

The *Mycobacterium tuberculosis* 65-Kilodalton Antigen Is a Heat Shock Protein Which Corresponds to Common Antigen and to the *Escherichia coli* GroEL Protein

THOMAS M. SHINNICK,^{1,2*} MICHAEL H. VODKIN,³ AND JIM C. WILLIAMS^{3,4}

Division of Bacterial Diseases, Center for Infectious Diseases, Centers for Disease Control, Atlanta Georgia 30333¹; Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, Georgia 30322²; Rickettsial Diseases Laboratory, Airborne Diseases Division, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21701³; and Office of the Director of Intramural Research Programs, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892⁴

Received 9 September 1987/Accepted 30 October 1987

Monoclonal hybridoma antibodies directed against a 65-kilodalton (kDa) mycobacterial protein could detect similarly sized antigens in many other bacterial species. In *Pseudomonas aeruginosa*, the cross-reacting protein corresponded to a 62-kDa antigen that has been called Common Antigen. The mycobacterial 65-kDa antigen and Common Antigen are similar in that both (i) are highly immunoreactive molecules, (ii) contain species-specific and genus-specific epitopes in addition to the broadly cross-reactive epitopes, (iii) can be isolated as homomultimers of greater than 240 kDa, and (iv) have similar amino acid compositions. In *Escherichia coli*, the cross-reactive protein corresponded to the GroEL protein. Both the GroEL protein and the mycobacterial 65-kDa protein are expressed as heat shock proteins.

The diseases caused by mycobacteria, primarily tuberculosis and leprosy, affect over 40 million persons worldwide (1). The pathogenicity of these infections and immunity to them are thought to be mediated by the host cellular immune response, which is directed against a variety of mycobacterial antigens (8, 9, 16, 23-25, 31, 32, 34, 36). One particular antigen, designated the 65-kilodalton (kDa) antigen, has received a great deal of attention recently because it appears to be one of the major, immunologically active mycobacterial antigens following infection or immunization (11, 20, 24, 26, 32, 34, 46). This antigen was originally identified in extracts of *Mycobacterium leprae* as the ~65-kDa target of monoclonal antibody MLIIIIE9 (15). In the tubercle bacilli, this antigen is found as an ~250-kDa multimeric structure composed of ~65-kDa subunits (10).

The 65-kDa antigen is a highly immunoreactive protein. Purified 65-kDa antigen can elicit a strong delayed-type hypersensitivity reaction in experimental animals (10, 14, 46). In immune mice, perhaps as many as 20% of the T cells that can be stimulated to proliferate in vitro by a sonicated extract of mycobacteria are actually stimulated to proliferate by the 65-kDa antigen (21). In humans, antibodies and T cells directed against this antigen can be isolated from persons infected with *M. leprae* or *M. tuberculosis* as well as from persons immunized with the vaccine strain *M. bovis* BCG or heat-killed *M. leprae* (4, 11, 20, 24, 26, 32, 34, 46). These immune responses recognize epitopes of the 65-kDa antigen that are unique to a given species as well as epitopes that are common to two or more mycobacterial species (11, 21, 23, 26, 32, 34, 46).

The reactivities of mouse monoclonal antibodies directed against the mycobacterial 65-kDa antigen range from binding to the 65-kDa antigen of only one species to binding to antigens of as many as 23 different mycobacterial species (5). Some of these antibodies also react with antigens present in gram-negative bacteria, gram-positive bacteria, spirochetes,

and rickettsiae (5, 42, 46). We report here that the cross-reactive proteins in gram-negative bacteria correspond to a family of conserved bacterial proteins that have been called Common Antigen (CAG) (19). This antigen was originally identified as an immunoreactive, ~62-kDa protein of *Pseudomonas aeruginosa* (19, 43). (This is not the enterobacterial common antigen, which is an amino sugar heteropolymer [28].) In addition, we show that the cross-reacting protein in *Escherichia coli* is the product of the *groEL* gene, which is an *E. coli* gene required for the growth of phage lambda (reviewed in reference 13). It has also been called *mopA* (morphogenesis of phage). Both the GroEL protein (33) and the mycobacterial 65-kDa protein are heat shock proteins. Heat shock proteins are a set of highly conserved proteins synthesized in response to a sudden increase in temperature or to other environmental stresses (reviewed in references 27 and 39).

MATERIALS AND METHODS

Plasmids, phage, and bacteria. *M. smegmatis* 607 was obtained from the American Type Culture Collection, Rockville, Md., and maintained on Middlebrook 7H9 medium plus ADC (Difco Laboratories, Detroit, Mich.). *Legionella pneumophila* gp1 (Phil-1) and *P. aeruginosa* KC1767 were obtained from the stock culture collection of the Immunology Laboratory, Centers for Disease Control, Atlanta, Ga. The *E. coli* strains carrying *groE* mutations were obtained from C. Georgopoulos, University of Utah, Salt Lake City. The *E. coli* strains were maintained in LB media or B broth (30). Phage λ cI857 was obtained from R. Shuster, Emory University, Atlanta, Ga.

Plasmid pTB27 is a pUC19 (49) derivative that contains a 5.4-kilobase-pair (kbp) DNA fragment that carries the coding sequences for β-galactosidase fused to the *M. tuberculosis* 65-kDa antigen open reading frame (40). In this construct, active β-galactosidase enzyme is synthesized by using the promoter of the 65-kDa antigen gene. Plasmid pTB28 is a similar construct in which this enzyme is expressed by using

* Corresponding author.

its own promoter (40). Plasmid pRL23 was constructed by inserting the 4.5-kbp *EcoRI* fragment from λ SK116 (40, 41) into *EcoRI*-cleaved pTB11 (40). This construction generated a plasmid carrying a 6.3-kbp fragment of the *M. tuberculosis* genome that contains the 65-kDa antigen gene plus ~1.8 kbp of flanking sequences upstream and ~3 kbp of flanking sequences downstream of the gene. These plasmids were propagated in *E. coli* MC1061 (6). Plasmid pOF39 contains the *E. coli groE* operon and was propagated in *E. coli* OF230 (12).

Antibodies and immunoprecipitations. Mouse monoclonal antibodies reactive with the 65-kDa antigen were obtained from the IMMLEP or IMMTUB Monoclonal Antibody Bank of the World Health Organization (H. D. Engers et al., Letter, Infect. Immun. 48:603–605, 1985; H. D. Engers et al., Letter, Infect. Immun. 51:718–720, 1986). Serum samples from patients with culture-confirmed *P. aeruginosa* infections were obtained from A. Highsmith, Hospital Infections Program, Centers for Disease Control.

For immunoprecipitations, ~10⁵ cpm of a crude lysate of [³⁵S]methionine-labeled cells was added to 0.5 ml of buffer (0.15 M NaCl, 50 mM Tris [pH 7.5], 1% sodium deoxycholate, 0.5% Nonidet P-40, 0.1% sodium dodecyl sulfate) and precleared by treatment with Formalin-fixed *Staphylococcus aureus* (ICN Immunologicals, Lisle, Ill.). Samples were reacted with 0.5 μ l of antiserum overnight at 4°C. Immune complexes were collected by using *S. aureus*, and the pellets were washed twice with 0.5 M LiCl–0.1 M Tris (pH 8.5). The pellets were suspended in gel loading buffer, boiled, centrifuged to remove *S. aureus*, and subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and autoradiography.

Gel electrophoresis and immunoblot analysis. SDS-polyacrylamide gel electrophoresis was performed by using the discontinuous buffer system of Laemmli (22). Typically, 10 μ g of a crude cell lysate or 1 μ g of purified protein was loaded per well. After the completion of electrophoresis, the proteins were transferred to nitrocellulose (47), stained with a silver stain kit used in accordance with the instructions of the manufacturer (Bio-Rad Laboratories, Rockville Center, N.Y.), or prepared for autoradiography by treatment with En³Hance in accordance with the instructions of the manufacturer (New England Nuclear Corp., Boston, Mass.).

Immunoblotting was performed as previously described (40, 47). Monoclonal antibodies were used at 1:200 to 1:1,000 dilutions, and human serum was used at a 1:100 dilution. The secondary horseradish peroxidase-antiimmunoglobulin conjugates (Bio-Rad Laboratories) were used at 1:1,000 dilutions. Dilutions were made in 0.15 M NaCl–50 mM Tris (pH 8)–0.05% Tween 20. Immune complexes were visualized by using 0.5 mg of 4-chloro-1-naphthol per ml in 0.15 M NaCl–50 mM Tris (pH 8)–20% methanol–0.1% H₂O₂.

Purification of *P. aeruginosa* CAg. CAg was purified as previously described (44). Briefly, cells were harvested from an overnight culture of *P. aeruginosa* in tryptic soy broth (Difco Laboratories) and lysed by sonication. Cell membranes and walls were removed by centrifugation at 48,000 $\times g$ for 30 min. The majority of proteins were removed by precipitation with 18% Na₂SO₄. The supernatant was dialyzed and then chromatographed on a column (1.5 by 75 cm) of Sephadex G-200. Fractions containing CAg were pooled and further purified by Sephadex-DEAE anion-exchange chromatography.

Heat shock response. Cells were grown to the mid-log phase, harvested by centrifugation, washed once in methionine assay medium (Difco Laboratories), and resuspended in

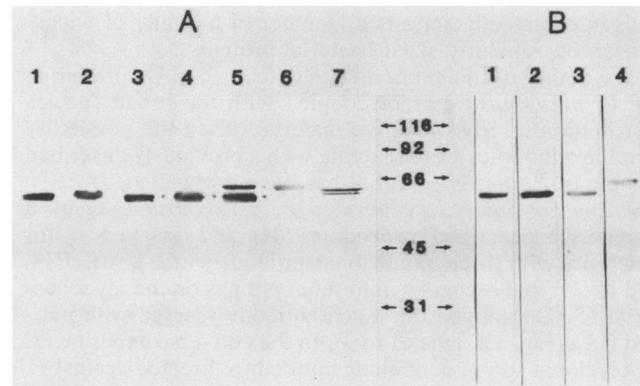


FIG. 1. Immunoblot assay. Proteins in crude lysates of bacteria (~10 μ g) were separated on 8% polyacrylamide-SDS gels and transferred to nitrocellulose as described in Materials and Methods. (A) The immobilized proteins were reacted with monoclonal antibody Y1.2. Lanes: 1, *P. aeruginosa*; 2, *L. pneumophila*; 3, *R. rickettsii*; 4, *E. coli* MC1061; 5, *E. coli* MC1061(pRL23); 6, *M. tuberculosis*; 7, *M. smegmatis*. (B) Lanes 1 to 3: Purified *P. aeruginosa* CAg (~1 μ g per lane) was reacted with monoclonal antibodies to the mycobacterial 65-kDa antigen. Lanes: 1, Y1.2; 2, ML11H9; 3, IT-33. In lane 4, antibodies directed against *P. aeruginosa* CAg were reacted with a crude lysate of *M. tuberculosis*. Numbers between panels indicate sizes in kilodaltons.

methionine assay medium at 1 A₆₀₀ unit per ml. After 30 min of incubation at either 30 or 46°C, 50 μ l of methionine assay medium containing 5 μ Ci of [³⁵S]methionine was added to each 1 ml of culture. After an additional 30 min of incubation, cells were harvested by centrifugation and washed three times with cold methionine assay medium. The final pellet was suspended in 0.15 M NaCl–10 mM Tris (pH 7.5), and the cells were disrupted by sonication. Cell debris was removed by centrifugation, and incorporation was assessed by determining the counts precipitable by 15% trichloroacetic acid. Samples were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

β -Galactosidase assays. *E. coli* MC1061 cells containing plasmid pTB27 or pTB28 were grown in B broth at 30°C to an A₆₀₀ of 0.1. Aliquots of the cultures were then incubated at 30, 37, or 42°C until the A₆₀₀ reached 0.4 to 0.5. The activity of β -galactosidase was assayed as described by Miller (30).

RESULTS

Correspondence of the 65-kDa antigen to CAg. To look more closely at the cross-reactivity of monoclonal antibodies directed against the mycobacterial 65-kDa antigen, we assayed extracts of bacteria representing five genera for reactivity with a panel of monoclonal antibodies directed against five different epitopes on the 65-kDa antigen. The results of an immunoblot assay with the most broadly reactive antibody, Y1.2, are shown in Fig. 1A. This antibody detected 60- to 65-kDa antigens in extracts of *P. aeruginosa* (lane 1), *L. pneumophila* (lane 2), *Rickettsia rickettsii* (lane 3), *E. coli* (lane 4), *M. tuberculosis* (lane 6), and *M. smegmatis* (lane 7). In an *E. coli* strain carrying a plasmid expressing the *M. tuberculosis* 65-kDa antigen, the antibody detected both the mycobacterial antigen and the cross-reacting *E. coli* 60-kDa antigen (lane 5). For each species, at least two of the monoclonal antibodies reacted with the 60- to 65-kDa antigens, although the patterns of reactivity did vary between the species (Table 1). These results suggest that the 65-kDa

antigen of mycobacteria is a member of a family of widely conserved, similarly sized bacterial proteins.

The ability of the antibodies to detect a 62-kDa protein in the *P. aeruginosa* extract coupled with the broad species distribution of cross-reactive material raised the possibility that the antibodies were reacting with a previously described protein of *P. aeruginosa* that has been designated CAg. To test this possibility, we purified *P. aeruginosa* CAg by a previously described procedure (44) and assayed it for reactivity with three monoclonal antibodies (Y1.2, MLIH9, and IT-33) that recognize different epitopes on the mycobacterial 65-kDa antigen (5). Each antibody reacted with purified CAg (Fig. 1B, lanes 1 to 3). In the converse experiment, a polyclonal serum containing antibodies directed against *P. aeruginosa* CAg reacted with the mycobacterial 65-kDa antigen (Fig. 1B, lane 4). Therefore, the cross-reactive species in the *P. aeruginosa* extract is CAg. In other words, the 65-kDa antigen is the mycobacterial counterpart to CAg of gram-negative bacteria.

Expression of the 65-kDa antigen as a heat shock protein. During the cross-reactivity studies, we noticed that the amounts of the *M. tuberculosis* 65-kDa antigen as well as the cross-reacting *E. coli* 60-kDa antigen appeared to be several-fold greater in extracts of cells grown at 37 or 42°C than at 30°C (data not shown). To investigate this further, we compared β -galactosidase activity in cells in which the enzyme was expressed by using the promoter of the *M. tuberculosis* 65-kDa antigen gene with that in cells in which the enzyme was expressed by using its own *E. coli* promoter. The level of β -galactosidase activity in cells harboring plasmid pTB28 (*lacZ* promoter) decreased with increasing temperature such that the level of activity at 42°C (~10,000 U/mg of protein) was about one-half that at 30°C (~20,000 U/mg of protein). On the other hand, the level of β -galactosidase activity in cells carrying plasmid pTB27 (65-kDa antigen gene promoter) was about fivefold higher at 37°C (~3,000 U/mg of protein) and eightfold higher at 42°C (~5,000 U/mg of protein) than at 30°C (~600 U/mg protein). Thus, the accumulation of β -galactosidase is temperature regulated when its expression is under the control of the 65-kDa antigen gene sequences.

To determine if the syntheses of the *E. coli* 60-kDa protein and the mycobacterial 65-kDa protein were increased in response to heat shock, we assayed de novo protein synthesis in cells of *E. coli* and *M. smegmatis* following a shift in temperature from 30 to 46°C (Fig. 2). (*M. smegmatis* was used since it is much easier to grow, radiolabel, and lyse than tubercle bacilli.) The major *E. coli* proteins synthesized in response to heat shock displayed the expected apparent molecular sizes of about 85, 70, 60, 25, 21, and 14 kDa (Fig.

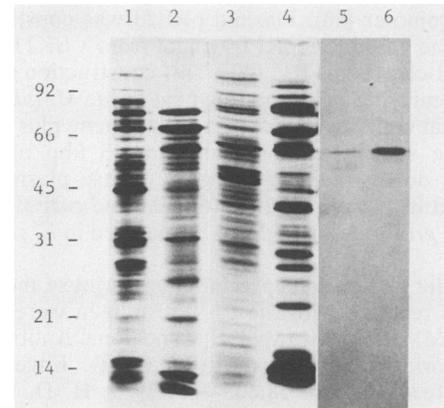


FIG. 2. De novo protein synthesis assay. Newly synthesized proteins in *E. coli* MC1061 or *M. smegmatis* grown at 30°C or following a shift to 46°C were labeled with [³⁵S]methionine as described in Materials and Methods. Portions of the labeled *M. smegmatis* extracts were immunoprecipitated with monoclonal antibodies directed against the 65-kDa antigen. Samples were electrophoresed on 10% polyacrylamide-SDS gels. Lanes: 1, *E. coli* MC1061, 30°C; 2, *E. coli* MC1061, 46°C; 3, *M. smegmatis*, 30°C; 4, *M. smegmatis*, 46°C; 5, *M. smegmatis*, 30°C, immunoprecipitated; 6, *M. smegmatis*, 46°C, immunoprecipitated. Numbers at left indicate sizes in kilodaltons.

2, lanes 1 and 2) (48). In *M. smegmatis*, the major proteins synthesized in response to heat shock displayed apparent molecular sizes of about 95, 72, 62, 40, 32, 28, 23, and 16 kDa (Fig. 2, lanes 3 and 4). One of the heat shock proteins of each species migrated with the same apparent molecular size as the antigen identified by the cross-reactivity studies. To determine if the labeled proteins were the 65-kDa antigens, we reacted portions of the lysates with monoclonal antibodies directed against the mycobacterial 65-kDa antigen. The antibodies precipitated the 62-kDa protein whose expression was much greater in the heat-treated *M. smegmatis* cells than in the cells incubated at 30°C (Fig. 2, lanes 5 and 6). Therefore, the rate of synthesis of the 65-kDa cross-reactive material is increased in response to heat shock, indicating that it is a heat shock protein.

Correspondence of the 65-kDa antigen to the *E. coli* GroEL protein. An *E. coli* heat shock protein that migrates with an apparent molecular size of ~60 kDa is the product of the *groEL* gene. The *E. coli* GroEL protein and the *M. tuberculosis* 65-kDa antigen are similar in that both are found in the cytoplasm as homomultimers of >250 kDa (10, 17). They also have similar amino acid compositions (10, 17). Furthermore, a plasmid containing the *groE* operon hybridizes at a

TABLE 1. Species specificities of the 65-kDa antigen monoclonal antibodies

Bacterial extract	Reactivity ^a with monoclonal antibody:				
	Y1.2	MLIHH9	MLIHC8	MLIIC8	MLIIE9
<i>Mycobacterium leprae</i>	+	+	+	+	+
<i>Mycobacterium tuberculosis</i>	+	+	-	+	-
<i>Mycobacterium smegmatis</i>	+	+	+	+	-
<i>Escherichia coli</i>	+	+	+	-	-
<i>Pseudomonas aeruginosa</i>	+	+	-	-	-
<i>Legionella pneumophila</i>	+	+	+	-	+
<i>Rickettsia rickettsii</i>	+	-	+	-	+

^a The reactivity of monoclonal antibodies directed against the mycobacterial 65-kDa antigen with antigens in bacterial extracts was measured in an immunoblot assay as described in Materials and Methods. +, a reacting species of ~60- to 65-kDa was observed in the immunoblot; -, no reacting species was present in this size range.

low stringency to DNA fragments isolated from the coding region of the *M. tuberculosis* 65-kDa antigen (data not shown), and the amino acid sequence of the GroEL protein displays excellent alignment and about 54% identity with the amino acid sequence of the *M. tuberculosis* 65-kDa antigen (C. Woolford and R. Hendrix, personal communication).

To determine if the *M. tuberculosis* 65-kDa antigen might have activities similar to those of the *E. coli* GroEL protein, we attempted to suppress defects in the GroEL protein with the *M. tuberculosis* 65-kDa antigen. To do this, we transformed either a plasmid (pRL23) that expresses the *M. tuberculosis* 65-kDa antigen or a plasmid (pOF39) that expresses the *E. coli* GroEL protein into mutant CG247 (*groEL140*). The *groEL140* mutation renders the cells unable to grow at 42°C and unable to support the growth of phage lambda at any temperature (12). The pOF39 transformants grew as well at the nonpermissive temperature (42°C) as at the permissive temperature (32°C). The plating efficiency of λ cI857 on CG247(pOF39) was about one-half that on wild-type strain CG25 (*groEL*⁺). Thus, as expected, the plasmid carrying the *groEL* gene can overcome the defects associated with the *groEL140* mutation. On the other hand, transformants expressing the mycobacterial 65-kDa antigen grew poorly at 42°C. Only about 1% of the cells that could form colonies at 32°C could form colonies at 42°C, and the colonies formed at 42°C were much smaller than those formed by the wild-type strain. This result was only slightly better than that found with the mutant strain, in which about 0.1% of the cells could form small colonies at 42°C. The plating efficiency of λ cI857 on CG247(pRL23) was indistinguishable from that on mutant CG247 (<10⁻⁶ of that on CG25). These observations suggest that the mycobacterial 65-kDa antigen cannot efficiently substitute for the *groEL* protein to overcome the defect in the *E. coli* GroEL protein that renders CG247 (*groEL140*) unable to grow at elevated temperatures and support phage growth.

In *E. coli*, the *groE* operon contains the coding sequences for the GroES (14-kDa) and GroEL (60-kDa) proteins preceded by a heat shock promoter (12). There is a reasonable match to the consensus heat shock promoter sequence (27) about 300 bases upstream of the open reading frame encoding the *M. tuberculosis* 65-kDa antigen: 7 of 10 nucleotides match in the -35 region and 7 of 9 match in the -10 region. However, there does not appear to be an open reading frame upstream or downstream of the 65-kDa antigen-coding sequences that could encode a GroES analog. A plasmid (pRL23) containing the intact 65-kDa antigen gene flanked by ~1,800 base pairs upstream and ~3,000 base pairs downstream of the coding sequences did not express a 12- to 18-kDa protein in minicells or in an in vitro procaryotic transcription-translation system but did express the 65-kDa antigen (data not shown). Also, pRL23 was unable to suppress the defects in mutants CG217 (*groES30*) and CG232 (*groES7*). These observations suggest that the 65-kDa antigen gene is not part of an operon with a GroES analog and that the portion of the mycobacterial genome in pRL23 does not contain a GroES analog. Two caveats here are that the mycobacterial analog of GroES might not be expressed in *E. coli* or might not function sufficiently well in *E. coli* to overcome the *groES* defects. However, there is an ~16-kDa protein in *M. smegmatis* that displays a strong heat shock response similar to that of the *E. coli* *groES* gene product. If this protein corresponds to the *E. coli* GroES protein, then the mycobacterial GroES and GroEL analogs are expressed from two heat-inducible genes instead of the one operon seen in *E. coli*.

DISCUSSION

The 65-kDa antigen appears to be the mycobacterial counterpart to a widely conserved bacterial protein—the *E. coli* GroEL protein. Analogous proteins have been described in plants (38), and monoclonal antibody Y1.2 can cross-react with proteins in mammalian cells (T. M. Shinnick, unpublished observations). In addition to the shared epitopes, these proteins also display common structural and immunologic features: (i) they can be isolated as homomultimers of 250 to 900 kDa composed of ~60-kDa subunits; (ii) they contain species-specific and genus-specific epitopes as well as cross-reactive epitopes; (iii) they can be either located in the cytoplasm or exposed on the cell surface, depending on which particular species within the genus is examined; and (iv) they are major immunogens and antigens during infections with the corresponding bacteria (10, 17, 19, 43, 44, 46). In addition, the amino acid compositions of the mycobacterial 65-kDa antigen (10, 29, 40, 46), *P. aeruginosa* CAg (43), and *E. coli* GroEL protein (17) are similar to each other. Finally, the amino acid sequences of the *M. tuberculosis* 65-kDa antigen and the *E. coli* GroEL protein display about 54% identity (40; C. Woolford and R. Hendrix, personal communication). The high degree of conservation of sequence and structure suggests that this antigen is a biologically important protein.

The role of *groE* in *E. coli* is not well understood (reviewed in references 13 and 27). The GroEL protein accounts for ~2% of the total cell protein at 37°C and up to 15% under heat shock conditions (27). Mutations in *groE* have pleiotropic effects on the bacteria, including (i) defects in the synthesis of DNA, RNA, and proteins; (ii) filamentation; and (iii) alterations in permeability (13, 27). These observations, coupled with the conservation of this protein and its heat-inducible expression, suggest that the *groE* gene products may provide some structural function that is necessary for cell integrity, especially in response to environmental stresses.

Certain phages, such as λ or T5, have evolved to use the GroEL multimer during the assembly of phage structures (13). Given the protein-protein interactions necessary for the assembly of these structures, it is not too surprising that the mycobacterial 65-kDa antigen cannot readily substitute for the *E. coli* GroEL protein to overcome the defect in phage growth in a *groEL* mutant. Along similar lines, suppression of the defect in the growth of CG247 (*groEL140*) at 42°C also requires protein-protein interactions (13). The ability of CG247(pRL23) to grow slightly better at 42°C than the mutant strain suggests that the mycobacterial 65-kDa antigen can perhaps weakly substitute for the *E. coli* GroEL protein in overcoming the *groEL* defects. Overall, though, given the conservation of structure and sequence of this protein, it seems likely that the 65-kDa antigen has functions in mycobacteria similar to those of the GroEL protein.

Previously, we reported that the *M. tuberculosis* 65-kDa antigen shows 50 to 60% amino acid sequence identity with a 158-residue amino acid sequence deduced from a portion of an open reading frame present on a 595-base-pair fragment of the *E. coli* genome (42). Expression of this *E. coli* sequence can overcome a defect in the *ams* (altered mRNA stability) gene (7, 35). Recent evidence suggests that the product of the full-length open reading frame is a 60-kDa protein that reacts with monoclonal antibody Y1.2 (T. M. Shinnick, unpublished results). In addition, the restriction map of a plasmid carrying the cloned sequence (7) is identical to that of a plasmid carrying the *groE* operon (12). Thus, it appears

that the *groEL* gene product (map location, 94 min) can suppress a defect in the *ams* gene (24 min), reminiscent of the observation that overproduction of the GroEL protein can suppress defects in *dnaA* (12).

A large portion of the immune response to infecting mycobacteria appears to be directed at two particular proteins, the 70-kDa and 65-kDa antigens. Peripheral T cells of the majority of infected individuals tested so far react with these two proteins (21; S. H. E. Kaufmann and M. E. Munk, personal communication). Both antigens also elicit strong humoral responses (2-4, 20, 26, 45, 46). The 70-kDa antigen shows homology to the highly conserved 70-kDa heat shock protein (2, 27, 39; R. Garsia, personal communication; R. A. Young, personal communication). Following heat shock, these two proteins appear to become the major protein species in *M. smegmatis* (Fig. 2) and *E. coli* (27). Other environmental stresses, such as exposure to H₂O₂ or a shift to anaerobiosis, can also induce the synthesis of these proteins (reviewed in reference 39). Indeed, accumulation of the *M. bovis* BCG 65-kDa antigen is much greater in zinc-deficient medium than in zinc-sufficient medium (10). Perhaps, then, the environmental stresses inherent in macrophages are sufficient to induce the synthesis of these two proteins, which may in turn play a role in the ability of *M. tuberculosis* to survive within macrophages. The apparent immunodominance of these two proteins may then be a consequence of their being abundant proteins in the bacterium. Alternatively, exposure to other bacteria or pathogens may prime the host for a strong immune response to these highly conserved proteins.

Finally, the 65-kDa cross-reactive proteins appear to be major immunoreactive proteins of several pathogenic bacteria and contain both genus-specific and species-specific epitopes. Despite the conservation of amino acid sequence, these proteins may still be sources of potentially useful immunodiagnostic reagents. Indeed, much work has focused on the development of immunodiagnostic procedures based on CAg, with particular emphasis on the generation of genus- or species-specific reagents (18, 19, 37). However, great care will have to be taken to analyze thoroughly the specificity of the reagents to avoid complications arising from the conserved nature of this protein.

ACKNOWLEDGMENTS

We thank J. Bardwell, P. Fields, R. Garsia, R. Hendrix, S. Kaufmann, B. Plikaytis, and C. Woolford for advice, discussions, and providing results before publication. We thank C. Georgopoulos, A. Highsmith, B. Plikaytis, R. Shuster, and the Committees for Research in the Immunology of Leprosy and Tuberculosis of the World Health Organization for generously providing us with antisera, phage, and bacterial strains. We gratefully acknowledge the technical assistance of M. Sammons, R. VanLandingham, and L. Walker.

Portions of this work were supported by Public Health Service grant AI22217 from the National Institute of Allergy and Infectious Diseases.

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