterically activates the KD, rather than promoting intradimer phosphorylation.

Our results support a model of PknD activation in which unphosphorylated, monomeric kinase subunits comprise the inactive state (Fig. 5A). Dimerization of the extracellular domains brings together the intracellular KDs, which promotes back-to-back dimerization and activates the kinase for auto...

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**FIGURE 3. Mutations in the predicted PknD dimer interface reduced activity and altered substrate specificity.** A, locations of two predicted PknD dimer-interface mutants (space filling representation) based on the PknE structural model. The sequences of the PknD and PknE KDs are 65% identical (79% similar). His-79 is predicted to be central to the dimer interface, whereas Tyr-81 is more peripheral. B, electrospray ionization-ion trap mass spectrum of the H79A PknD KD variant. This representative spectrum revealed only a single species with a mass equal to the predicted mass of unphosphorylated protein. Mass spectra indicating the autophosphorylation states of the PknD KD mutants purified from *E. coli* are summarized in Table 1. C, activity of the phosphorylated Y81A KD was not markedly stimulated by dimerization. The mutant KD fusions were purified from *E. coli*, and activity of a 1:1 mixture of the FKBP and FRAP fusions was assessed on MyBP and Rv0516c substrates. The H79A variant was inactive under identical conditions (data not shown). D, after treatment with the PstP phosphatase, dimerization of the Y81A variant stimulated phosphorylation of Rv0516c. Neither autophosphorylation nor MyBP phosphorylation was observed. For the Y81A mutant, autophosphorylation was not required for dimerization to stimulate phosphorylation of Rv0516c. This autoradiogram is displayed to illustrate the effects of rapamycin (Rap), and the band intensities cannot be compared directly with those in other panels. The dephosphorylated H79A variant was inactive under all conditions tested (data not shown).
TABLE 1
Mutational effects on PknD autophosphorylation
For each of the KD variants, the predicted mass of the unphosphorylated sequence was compared with the mass(es) observed using electrospray ionization-ion trap mass spectrometry. Peaks differing by multiples of 80 Da from the predicted mass were interpreted as phosphorylated forms. Neither the H79A (dimer interface mutant) nor the D138N (catalytically inactive) mutant of PknD was phosphorylated as isolated from E. coli. The Y81A mutant contained between 5 and 12 phosphates, with the most abundant species (in parentheses) containing eight phosphates. In contrast, WT PknD contains up to 14 phosphates (with the most abundant species containing 11 phosphates) as isolated from E. coli (23).

<table>
<thead>
<tr>
<th>PknD mutant</th>
<th>Predicted mass</th>
<th>Observed mass</th>
<th>No. of phosphates</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (23)</td>
<td>Da</td>
<td>Da</td>
<td></td>
</tr>
<tr>
<td>H79A</td>
<td>40,872.0</td>
<td>40,872.1</td>
<td>0</td>
</tr>
<tr>
<td>Y81A</td>
<td>40,846.0</td>
<td>41,254–41,805</td>
<td>5–12 (8)</td>
</tr>
<tr>
<td>D138N</td>
<td>40,937.1</td>
<td>40,937.3</td>
<td>0</td>
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</tbody>
</table>

FIGURE 4. Catalytically inactive D138N PknD KD activated the WT KD in rapamycin-induced heterodimers. A, dephosphorylated WT PknD fused to FKBP was paired with catalytically inactive D138N PknD KD fused to FRAP. Rapamycin (Rap)-induced dimerization increased phosphorylation of the kinase domains, MybP and Rv0516c. B, rapamycin failed to stimulate phosphorylation by a 1:1 mixture of WT and D138N KDs fused to FKBP, which do not dimerize upon rapamycin addition. These results demonstrate that PknD KD activity was stimulated by dimer formation.

and trans-phosphorylation. The findings that dimerization directly activated the dephosphorylated KD (Fig. 2D) and the catalytically inactive KD activated the WT KD (Fig. 4A) provide direct support for this allosteric mechanism. The hypophosphorylation of the Y81A variant (Table 1) suggests that KD dimer formation also is required for full autophosphorylation. As seen with other STPKs (2, 23, 24), autophosphorylation activated the PknD KD (Fig. 2C). We observed little or no further activation upon rapamycin-induced dimerization of the autophosphorylated KD (Fig. 2B), suggesting that the phosphorylated monomeric subunits may be maximally active on some substrates. Once the appropriate sites are phosphorylated, dimer formation may be less important for activity. Thus, phosphorylation provides an additional mechanism to activate the PknD KD. Consequently, our data support a model in which loss of dimerization signal and dephosphorylation are both required to return the activated, phosphorylated kinase to the inactive state.

Consistent with the finding that a catalytically inactive mutant KD activates WT subunits in vitro (Fig. 4A), overexpression of full-length D138N PknD in M. tuberculosis increased Thr phosphorylation of specific proteins in vivo (16). These phospho-Thr proteins (which are rapidly phosphorylated upon expression of full-length WT PknD) are thought to include PknD itself as well as a number of putative substrates. This seemingly paradoxical increase in phosphorylation upon expression of a catalytically inactive kinase could be an indirect effect of protein overexpression or a result of the activities of other STPKs. The specific nature of the Thr phosphorylation, however, may be best explained by activation of the endogenous WT PknD by dimerization with the excess of inactive mutant subunits in vivo.

The role of dimerization in activating PknD raises the question of how general this mechanism might be. The conservation of the dimer interface in orthologs of PknD and PknB (5) and the structural conservation of the back-to-back dimer in PknE (3) support the idea that dimerization regulates these kinases in numerous bacterial genera. Consistent with the allosteric model, M. tuberculosis PknB, PknD, PknE, PknG, and PknK all contain recognizable interaction domains fused to the KD (25). Moreover, a fragment comprising the transmembrane and ligand-binding domains of the B. subtilis PknB ortholog, PrkC, was sufficient to form dimers in vivo (26). Although several of the M. tuberculosis KDs have weak affinities (3), this low dimer stability is suitable for membrane-tethered systems in which diffusion is two-dimensional and in which signaling ligands drive dimerization.

The simple ligand-promoted dimerization mechanism (Fig. 5A), however, is unlikely to explain the activation of all the M. tuberculosis STPKs, because six of these proteins lack a ligand-binding domain with a recognizable fold or function. The predicted extracellular domains of PknA, PknF, PknH, PknI, and PknJ include sequences with low complexity, and PknL has only six amino acids predicted to occur outside the cell. Although it is possible that binding proteins may engage these extracellular domains, these latter kinases may not recognize activating ligands. Instead, they may be regulated either by trans-phosphorylation by another activated STPK or by dimerization by which mass action to the STPK protein concentration. The importance of a complementary dimer interface in enabling maximal phosphorylation (Fig. 3B) suggests that the STPKs that are regulated by phosphorylation may pair exclusively with kinases with similar dimer interfaces. Because of the divergence of the interface residues of the three groups of M. tuberculosis receptor STPKs (PknA/B/L, PknD/E/H, PknF/I/J) (3), it is unlikely that heterodimers are formed between members of the divergent groups. These considerations support the hypothesis that these three groups of STPKs form hierarchical networks that are influenced by both the presence of extracellular signaling ligands and the concentrations of each STPK. Unless signaling ligands can bring together different sensor domains, ligand-stabilized homodimers of the receptor kinases are likely to play the roles of master STPKs at the tops of the signaling hierarchies.

The allosteric activation mechanism of PknD differs from the direct intradimer cross-phosphorylation of subunits promoted by dimerization of various eukaryotic receptor tyrosine kinases (27). Instead, PknD activation shares features with the allosteric regulation mechanisms of the human dsRNA-dependent PKR (7, 8) and the EGF receptor kinase (28). In response to binding of dsRNA to the N-terminal ligand-binding domain, the PKR KD forms a structurally similar back-to-back symmetric dimer (Fig. 1A and Fig. 5B), which activates the kinase domains by inter-dimer phosphorylation of the activation loops (7, 8). Mutation of PKR dimer interface residues, far from the active site, resulted in a loss of activation and substrate phosphorylation (8). Both KDS in the PKR dimer act simultaneously as
enzymes and allosteric effectors. A similar mechanism was suggested by the dimer structure of yeast GCN2, which forms a distinct dimer through the N-terminal lobe (29). PknD displays the added features that the unphosphorylated dimer may be active in phosphorylating a cognate substrate (Fig. 3), and the phosphorylated monomeric kinase is active (Fig. 2B).

Signaling through transmembrane approximation also resembles the mechanism of activation for human epidermal growth factor receptor (EGFR; Fig. 5C). EGF binding to the extracellular receptor domain activates the intracellular EGFR KD by favoring an asymmetric dimer in which one KD monomer stabilizes the C-helix of the other subunit in the active conformation (28). This association, however, involves a surface distinct from the back-to-back dimer interface in PKR (Fig. 5B) and PknD (Fig. 5A). In the EGFR asymmetric dimer, only one kinase domain is expected to be active.

Despite differences between the PknD and PKR activation models, a number of similarities are apparent. Strikingly, the location of the allosteric interface is structurally conserved between PKR and the M. tuberculosis receptor kinases (Fig. 1A).

Dimerization Activates a Bacterial Ser/Thr Kinase

**FIGURE 5.** Similar regulatory mechanisms in bacterial and eukaryotic protein kinases. A, model of PknD activation. The monomeric, dephosphorylated kinase (dark blue) is the inactive form (upper left). Extracellular ligand (light blue) binding to the extracellular domain (maroon) dimerizes the kinase, forming an active complex (upper right). Phosphorylation of the kinase domain ensues (lower right), further stimulating activity. The phosphorylated kinase monomers remain active in the absence of the extracellular ligand (lower left). The activity of the phosphorylated monomers implies that trans-phosphorylation by other STPKs may provide a distinct mechanism of regulation capable of activating the kinase in the absence of dimerizing signal. The M. tuberculosis Ser/Thr phosphatase PstP (middle) dephosphorylates the kinase, returning the system to the inactive state only in the absence of the extracellular ligand. B, mycobacterial receptor Ser/Thr kinase activation resembles the activation mechanism of PKR (7, 8). Ribbon diagram showing the activated PKR dimer, with the C-helix of each kinase domain colored red to show the locations of allosteric sites (additional domains left out for clarity). The back-to-back PKR dimer stabilizes the active conformation of the KD and stimulates interdimer autophosphorylation, which fully activates the kinase. C, transmembrane signaling by dimerization of EGFR (blue). EGF (red) binding to the extracellular receptor stabilizes an intracellular KD dimer. The KDs form an asymmetric dimer that activates one monomer. Much like a pseudo-kinase (31), the other monomer serves as an inactive allosteric effector. Like PknD and PKR, either monomer in EGFR can become activated.
In the Src tyrosine kinase, binding of Trp-260 of the linker domain to the analogous KD surface helps stabilize the inactive state (30). These similarities suggest that this region of the N-lobe distal to the active site serves as an allosteric regulatory site in a variety of protein kinases. The utilization of this interface in diverse STPKs implies that back-to-back dimerization represents a widespread allosteric regulatory mechanism. The appearance of this regulatory strategy in different kinases suggests that the mechanisms of structural communication or coupling from the allosteric surface to the active site also are likely to be preserved.

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