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Components of Eukaryotic-like Protein Signaling Pathways in *Mycobacterium tuberculosis*

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ABSTRACT

Eukaryotic-like protein kinases have been identified recently in several prokaryotes by comparative studies of DNA sequences and Western blotting techniques using antiphosphoprotein antibodies. Examination of the *Mycobacterium tuberculosis* genome by means of PCR amplification with consensus primers, Southern hybridization, and comparative analysis of DNA sequences with genomic databases revealed the existence of at least seven eukaryotic-like protein kinases in this pathogen. In addition, we report the biochemical identification of phosphorylated proteins in *M. tuberculosis*. Taken together, these findings show that *M. tuberculosis* possesses elements of cell signaling similar to those observed in eukaryotic organisms. We suggest that some of these processes may play roles in the pathogenesis of *M. tuberculosis*.

INTRODUCTION

Among infectious diseases, tuberculosis (TB) is considered to be the leading cause of death in the world, and since the late 1980s, there has been a significant reemergence of TB in industrialized nations. Many of these cases were shown to involve multiple antibiotic-resistant forms, which clearly emphasizes the urgent need for effective new treatments based on an understanding of the disease. The causative pathogen of TB, *Mycobacterium tuberculosis*, is a facultative intracellular pathogen, and its incorporation into macrophages and subsequent ability to avoid host-determined destruction is believed to be critical to its virulence. The response of the host immune system to *M. tuberculosis* infection is complex and involves T cell lymphocytes, mononuclear phagocytes, and cytokines (McDonough et al., 1993). In response, *M. tuberculosis* has been shown to have the ability to subvert the normal immune response mechanisms of the host such that the survival of the pathogen may be enhanced. Preventing lysosomal acidification (Sturgill-Koszycki et al., 1994), inhibition of antigen processing (Moreno, 1988), inhibition of interferon-γ (IFN-γ) activation (Sibley et al., 1988), and inhibition of protein kinase C (PKC) (Chan et al., 1991) are all considered to be survival strategies of pathogenic mycobacteria. Such mechanisms of protection are likely to rely on adaptive responses to external signals.

Protein phosphorylation is a major posttranslational modification used by organisms to transduce extracellular signals into cellular functions. Currently, the best defined molecular system responsible for stimu-

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lus response in bacteria is the two-component system consisting of receptor histidine protein kinase sensors and their associated response regulators (Stock et al., 1989). These two-component systems regulate a variety of bacterial responses, including chemotaxis, phototaxis, osmosis, and nitrogen fixation (Stock et al., 1989). These systems have been identified in *M. tuberculosis* (Philipp et al., 1996; Via et al., 1996). In comparison, eukaryotic protein phosphorylation occurs predominantly on serine, threonine, or tyrosine residues. These covalent modifications have been shown to regulate cellular processes, such as cell signaling, mitosis, differentiation, survival, and cell death (Hunter, 1995; Sun and Tonks, 1994). Although serine/threonine and tyrosine kinases were once considered to be unique to eukaryotes, evidence from phosphoprotein antibody studies and the recent accumulation of bacterial nucleotide sequence information have shown increasing number of prokaryote species to contain eukaryotic-like protein kinases (Cortay et al., 1986; Kennelly and Potts, 1996; Chow et al., 1994; Frasch and Dworkin, 1996; Waters et al., 1994; Zhang et al., 1996; Zhang, 1996).

It was shown previously that *M. tuberculosis* and other pathogenic mycobacteria possess proteins phosphorylated on tyrosine, as detected with a monoclonal antibody specific for phosphotyrosine (Chow et al., 1994). These findings provided some of the first evidence of eukaryotic-like cell signaling in bacteria. Using the same approach, tyrosine-phosphorylated proteins were subsequently identified in several species of *Streptomyces* (Waters et al., 1994) and in *Myxococcus xanthus* (Frasch and Dworkin, 1996). Eukaryotic-like protein phosphorylation has been shown to be an essential component of virulence in *Yersinia pseudotuberculosis*, where a secreted serine/threonine kinase has been identified (Hakansson et al., 1996). In this report we show, by analysis of available *M. tuberculosis* genome sequences, that there are at least seven genes likely to be involved in mycobacterial signal transduction based on their similarity to eukaryotic-like protein kinase genes. These studies strengthen the notion that phosphorylated cell signaling elements, once considered to be unique to eukaryotes, are present in *M. tuberculosis*. It is thus tempting to speculate that the pathogenesis of tuberculosis may involve modification of host cell signaling processes and, more specifically, that the signal transduction mechanisms of macrophages required for their activation are disrupted or altered by *M. tuberculosis* infection.

MATERIALS AND METHODS

Strains, Growth Medium, and Sample Preparation

Cultures of *M. tuberculosis* H37Rv (strain 107) were grown in Proskauer-Beck plus Tween (PB&T) medium (0.05% Tween-80) and expanded fivefold for 2 weeks. Cells were harvested by centrifugation for 5 min at 3750*g*. Cell culture medium was filtered twice through Nunc 0.22 μm filters and stored at -70°C until assayed. Cell pellets were washed once in lysis buffer (20 mM Tris, pH 7.5, 2 mM EDTA, 150 mM NaCl, 0.2 mM PMSF, 1 μg/ml pepstatin A, 1 μg/ml aprotinin, 0.5 mM sodium orthovanadate, 0.2 mM sodium molybdate), resuspended in the same buffer, and then subjected to mechanical bead breakage (Mini-Beadbeater, Biospec Products) for 5 min at ambient temperature using 100 μm zirconium beads (Sigma). Cell debris was removed by centrifugation at 10000*g* for 5 min. The resulting supernatant was filter sterilized (Millex 0.22 μm, Millipore) and stored in aliquots at -70°C.

Gel Electrophoresis

SDS-PAGE gels (12%) were prepared according to the method of Laemmli (1970). The gels were stained with Coomassie Blue R-250 or silver staining methods. Protein concentrations were determined by bicinchoninic acid method (Sigma).

In Vitro Kinase Assay

Mycobacterial culture medium (500 μl) or a volume of cell extract corresponding to approximately 20 μg total protein was filter centrifuged for 70 min at 14,000*g* in 10,000 Da cutoff microconcentrators (Microcon 10, Amicon). Proteins were resuspended in 15 μl kinase buffer (20 mM Pipes, pH 7.2, 10 mM $MnCl_2$, 10 mM $MgCl_2$), and the reaction was initiated by the addition of 10 μCi [γ-^{32}P]ATP. Incubation

at ambient temperature for 15 min was stopped by the addition of 3× Laemmli sample buffer, followed by boiling for 5 min and resolution on 12% SDS-PAGE gels. The gels were washed and dried and exposed to Kodak X-OMAT/AR film.

Cloning and Gene Manipulations

Genomic DNA of mycobacteria was prepared as described (Marklund et al., 1995). Plasmid DNA was isolated using the alkaline lysis procedure. Cosmid DNA preparations employed standard procedures (Sambrook et al., 1989). Analysis of restriction digest products was carried out on agarose gels as described (Sambrook et al., 1989). Competent *Escherichia coli* cells were prepared using the $CaCl_2$ procedure (Sambrook et al., 1989). Electrocompetent cells were prepared and transformed with the Gene Pulser apparatus according to the manufacturer's protocol (BioRad).

Oligonucleotide Primers

The following primers were designed to amplify *M. tuberculosis* protein kinase genes: PKI, 5′-GT[C/G]CACC[G/T/C]GGACCT[C/G]AA-3′, PKII, 5′-A[G/C][C/T]TC[G/C] GG[G/C] GGC ATG TA-3′. Oligodeoxynucleotides were synthesized on a Beckman Oligo 1000 synthesizer.

DNA Labeling and Hybridization

DNA was transferred to nylon filters (Hybond N, Amersham) and hybridized to probes obtained from PCR reactions. Probes were labeled using the digoxigenin random priming method (Boehringer-Mannheim).

DNA Sequencing and Sequence Analysis

Nucleotide sequencing was performed using the dideoxy chain termination procedure by direct sequencing from double-stranded DNA using an ABI 373A automated DNA sequencer. Protein sequences were accessed from the PIR or Swiss-Prot data banks using the EBI and NCBI search engines. Sequence analysis was performed with the Genetic Data Environment (GDE) software package, version 2.2 (S. Smith, University of Illinois and Harvard University). Paired sequence alignments and multiple sequence alignments were carried out using the FASTA program or the Clustal-V algorithm in the GDE package.

Nucleotide Sequence Accession Number

The sequence reported in this work has been submitted to EMBL and assigned the accession number X99342 for PKI of *M. tuberculosis*.

Polymerase Chain Reaction (PCR)

Taq DNA polymerase was purchased from BRL. Unless otherwise mentioned, reaction conditions were as follows: manufacturer's buffer, 200 nM each dNTP, 50–100 pmol of each primer, 10% DMSO, 1–50 ng template DNA, and 1–5 U Taq polymerase. Cycle conditions: 3′ 95°C (1×); 1′ 94°C; 2′ 55°C; 3′ 73°C (30×); 10′ 72°C (1×). The reaction mixture (10 μl) was electrophoresed to identify amplified products.

RESULTS

Identification of M. tuberculosis *Phosphorylated Proteins*

Phosphorylated proteins were detected by using a radioactive protein kinase assay. This assay showed that the culture medium of *M. tuberculosis* contained a 30 kDa phosphoprotein that was absent in *Mycobacterium intracellulare* and BCG (Fig. 1). Cell-free extracts of *M. tuberculosis* contain four phosphorylated proteins of 18, 38, 55, and 66 kDa. Those less than 55 kDa differ in size from those of *M. intracellulare* and BCG. The fact that the 30 kDa protein appears predominantly in the culture medium strongly

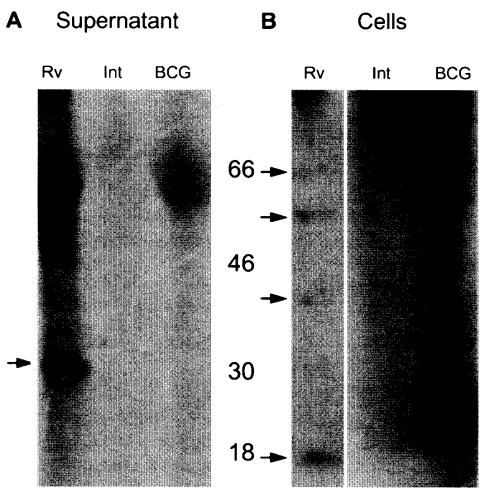

FIG. 1. In vitro kinase assay of *M. tuberculosis* cell extract and culture medium. **A.** PB&T medium after 21 days growth of *M. tuberculosis* H37 (Rv), *M. intracellulare* (int) and BCG. **B.** Cell-free extract of the same strains. The samples were incubated with [γ-^{32}P]ATP as described in Materials and Methods.

suggests that it is a secreted protein and not an artifact of cell lysis. This 30 kDa protein was subsequently identified as a serine phosphorylated protein (D. Ng and K. Chow, unpublished observations).

M. tuberculosis *Genome Analysis by PCR and Southern Hybridization*

The catalytic domain of eukaryotic protein kinases has been identified to be about 300 amino acids long and lies near the carboxyl terminus in most single subunit enzymes, with the amino terminus being devoted mainly to regulatory and subcellular positioning roles. Within the catalytic domain, 11 major conserved subdomains are evident (Hanks and Lindberg, 1991). We have used PCR to identify genes coding for eukaryotic-like protein kinases in *M. tuberculosis*. Mixed degenerate oligonucleotides, designed for a ~200 bp region in domains VI and VII (Hanks and Lindberg, 1991), taking into consideration the sequences of known bacterial ser-thr kinases and the mycobacterial high GC content, were used as primers to amplify the phosphoprotein gene fragments (Fig. 2). Of the three PCR products detected, two, the 150 bp and the 450 bp fragments, were cloned and sequenced. The 150 bp fragment (PKI) showed 97% identity to *afs*K from *Streptomyces coelicolor* and can be considered a mycobacterial homolog of this signaling kinase (accession No. X99342). The 450 bp fragment was identified as a fragment of cholesterol oxidase (*cho*D, accession No. X99343). The homology between PKI and *cho*D is limited to the DNA level. Southern hybridization with the PCR fragment, PKI, confirmed its presence in the *M. tuberculosis* chromosome and the cosmid library used for the *M. tuberculosis* genome sequencing project (data not shown).

Other Eukaryotic-Like Protein Kinases in Mycobacteria

A cluster of two putative eukaryotic-like serine/threonine protein kinases genes, *pkn*A and *pkn*B (Table 1) can be identified in the *Mycobacterium leprae* genome sequence database. They code for proteins of approximately 25 kDa and 32 kDa, respectively. This gene cluster is located on cosmid B1770, which was provided by Dr. S. Cole. The *pkn*A–B gene cluster was amplified from the cosmid DNA by PCR and used as a hybridization probe to detect homologous sequences in several mycobacterial genomes. As seen in Fig-

FIG. 2. PCR amplification of *M. tuberculosis* genomic DNA using PKI and PKII mixed oligonucleotide primers. DNA agarose electrophoresis of (1) reaction mix containing both primers with no template DNA, (2) complete reaction mix with *M. tuberculosis* H37Rv genomic DNA, (3) complete reaction mix without PKI primer, and (4) complete reaction mix without PKII primer.

ure 3, mycobacterial genomic DNAs hybridized under stringent conditions to the *M. leprae* probe, identifying the presence of at least one additional eukaryotic-like protein kinase in these organisms.

Scrutiny of the ongoing *M. tuberculosis* genome sequencing project (B. Barrell, Sanger Center Cambridge, UK) has thus far provided evidence of the presence of seven eukaryotic-like protein kinases. Table

TABLE 1. SUMMARY OF GENES FROM *MYCOBACTERIUM* SPECIES BELONGING TO EUKARYOTIC-LIKE PROTEIN KINASE FAMILY

Organism	*Name*	*Accession No.*	*P site*	*Size (aa)*	*Source*
M. tuberculosis	PKY38	Z74697	ser-thr	586	Sanger Center
M. tuberculosis	PKY50	Z77137	ser-thr	616	Sanger Center
M. tuberculosis	PKY49	Z73966	ser-thr	590	Sanger Center
M. tuberculosis	PK1	X99342	ser-thr-tyr	n/d	This work
M. tuberculosis	PknB	Z80233	ser-thr	626	Sanger Center
M. tuberculosis	PknA	Z80233	ser-thr	431	Sanger Center
M. tuberculosis	Pkn12A	Z81360	ser-thr	566	Sanger Center
M. tuberculosis	Pkn12B	Z81360	ser-thr	476	Sanger Center
M. leprae	PknB	Z70722	ser-thr	315	Philipp et al.
M. leprae	PknA	Z70722	ser-thr	253	Philipp et al.

n/d, not determined.

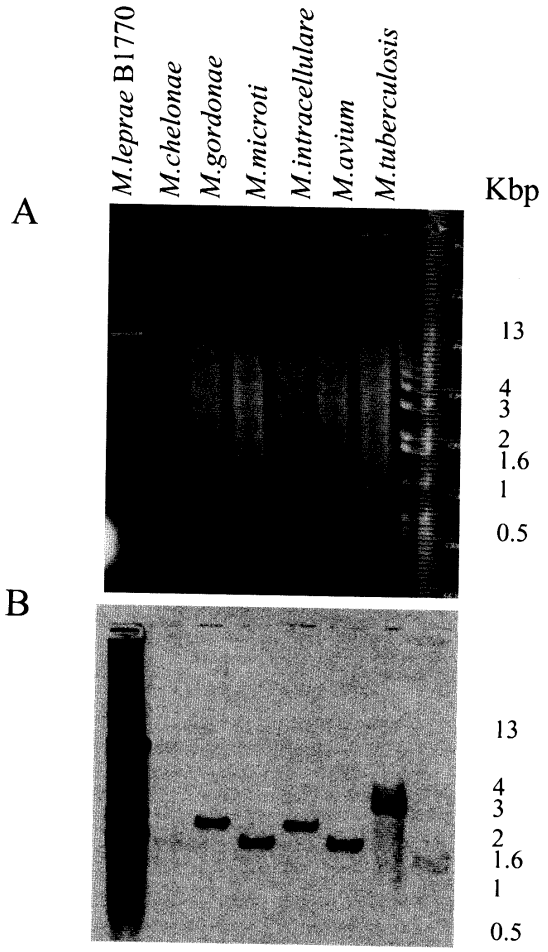

FIG. 3. Southern blot analysis of mycobacterial genomic DNAs using *pkn*A and *pkn*B from *M. leprae* as probes. A 2 kb PCR product of the *M. leprae* protein kinase gene cluster containing both genes was used as a probe for identification of homologous genes in other mycobacterial intracellular pathogens. Hybridization was performed at 65°C, and washings and detection were performed as described in Materials and Methods. **A.** Mycobacterial genomic DNAs digested with the restriction enzyme *Pst*I. **B.** The corresponding Southern blot.

1 summarizes the derived protein sequences. Five genes code for proteins of approximately 60 kDa, and two code for 45 kDa proteins. The amino acid sequences encoded by these genes do not correspond to PKI, mentioned previously or, in size, to the 30 kDa phosphoprotein detected in the culture medium. Figure 4 shows multiple alignments of the catalytic domains of the putative *M. tuberculosis* protein kinases relative

to known bacterial protein kinases. This comparison demonstrates that these kinases share significant regions of similarity. Furthermore, a phylogenetic comparison (Fig. 5) indicates that there are at least four major structural classes among the bacterial eukaryotic-like kinases.

DISCUSSION

It was shown previously that protein phosphorylation occurs in pathogenic mycobacteria, and it was, therefore, proposed that this process may play a role in host cell infection (Chow et al., 1994). In this re-

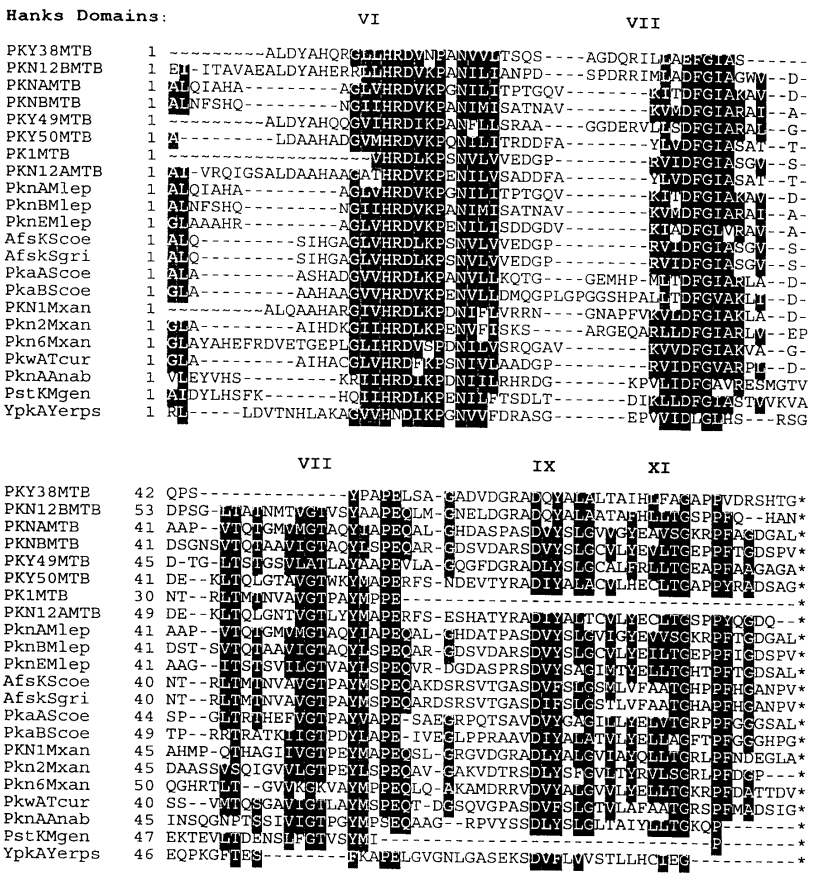

FIG. 4. Compiled protein alignment of *M. tuberculosis* protein kinases and various eukaryotic-like protein kinases from other bacteria. Domains 1–10 according to Hanks nomenclature were aligned using the CLUSTALV program with PAM 250 weighting maxtrix and with fixed and floating gap penalties of 10. Protein kinases shown in this figure can be found in the data banks.

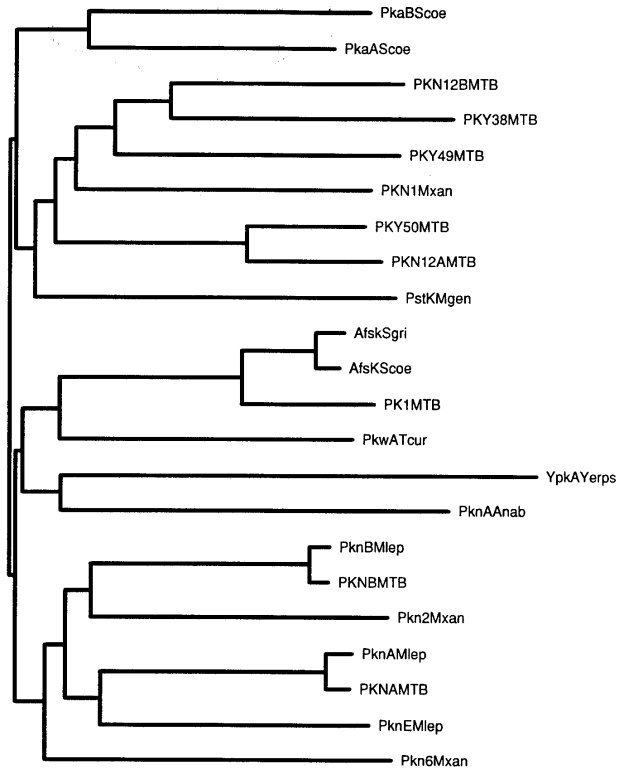

FIG. 5. Catalytic domain phylogeny of the eukaryotic-like protein kinases from prokaryotes. The alignment file presented in Figure 4 was used to create a phylogenetic tree using DeSoete tree fit with no distance corrections.

port, we provide further evidence for the presence of an extensive group of eukaryotic-like protein kinases and related signaling pathways in *M. tuberculosis*. Biochemical assays and genomic data analysis provide clear evidence for the existence of phosphoproteins in *M. tuberculosis* at both the genetic and protein levels. These findings extend the list of eukaryotic-like protein functions described in prokaryotes (Kennelly and Potts, 1996; Zhang, 1996).

It has been shown that eukaryotic-like phosphoproteins are present in a variety of bacteria. In *Streptomyces* and *Myxococcus* spp., protein tyrosine and serine phosphorylation have been associated with the growth and developmental cycle (Ueda et al., 1996; Waters et al., 1994; Zhang et al., 1996). Interestingly, mycobacteria (although members of the Actinomycetaceae family) do not possess the complex developmental growth cycle typical of this family. Consequently, the protein kinases we have identified may be involved in other aspects of gene regulation. Specifically, we hypothesize a role for these signaling elements in the pathogenicity or dormancy of *M. tuberculosis*. In particular, these elements may provide a means for *M. tuberculosis* to adapt to a hostile host environment by subverting normal immunologic responses.

We have described the identification of eukaryotic-like protein kinases by PCR, Southern hybridization, and data analysis of sequences provided by the *M. tuberculosis* genome sequencing project. Seven genes coding for deduced phosphoproteins were detected in the *M. tuberculosis* nucleotide database. This supports the biochemical demonstration that at least five phosphorylated proteins are present in *M. tuberculosis* extracts when in vitro tests are performed. Because of the limitations of the in vitro assay, we believe that there are more proteins capable of being phosphorylated in vivo. Southern hybridization showed that *pkn*A/B homologs exist in *M. tuberculosis* as well as in other intracellular mycobacterial pathogens. This is confirmed by results from the *M. tuberculosis* genome sequencing project. PCR amplification using degenerate primers for protein kinase conserved domains identified a homolog of *afs*K, a eukaryotic-like *Streptomyces* kinase. There is a striking similarity between the PCR product PKI and *afs*K. The latter is known to be involved in differentiation and secondary metabolite production in *Streptomyces*, and one could assume a related regulatory role in mycobacteria. Evaluation of the protein alignment data (Figs. 4 and 5) shows that there is considerable diversity among the bacterial protein kinases, not only between species but also between different kinases in the same organism (Fig. 5). The protein kinases of the two mycobacterial pathogens, *M. tuberculosis* and *M. leprae*, fall into two different clusters, as do those of *M. xanthus*. In addition, alignments appear to indicate relationships based on biochemical function rather than on taxonomic relationships. The consensus sequence patterns of the *M. tuberculosis* PKI PCR fragment and *Streptomyces griseus afs*K are more typical of eukaryotic dual specificity kinases that are phosphorylated on both serine and tyrosine residues. There is still no direct genetic evidence of tyrosine-specific kinase genes in bacteria, although there are increasing reports of proteins phosphorylated on tyrosine.

In the cases of pathogens, such as *Yersinia pseudotuberculosis, Salmonella typhimurium*, and enteropathogenic *E. coli* (EPEC), protein phosphorylation has been shown to affect host cell signal transduction pathways (Bliska and Falkow, 1993). The serine/threonine kinase, YpkA, of *Y. pseudotuberculosis* is translocated to the inner surface of the host cell plasma membrane (Hakansson et al., 1996). YpkA is a plasmid-encoded virulence factor involved in interference with the host cell signaling pathways. Another invasive bacterium, *Listeria monocytogenes*, alters host signaling by stimulating MAP kinase directly on attachment to epithelial cells (Tang et al., 1994). The effectiveness of *M. tuberculosis* as an intracellular pathogen likely depends on its ability to maintain infection by biochemical modification of the hostile host environment. Although the mode of infection by *M. tuberculosis* is quite different from that of the aforementioned pathogens, the involvement of intracellular and extracellular phosphorylation mechanisms in *M. tuberculosis* infection is highly likely. Phosphorylation may mediate *M. tuberculosis* inhibition of lysosome fusion with the phagosome (Hart et al., 1972) and vesicular proton-ATPase acidification (Sturgill-Koszycki et al., 1994). Interference with the host's normal immune response during mycobacterial infection may also be mediated by diversion of the host's signaling pathways. For instance, it has been shown that the addition of mycobacterial lipoarabinomannan to macrophage cultures causes inhibition of IFN-γ-mediated activation (Chan et al., 1991; Sibley et al., 1988). As IFN-γ-induced signal transduction is dependent on protein tyrosine phosphorylation (Müller et al., 1993), it is possible that a mycobacterial tyrosine kinase interferes in this pathway. Previous studies have also shown that priming/activation of macrophages by bacterial lipopolysaccharides (Weinstein et al., 1992) or the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (Pollock and Withnall, 1993) is mediated by tyrosine phosphorylation events executed by protein tyrosine kinases. In addition, identification of a serine-phosphorylated protein in cell culture medium of *M. tuberculosis* provides evidence for the possibility of the involvement of other *M. tuberculosis* phosphorylated proteins in interactions with the host cell. Serine-phosphorylated proteins are implicated in middle and late signal transduction events in eukaryotic cells (Hunter, 1995; Sun and Tonks, 1994). Interference with such stages has not been observed to date in macrophages infected with *M. tuberculosis*. This study identifies a potential *trans*-acting protein produced by *M. tuberculosis*.

Our results suggest that *M. tuberculosis* possesses signal transduction pathway related to those of eukaryotes, in addition to the known two-component system of bacteria. Identification of the mechanisms, receptors, and biochemical activities in both the bacteria and the host cell signaling pathways is required to determine if these cell signaling elements are involved in *M. tuberculosis* pathogenicity. We are in the process of examining *M. tuberculosis* phosphoprotein genes by means of their expression and biochemical characterization to further assess their role in *M. tuberculosis* physiology. Continued scrutiny of the *M. tu-*

berculosis nucleic acid sequence database will likely reveal additional protein kinases and associated phosphatases that play important roles in the virulence mechanisms of this major pathogen.

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