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Mycobacterium tuberculosis transporter MmpL7 is a potential substrate for kinase PknD ☆

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Abstract

The *Mycobacterium tuberculosis* serine/threonine protein kinases are attractive potential drug targets, and protein kinase D (PknD) is particularly interesting, as it is autophosphorylated on 11 residues, binds proteins containing forkhead associated domains, and contains a β-propeller motif that likely functions as an anchoring sensor domain. We created a *pknD* knockout of a clinical *M. tuberculosis* isolate, and found that on *in vitro* phosphorylation of cell wall fractions it lacked a family of phosphorylated polypeptides seen in the WT. Mass spectrometry identified the phosphorylated polypeptides as MmpL7, a transporter of the RND family. MmpL7 is essential for virulence, presumably because it transports polyketide virulence factors such as phthiocerol dimycocerosate (PDIM) to the cell wall. Phosphorylation of the MmpL family of transporters has not been previously described, but these results suggest that PknD, and perhaps other serine/threonine kinases, could regulate their critical role in the formation of the *M. tuberculosis* envelope.

Keywords: Tuberculosis; MmpL7; Serine/threonine kinases; Phosphorylation; RND transporters; PknD

Until recently, it was thought that signal transduction involving serine/threonine kinases was limited to eukaryotes. Subsequently, serine/threonine kinases were found in many, but not all, bacteria [1], and genomes of some Actinomycetes and Cyanobacter were found to encode several putative examples [2]. Work on inhibitors of eukaryotic serine/threonine kinases has resulted in new anti-cancer agents [3], so it is possible that the 11 serine/threonine kinases in the Actinomycete *Mycobacterium tuberculosis* [4] could be potential targets for new, anti-tuberculosis drugs. All but two of the *M. tuberculosis* serine/threonine kinases appear to contain N-terminal phosphoactivation regions followed by transmembrane segments and putative extracellular sensor domains that may activate the kinase activity and initiate signal transduction pathways. Although no clear phosphorylation cascade has been defined for any of the *M. tuberculosis* kinases, possible substrates and biological roles have been deduced with both experimental screens and examination of adjacent genes [5–8].

PknD is particularly interesting because C-terminal to the transmembrane region it contains a β propeller, a motif with six blades, each containing four anti-parallel β strands around a central cup-like pore [9]. In eukaryotes this motif has been associated with a number of different types of proteins, including the receptor tyrosine kinase *sevenless* [10].

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In prokaryotes the β propeller motif appears to be less common, but it has been found on proteins containing serine/threonine kinase motifs in the Actinomycetes *Streptomyces coelicolor* and *Thermomonospora curvata* [2]. The PknD genes in *Mycobacterium bovis* and *M. bovis* BCG lack the transmembrane segment and β propeller motif [11] due to an additional adenine after C829, which results in a frame shift that truncates the 70 kDa *M. tuberculosis* protein into a 31 kDa protein. The N-terminal, cytoplasmic portion of the *M. tuberculosis* PknD, was found to autophosphorylate on 11 residues, predominantly threonines, which were susceptible to PstP, the only *M. tuberculosis* Ser/Thr protein phosphatase [12]. PknD has also been shown to associate with two proteins containing phospho-threonine-binding forkhead associated domains—Rv 0020c and ABC transporter Rv1747, the latter of which is required for full virulence [13]. Because the *pknD* gene is adjacent to genes encoding phosphate transporters and phosphate-binding proteins [14,15], it was assumed to be involved with phosphate transport [4]. However, this has not been demonstrated, and normal phosphate uptake has been reported in a *pknD* mutant [15].

To study the role of PknD in *M. tuberculosis* physiology and to identify its potential substrates, we constructed a mutant strain of *M. tuberculosis* in which the kinase region of PknD was deleted, and after *in vitro* phosphorylation of protein fractions, we looked for changes in the pattern of phosphorylated proteins. The results suggest that PknD may play a role in the phosphorylation of MmpL7, a transporter associated with the deposition of components of the complex *M. tuberculosis* cell wall.

Experimental procedures

Bacterial strains and culture conditions. Mycobacterium tuberculosis 2*D*3 (MT2D3) is a virulent clinical strain isolated from a patient in Caracas, Venezuela. Its spoligotype is one of the most common found in Caracas isolates (unpublished results) and belongs to the LAM family [16]. *M. smegmatis* strain mc^2155 was used for electroduction of plasmid DNA [17]. *Escherichia coli* XL1-Blue and *E. coli* HB101 were used for plasmid transformation and phage transduction, respectively.

Mycobacterial strains were grown at 30 °C or 37 °C in Middlebrook 7H9 Tween medium (Himedia) or 7H11 agar supplemented with 10% OAD (0.05% Oleic Acid, 5% bovine serum albumin, fraction V (Roche), 2% glucose, and 0.85% NaCl) and 0.2% glycerol. *E. coli* strains were grown at 37 °C in Luria–Bertani (LB) broth or on LB agar plates. When required, Hygromycin B was used at a concentration of 50–75 μg/ml for mycobacteria and 150 μg/ml for *E. coli*.

PknD mutant construction. Enzymes and molecular biology reagents were purchased from commercial sources and used according to the manufacturers' instructions. Basic DNA manipulations were done according to standard protocols [18]. The H37Rv cosmid library was the kind gift of Lisa Pascopella [19].

The PknD mutant was constructed by allelic exchange using the specialized transducing phage system as described [17]. To replace a 442 bp section of *pknD* encoding Hank's domains [20] VIa, VIb, VII, VIII, IX (amino acids 100–247), with the hygromycin resistance cassette, two DNA fragments, 1-2DKO (0.691 kb) and 3-4DKO (0.972 kb), flanking this region were amplified by PCR from an *M. tuberculosis* H37Rv cosmid library and ligated into plasmid pYUB854 on the sides of the *res-hyg-res* cassette. The primers for amplifying the 1-2DKO fragment were D1 (5′-AATTTCTAGAAAGAGGTGCCGTCGATCATGC-3′) and D2 (TATAGGTACCTCGTTTGCGGTGGGTAAGC-3′), and those for 3-4DKO were D3 (ATTAATGCATTCGGTGTCGGTGACATAGACG-3′) and primer D4 (ATTAAAGCTTTCAGGTGATCGCCAAAGGC-3′). The subsequent steps to produce the specialized transducing phage used for allelic exchange were carried out as described [17].

Southern blot analysis. To identify PknD mutants, chromosomal DNA was isolated from Hyg^R colonies using the CTAB method [17], without glycine. The DNA (1–2 μg) was digested with *Bam*HI and fragments were separated on 0.7% agarose gels without ethidium bromide and transferred to Hybond-N^+ membranes (Amersham) by semidry electroblotting (Bio-Rad). The probe (fragment 1-2DKO) was labeled with the AlkPhos Direct system (Amersham Biosciences) and hybridized overnight at 60 °C. Signal detection was done with the CDP-*Star*™ system (Amersham Biosciences).

Preparation of protein fractions. Log phase liquid cultures of a *pknD* mutant and the WT parental strains were harvested by centrifugation at 2500*g* at 4 °C for 15 min. The pellets were washed three times in ice-cold sterile PBS (pH 7.5) and resuspended (∼1–2 ml/g) in lysis buffer [20–50 mM Tris, pH 7.5, and complete protease inhibitor cocktail (Roche)]. The suspension was mixed (1:1) with zirconium beads and then subjected to three rapid freezing cycles in liquid nitrogen. This was followed by three, 3-min vortex cycles, each separated by 1 min intervals on ice, or three cycles of 25 s using a Mini-Beadbeater Cell Disruptor (Biospec Products) separated by 3-min intervals on ice. The total protein extract (E) fraction was sterilized by passage through 0.45 μm filters, and the cell wall associated protein (CW) fraction was subsequently separated as the insoluble fraction by centrifugation at 20000*g* at 4 °C for 20 min. The CW fraction was resuspended in lysis buffer in a volume equal to that of the E fraction. The Bradford assay [21] was used for protein quantification with BSA as protein standard. Samples were stored at −70 °C until use.

Western blots. The E fractions (40 μl) were separated on a 12% SDS–PAGE gel and transferred to nitrocellulose membrane by semidry electroblotting. The membrane was blocked overnight with 5% skim milk in PBS (pH 7.5) + Tween 20 (0.01%) (PBST) with mild shaking at 4 °C. The blot was washed with PBST and then incubated for 2–3 h at room temperature in PBST + 1% skim milk with a 1:1000 dilution of rabbit anti-PknD antibody, the generous gift of Drs. Pedro Alzari and Stewart Cole, Pasteur Institute, Paris. The blot was then washed three times, 20 min each, in PBST and then incubated in PBST with 1/5000 goat anti-rabbit IgG HRP-conjugated antibody for 1 h. After three 15 min-washes with PBST, the blot was developed with 1-Step TMB-blotting (Pierce).

In vitro protein kinase assays. The kinase assay was performed at room temperature for 30 min in a 50 μl total volume containing 40 μl of protein sample (E and CW fractions), with or without Histone IIA (2 mg/ml) (Sigma), and 10 μl kinase buffer [20 mM Tris, pH 7.5, 2.5 mM $MnCl_2$, and 50 μM [γ-^{32}P]ATP (3000 cpm/pmol, Amersham or New England Nuclear)] [22]. The reaction was stopped by addition of Laemmli sample buffer [23], heated at 100° for 5 min, and then resolved by SDS–PAGE (8–15% continuous gradient gels). Following electrophoresis, gels were stained with 0.5% Coomassie brilliant blue and dried. Radioactively labeled, presumably phosphorylated, proteins were visualized by autoradiography.

Two-dimensional analysis of kinase activity of cell wall associated protein fraction. One hundred microliter of the CW fractions (in lysis buffer) was added to 2.5 mM $MnCl_2$ plus 50 μCi [γ-^{32}P]ATP. After 30 min at room temperature, the reaction was stopped with 150 μl of rehydration buffer [8 M urea; 0.5% (p/v) CHAPS; 0.2% (p/v) DTT; 0.5% (v/v) Buffer IPG 4-7; 0.002% bromophenol blue]. The first dimension electrophoresis was performed on a Ettan™ IPGphor Isoelectric Focusing system (Pharmacia Biotech) using pH 4–7 Immobiline Drystrip (13 cm) (Amersham Biosciences) and following standard first dimension conditions (rehydration for 14:00 h at 20°C; 50 μA/strip followed by 150V, 300Vh; 500V, 500Vh; 1000V, 1000Vh; separation at 8000V, 24000Vh).

The second dimension electrophoresis was performed on 10% SDS–PAGE gels: the strips were equilibrated for 15 min in 10 ml of buffer [2% SDS; 50 mM Tris–HCl, pH 8.8; 6 M urea; 30% (v/v) glycerol; 0.002% bromophenol blue; 100 mg DTT], placed on the gels, and sealed with 1% agarose. After electrophoresis the gels were soaked for 30 min (20%

methanol, 10% acetic acid, 2% glycerol) and dried. The radioactivity was detected with a PhosphorImager® SI system (Molecular Dynamics).

Protein spots digestion. Spots were excised from a dried 2D gel, cut into smaller pieces and washed overnight with 1 M NH_4HCO_3 in 200 µl of 20% (v/v) acetonitrile, at room temperature. On the following day, the wash solution was discarded and 200 µl of 50% methanol (v/v) and 5% (v/v) acetic acid were added for 2 h. Afterwards, the pieces were repeatedly washed with 20% (v/v) acetonitrile, 1 M NH_4HCO_3 until staining was removed. Subsequently, 200 µl of 100% acetonitrile solution was added for 5 min and the pieces were completely dehydrated in a vacuum centrifuge for 2–3 min. Then, 20 µl of 10 mM DTT was added for 30 min at room temperature followed by protein alkylation for 30 min using 30 µl of 100 mM iodoacetamide in 100 mM NH_4HCO_3. Dehydration of the pieces was carried out by dissolving again in 200 µl of 100% acetonitrile for 5 min at room temperature. The gel pieces were rehydrated on ice for 10 min in 50 mM NH_4HCO_3, and then 30 µl of 20 ng/µl trypsin was added for overnight tryptic digestion at 37 °C. Extraction of peptides was performed by microcentrifugation using 30 µl NH_4HCO_3. Afterwards 30 µl of 10% formic acid was used for washing the remnant gel pieces. The volume of the extracts was reduced to 20 µl by evaporation in a vacuum centrifuge at room temperature.

Peptide analysis. Peptides obtained from the digestion were subjected to ESI by direct injection in a 4000 Q Trap® LC/MS/MS (Applied Biosystems). Results were analyzed using Mascot Software (www.matrixscience.com). ScanProsite (http://ca.expasy.org/tools/scanprosite/) was used to predict phosphorylation sites of MmpL7, and transmembrane segments were predicted with the TMHMM Server v 2.0 (www.cbs.dtu.dk/services/TMHMM/).

Results

Construction and confirmation of a pknD mutant strain of M. tuberculosis

Using the specialized transducing phage system [17], the kinase domain of *pknD* (amino acids 100–247) was replaced with a hygromycin resistance cassette in strain 2D3, a clinical isolate of *M. tuberculosis*. The presence of the inserted cassette was confirmed by DNA amplification and Southern blotting (Fig. 1). Western blotting with polyclonal anti-PknD antibody showed that the mutant, strain KOD5, lacked the expected 69 kDa band, corresponding to the PknD seen in the parent MT2D3 (results not shown).

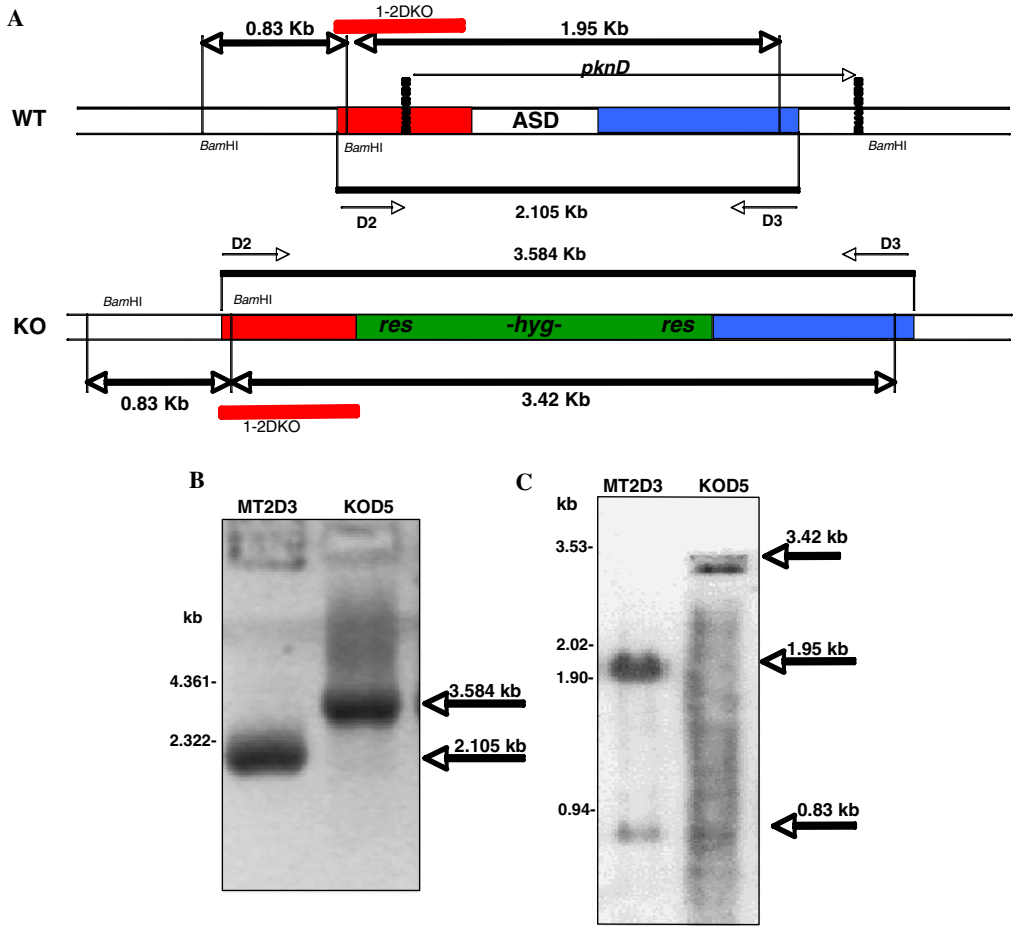

Fig. 1. Construction and demonstration of *pknD* mutant strain. (A) The kinase (ASD) domain of *pknD* was replaced with a hygromycin resistance cassette using a specialized transducing phage [17]. The red and blue segments were cloned into the transducing phage for allelic exchange. The dotted vertical lines delineate the *pknD* coding region in the WT. (B) DNA amplification of a lysate of WT MT2D3 with primers D2 and D3 yielded the expected 2.1 kb fragment, while the 3.58 kb fragment obtained from an KOD5 lysate confirmed the insertion of the hygromycin resistance cassette. (C) Southern blot of chromosomal DNA digested with *Bam*HI hybridized to the 691 bp fragment 1-2DKO. WT strain MT2D3 and mutant KOD5 both show the expected 0.83 kb fragment, while the WT 1.95 kb fragment in MT2D3 increases to 3.42 kb in KOD5 due to the presence of the hygromycin resistance cassette.

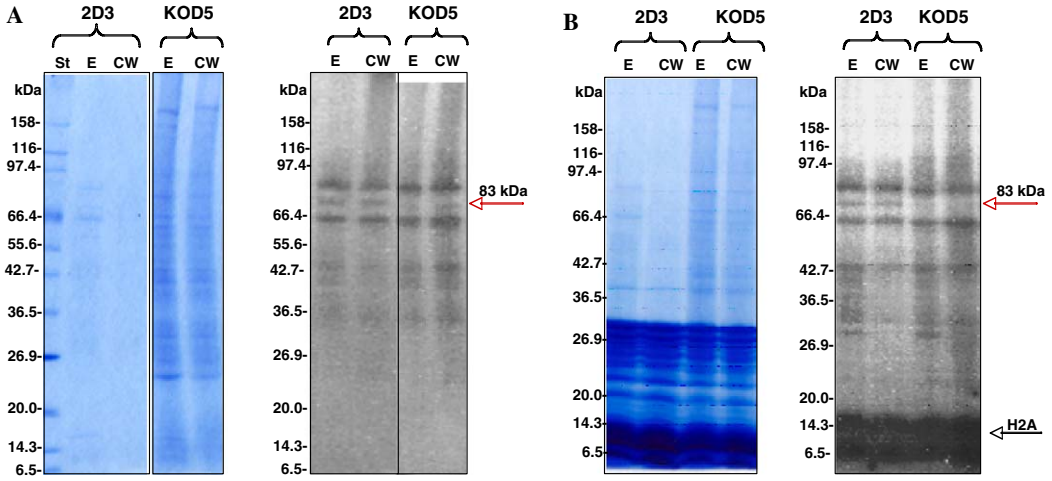

Fig. 2. One-dimensional analysis of the phosphorylation of endogenous substrates and histone IIA in protein fractions of MT2D3 and KOD5. Protein lysates of the total extract (E) or cell wall associated fraction (CW) were incubated with [γ-^{32}P]ATP and 2.5 mM $MnCl_2$ for 30 min at room temperature and then separated on gradient PAGE gels (8–15%). (A) Shows phosphorylation of endogenous proteins; (B) shows phosphorylation of added substrate histone IIA. On the left are the Coomassie blue stained gels, whose autoradiographs are on the right. The total proteins loaded were: MT2D3 (70 μl): 2.1 μg (E); 0.22 μg (CW). KOD5 (40 μl): 4.7 μg (E); 3.9 μg (CW).

Protein phosphorylation

In order to identify substrates for PknD, phosphorylation reactions were carried out with the total lysates and also the cell wall fractions of both the WT and PknD mutant strain (Fig. 2). Several phosphorylated bands were seen in the protein fractions from both strains, but the intensity of a band of approx. 83 kDa, present in the cell wall fraction of the WT, was considerably decreased in that of the PknD mutant strain KOD5, suggesting that it is a possible substrate for this kinase. Added histone IIA, a common substrate for kinases, was equally phosphorylated in the WT and mutant strains, indicating that, if phosphorylated by PknD, it is also efficiently phosphorylated by other *M. tuberculosis* kinases.

The band corresponding to the phosphorylated protein(s) of circa 83 kDa was less intense in the *pknD* mutant than in the wild-type strain, but was still visible. To further analyze this result, and determine whether the residual band seen in the mutant represented the same protein, phosphorylation reactions using the cell wall fractions were run on two-dimensional gels and detected with a Phosphorimager (Fig. 3). Several phosphorylated protein spots were less intense in the mutant compared to the WT (blue circles), while one group of spots appeared more intense in the mutant (green circles). However, the most striking difference was the absence of a group of spots corresponding to proteins with a size of approx. 80–90 kDa, (red circles) which is likely to be the same protein that was decreased in the 1D gel analysis of the mutant lysate phosphorylation.

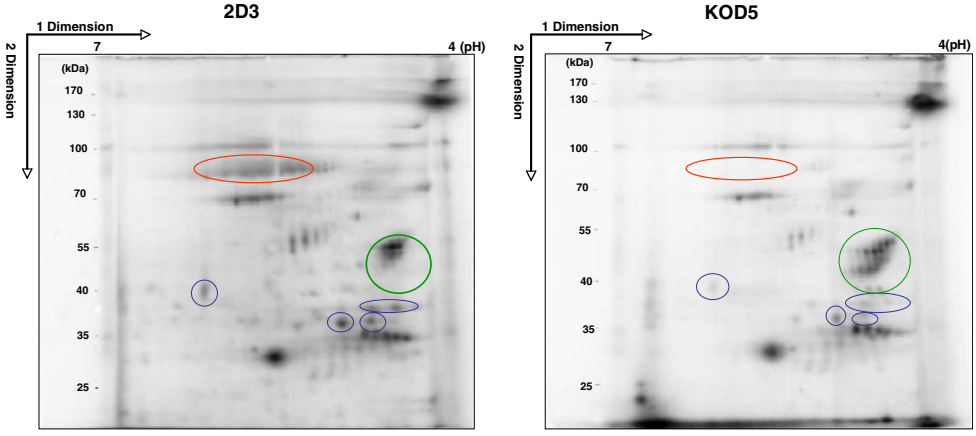

Fig. 3. Comparison of two-dimensional analysis of the phosphorylation of endogenous substrates. Kinase reactions using cell wall associated protein fractions (5–10 μg) of the WT MT2D3 and mutant KOD5 were separated on 2D gels (pH 4–7; 10% SDS–PAGE) and the autoradiographs compared. Phosphorylated proteins completely absent in the mutant are circled in red, those that decreased in the mutant are circled in blue, and those that were increased in the mutant are circled in green.

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  1 MPSPAGRLHR IRYIRLKKSS PDCRATITSG SADGQRRSPR LTNLLVVAAW
 51 VAAAVIANLL LTFTQAEPHD TSPALLPQDA KTAAATSRIA QAFPGTGSNA
101 IAYLVVEGGS TLEPQDQPYY DAAVGALRAD TRHVGSVLDW WSDPVTAPLG
151 TSPDGRSATA MVWLRGEAGT TQAAESLDAV RSVLRQLPPS EGLRASIVVP
201 AITNDMPMQI TAWQSATIVT VAAVIAVLLL LRARLSVRAA AIVLLTADLS
251 LAVAWPLAAV VRGHDWGTDS VFSWTLAAVL TIGTITAATM LAARLGSDAG
301 HSAAPTYRDS LPAFALPGAC VAIFTGPLLL ARTPALHGVG TAGLGVFVAL
351 AASLTVLPAL IALAGASRQL PAPTTGAGWT GRLSLPVSSA SALGTAAVLA
401 ICMLPIIGMR WGVAENPTRQ GGAQVLPGNA LPDVVVIKSA RDLRDPAALI
451 AINQVSHRLV EVPGVRKVES AAWPAGVPWT DASLSSAAGR LADQLGQQAG
501 SFVPAVTAIK SMKSIIEQMS GAVDQLDSTV NVTLAGARQA QQYLDPMLAA
551 ARNLKNKTTE LSEYLETIHT WIVGFTNCPD DVLCTAMRKV IEPYDIVVTG
601 MNELSTGADR ISAISTQTMS ALSSAPRMVA QMRSALAQVR SFVPKLETTI
651 QDAMPQIAQA SAMLKNLSAD FADTGEGGFH LSRKDLADPS YRHVRESMFS
701 SDGTATRLFL YSDGQLDLAA AARAQQLEIA AGKAMKYGSL VDSQVTVGGA
751 AQIAAAVRDA LIHDAVLLAV ILLTVVALAS MWRGAVHGAA VGVGVLASYL
801 AALGVSIALW QHLLDRELNA LVPLVSFAVL ASCGVPYLVA GIKAGRIADE
851 ATGARSKGAV SGRGAVAPLA ALGGVFGAGL VLVSGGSFSV LSQIGTVVVL
901 GLGVLITVQR AWLPTTPGRR
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Fig. 4. Peptides identifying MmpL7. The amino acid sequence of MmpL7 is shown with the approximate domains 1 and 2 underlined (predicted by TMHMM). The amino acids in green are the phosphorylated polypeptides identified by ESI from spots cut from a 2D gel of an *in vitro* phosphorylation reaction using the cell wall fraction of WT 2D3. The amino acids in red were identified by ESI but not phosphorylated. The spots analyzed were not observed in the 2D gel of an *in vitro* phosphorylation reaction using the cell wall fraction of the mutant KOD5. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

The complete absence of this cluster of spots, presumably different phosphorylation states of the same protein, on the 2D gels of the mutant, suggested that the residual band seen in the region of 83 kDa in the 1D gel of the mutant represented a different protein.

Substrate identification

The 80–90 kDa phosphorylated spots were excised from the 2D gel of a cell wall fraction of the WT 2D3 strain, digested with trypsin and subjected to ESI-MS. *In silico* analysis showed that five distinct, non-overlapping peptides, four of which were phosphorylated, matched a conserved transmembrane transport protein termed MmpL7 (Accession No. gi:15610079), giving a Mascot score of 60 ($p < 0.05$) (Fig. 4). This protein is predicted to contain 12 transmembrane segments, but the phosphorylated peptides identified correspond to the N-terminal extreme or domain 2, neither of which is predicted to form part of a transmembrane region.

Discussion

Starting with a virulent clinical isolate of *M. tuberculosis*, we constructed a mutant lacking the *pknD* kinase domain, and used protein fractions of the wild-type and of the mutant strains to perform *in vitro* phosphorylation reactions. Compared to the cell wall fraction of the WT parent, a series of phosphorylated spots, likely different phosphorylation states of the same protein, was notably absent on 2D gels of the *pknD* mutant. This family of spots was identified by mass spectrometry as MmpL7, a membrane bound transporter. Although the MmpL7 peptides were obtained from a cell wall fraction, this fraction was probably heavily contaminated with membrane proteins.

MmpL7 and the other members of the MmpL (mycobacteria membrane protein large) family [24] belong to the RND (resistance, nodulation, and cell division) family of transporters [25]. These proteins consist of 12 transmembrane domains (TMD) and two loops located between the first and second, and seventh and eighth TMDs. Structural analysis of the RND transporter AcrB found that it associates as trimers to form a transmembrane pore [26]. The *M. tuberculosis* genome contains 13 genes encoding MmpL family proteins. Five of these are located adjacent to genes coding for proteins involved with either fatty acid or polyketide synthesis, suggesting that they may be involved in the transport of the products of these genes. MmpL7 was determined to be essential for virulence [27], presumably because it transports phthiocerol dimycoceroserate (PDIM) [28] and a related but distinct phenolic glycolipid [29]. MmpL8 transports sulfolipid –N, which is a precursor of sulfolipid 1, and likely also transports other lipidic elements of the cell wall [30] that are important for virulence [31]. A study of the various MmpL proteins identified four (MmpL7, MmpL4, MmpL11, and MmpL8) that were essential for full virulence [24], while a separate genome-wide screen determined that mutants in any of six members of the family had compromised virulence (MmpL7, MmpL4, MmpL11, MmpL8, MmpL5, and MmpL10 [32].

Mass spectrometry found that four of the five MmpL7 peptides identified were phosphorylated. Neither the MmpL proteins, nor other RND transporters have been reported to be phosphorylated, but analysis with the protein motif finding program ScanProsite, even at high stringency, predicts that MmpL7 and the other MmpL transporters contain serine or threonine phosphorylation sites. This suggests that phosphorylation could regulate the deposition of the complex mycobacterial cell wall, or at least the transport of its components through the cell

membrane. In *Streptomyces*, some of the 34 S/T kinases appear to regulate the synthesis of polyketide secondary metabolites [33]. In the closely related mycobacteria, many of the abundant polyketide synthesis-like enzymes appear to be involved in the synthesis of the fatty acid components of the cell wall, so some regulation by the S/T kinases of cell wall synthesis or assembly would not be surprising. The interaction of the forkhead region of ABC transporter Rv1747 with different kinases also sets a precedent for the regulation of transporters by the S/T kinases in mycobacteria [34].

Three of the four phosphorylated peptides identified by mass spectrometry were located in MmpL7 domain 2, which lies between the seventh and eighth TMDs. Based on the solved structure of the RND transporter AcrB [26], MmpL7 domain 2 is predicted to be external to the membrane, so it is hard to envision how it can be phosphorylated by the cytoplasmic kinase domain of PknD, or by any other Ser/Thr kinases of *M. tuberculosis*. It was recently reported that MmpL7 domain 2 associates with PpsE, one of the polyketide synthases involved in the production of PDIM [35]. As the PpsE enzyme is presumed to be cytoplasmic, Jain and Cox [35] proposed different ways in which the two proteins might associate. Based on the structure of AcrB, an MmpL7 trimer creates a pore in the membrane, through which domain 2 of MmpL7 could possibly reenter the cytoplasm to interact with PpsE. Alternatively, the PpsE protein could traverse the pore to gain access to the extracellular domain 2 of MmpL7. Lastly, the structure of MmpL7 may differ from that of the model RND transporter AcrB, and domain 2 could perhaps be located in the cytoplasm. These possibilities could also apply to a putative association of MmpL7 with PknD, but if domain 2 stably remains outside of the membrane, the regulatory pathway must be more complex [36]. As yet, we have no confirmatory evidence that PknD directly phosphorylates MmpL7—it could be indirect through a phosphorylation cascade, or the KOD5 mutant strain could contain another, unidentified mutation affecting the true MmpL7 kinase. Studies to define the relationship of MmpL7 and PknD are currently in progress.

Any regulatory aspect of phosphorylation depends on the kinase activity of PknD being modulated, presumably when a ligand binds its extracellular β propeller sensor. While it is tempting to imagine that the PDIM content of the cell wall could be detected, the PknD ligand remains to be defined, as is the structural basis explaining how binding to this domain would modulate the kinase activity. While the functional significance of the apparent phosphorylation of MmpL7 is unknown, the many reports that MmpL7 plays a key role in the transport of important cell wall components, and the evidence presented here that it is phosphorylated, suggest that the serine/threonine protein kinases of *M. tuberculosis* could play a regulatory role in the construction, and perhaps composition of its complex cell wall.

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