Induction of mycobacterial proteins during phagocytosis and heat shock: a time interval analysis

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Abstract: Mycobacterium tuberculosis survives macrophage bactericidal activities by mechanisms that may include induction of stress proteins. We sought to determine whether the synthesis of any mycobacterial proteins is increased during phagocytosis and whether any of these proteins are also up-regulated during heat shock. Protein synthesis by M. tuberculosis H37Ra during phagocytosis by the mouse macrophage cell line IC-21, and during heat shock at 45 and 48°C, was monitored at various time intervals using $^{35}$S-labeled methionine/cysteine and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Our data suggest the existence of certain common elements in the stress response of mycobacteria to the three stress stimuli. This apparent similarity was best characterized by the up-regulation of a 25-kDa protein after exposure to each of the stress conditions. Furthermore, this 25-kDa protein and a 37-kDa protein that was also synthesized during phagocytosis appeared to be extracellular because they were preferentially solubilized when infected macrophages were lysed with 0.5% NP-40. J. Leukoc. Biol. 55: 633–641; 1994.

Key Words: protein synthesis • heat shock protein • macrophages

INTRODUCTION

Mycobacterium tuberculosis, an intracellular pathogen, adapts to the stressful environment of the macrophage by modifying its metabolism. This, in turn, may lead to its unique ability to resist the killing mechanisms of the macrophage. Induction of certain evolutionarily conserved proteins—known as heat shock proteins—during infection may account for the virulence of some intracellular pathogens [1, 2]. Heat shock proteins belong to a large family of stress proteins that can be induced in response to a variety of environmental stresses [3–5]. The fundamental importance of stress proteins is underscored by the fact that not only are these proteins induced in all animals, plants, and bacteria during stress but also, at low levels, they are needed for vital and basic functions of the cell [6–9]. It has been hypothesized that some of the mycobacterial proteins synthesized in response to phagocytosis by the macrophage could be identical to mycobacterial heat shock proteins [10] and that they may have an important role in infection and immunity.

In an attempt to clarify this hypothesis and to further characterize the behavior of M. tuberculosis during macrophage infection, we compared mycobacterial proteins synthesized during phagocytosis with those synthesized during heat shock at 45 and 48°C. Mycobacterial proteins were labeled biosynthetically by $^{35}$S-methionine/cysteine at various time intervals and subsequently analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), autoradiography, or fluorography.

MATERIALS AND METHODS

Bacterial culture and controls

M. tuberculosis H37Ra was grown to logarithmic phase (OD$_{600}$=0.7) at 37°C with continuous shaking in Proskauer-Beck (P-B) medium containing 0.05% Tween-80. Aliquots were then used for in vitro infection and heat shock experiments and to determine the effects of different culture media and gentamicin on mycobacterial protein synthesis.

To assess the effects of the culture medium on mycobacterial protein synthesis, $6 \times 10^8$ colony-forming units (CFU) of mycobacteria were incubated with 1 ml of one of the following four media: (1) RPMI 1640, (2) methionine/cysteine-deficient RPMI 1640 supplemented with 5% dialyzed fetal bovine serum (FBS; amino acid-deficient medium), (3) P-B and, (4) P-B containing 5 µg gentamicin/ml. After 1/2 h of incubation, 50 µCi of $^{35}$S-methionine/cysteine (Tran $^{35}$S-label, ICN) was added and protein synthesis was allowed to continue for 2 h.

The inhibitory effects of different gentamicin concentrations were tested on $6 \times 10^8$ mycobacterial CFU—equivalent to the approximate number of mycobacteria that remain associated with macrophages after 1/2 h incubation during the in vitro infection assay. Mycobacteria were incubated with 1 ml of P-B medium containing gentamicin at a concentration of 2.5, 5, 10, 15, 20, 25, 30, or 35 µg/ml. After 1/2 h, 50 µCi of the radioactive label was added and labeling was allowed for 2 h.

To determine the minimum concentration of cycloheximide needed to inhibit macrophage protein synthesis, the cells were grown to confluence in 175-cm$^2$ tissue culture flasks in RPMI 1640 supplemented with 10% FBS (Sigma). One-half hour before addition of the label, the medium was replaced with amino acid-deficient medium containing 25, 50, 100, 150, or 200 µg cycloheximide/ml. Labeling was carried out for 2 h in the presence of 100 µCi of the radioactive label.

In vitro assay for infection of macrophages by mycobacteria

Mouse macrophage cell line IC-21, derived from normal C57BL/6 peritoneal macrophages, was obtained from American Type Culture Collection (ATCC). Macrophages

Abbreviations: BHI, brain-heart infusion; CFU, colony-forming unit; EDTA, ethylenediamine tetraacetic acid; FBS, fetal bovine serum; M. tuberculosis, Mycobacterium tuberculosis; NF-40, Nonidet P-40; P-B, Proskauer-Beck; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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were grown in RPMI 1640 supplemented with 10% FBS to confluence in 175-cm² flasks (1.4 × 10⁷ cells) to be used for in vitro infection assays.

Before the infection, the macrophage monolayer was cooled on ice for 5 min to synchronize phagocytosis, and the mycobacteria, grown to logarithmic phase, were passed 10 times through a 27-gauge needle to disaggregate mycobacterial clumps. Mycobacteria were incubated with macrophages at a ratio of 40–50 CFU per macrophage in 6 ml of culture medium at 37°C in a CO₂ incubator. Following 1/2 h of incubation, the supernatant was removed and the monolayer was rinsed three times with phosphate-buffered saline (PBS) to remove any extracellular bacteria.

To determine the number of mycobacterial CFU not taken up by macrophages after the 1/2 h of incubation, the supernatant was pooled with the three PBS washes and centrifuged. The pellet was resuspended in 1 ml of PBS by passage through a 27-gauge needle. The suspension was diluted to 1:30,000 in PBS (all dilutions were done in PBS) and 10 µl of this was plated on brain-heart infusion (BHI) agar. The number of mycobacterial CFU taken up by macrophages was determined by lysing the infected cells with 0.5% NP-40. Following centrifugation, the pellet was resuspended in 1 ml of PBS, and 10 µl of a 1:20,000 dilution of this suspension was plated. The number of mycobacterial CFU originally used for infection was determined by plating 10 µl of a 1:50,000 dilution of the bacterial culture.

To assess the effect of cycloheximide on phagocytosis, macrophages were incubated with amino acid-deficient medium containing 200 µg cycloheximide/ml for 1/2 h before infection with mycobacteria. To determine the number of mycobacterial CFU taken up by cycloheximide-treated macrophages, infected cells were lysed with 0.5% NP-40, the contents were resuspended in 1 ml of PBS, and 10 ml of a 1:12,000 dilution of this suspension was plated on BHI agar. The number of mycobacterial CFU remaining in the supernatant was also determined as described.

**Metabolic labeling of intracellular mycobacteria**

Mycobacterial protein synthesis during phagocytosis was monitored by metabolic labeling using [³⁵S]methionine/cysteine. Labeling was carried out at time intervals of 0–1/2 h, 1/2–1 h, 1–2 h, 2–4 h, 4–7 h, 7–9 h, 20–23 h, and 45–48 h after infection, followed by a chase with excess cold methionine/cysteine for 10 min.

For the 0–1/2 h time interval, macrophages were incubated for 1/2 h with 6 ml of amino acid-deficient medium containing 200 µg cycloheximide/ml. After cycloheximide treatment the monolayer was infected with mycobacteria (40–50 CFU per macrophage) for 1/2 h in presence of 240 µCi of radioactive label. The labeling was terminated by a 10-min chase with cold amino acids, after which the monolayer was washed thoroughly with PBS.

For the 1/2–1 h time interval, macrophages were incubated with amino acid-deficient medium containing 200 µg cycloheximide/ml and mycobacteria (40–50 CFU per macrophage). After 1/2 h the monolayer was rinsed with PBS and incubated with fresh amino acid-deficient medium containing 200 µg cycloheximide/ml, 2.5 µg gentamicin/ml, and 240 µCi of radioactivity. The labeling was carried out for 1/2 h, followed by a 10-min chase.

For the remaining time intervals, macrophages were infected with mycobacteria for 1/2 h in RPMI 1640 supplemented with 10% FBS, after which the monolayer was rinsed with PBS and incubated with fresh medium. In each case, before addition of the label, the infected monolayer was treated for 1/2 h with 200 µg cycloheximide/ml and 2.5 µg gentamicin/ml in 6 ml of amino acid-deficient medium.

**Preparation of lysates from infected macrophages**

The infected macrophages were detached from the culture flasks and transferred to microcentrifuge tubes. The cells were lysed with 300 µl of 0.5% NP-40 and following centrifugation the NP-40-soluble material was removed and saved on ice. This fraction was thought to contain any mycobacterial protein that may be extracellular. The NP-40-insoluble material containing mycobacteria was resuspended in 100 µl of Tris-EDTA buffer, pH 8, and treated with 1% lysozyme at 37°C for 1/2 h. The lysozyme-treated bacteria were sonicated at full power using a microtip (Sonifier Model W185; Branson Ultrasonics Corporation) for 3 min followed by treatment with 2% SDS at 60°C for 15 min. The NP-40–soluble and -insoluble fractions were centrifuged at 11,000g for 10 min to remove the cell debris, filtered through a 0.45-µm filter (Millex-Hv, Millipore), and concentrated by ultrafiltration (Centricon-10; Amicon). The fractions were either analyzed separately or pooled together to be analyzed as the whole cell lysate.

**Heat shock**

Mycobacteria, grown in P-B medium to logarithmic phase, were aliquoted into microcentrifuge tubes and transferred to a water bath at either 45 or 48°C. Protein synthesis during heat shock was monitored using [³⁵S]methionine/cysteine at the same time intervals described for phagocytosis. Upon termination of labeling by a cold amino acid chase, mycobacteria were lysed and the lysate was pooled with its corresponding extracellular medium to include any extracellular protein. The lysate was then concentrated after removal of the cell debris. Mycobacteria were also metabolically labeled at 37°C and the lysate was used as control.

**SDS-polyacrylamide gel electrophoresis**

Labeled proteins were resolved on a 10–20% gradient minigel by the Laemmli procedure [11] and subsequently detected by autoradiography or fluorography. Samples for SDS-PAGE were prepared by precipitating the proteins with ice-cold acetone. The precipitate was dissolved in sample buffer containing 10% SDS and 2.5% 2-mercaptoethanol by heating at 60°C for 15 min. For autoradiography, gels were dried and exposed to an X-ray film (hyperfilm; Amersham) at −20°C for 3–5 days. For fluorography, gels were treated with a fluorography solution (Amplify; Amersham) and then dried and exposed to a preflashed X-ray film at −70°C for 5–7 days.

**RESULTS**

**Phagocytosis of M. tuberculosis H37Ra by macrophage cell line IC-21**

After infection of macrophages with mycobacteria, the infected monolayer was washed thoroughly with PBS to remove the extracellular bacteria. Phagocytosis could be confirmed by acid-fast staining of the infected cells (Fig. 1) and by appearance of colony-forming units on BHI agar after the infected cells were lysed with 0.5% NP-40 and their content were plated (Fig. 2A).

During labeling of the intracellular mycobacteria, cycloheximide was used to prevent macrophage protein synthesis.
In most cases macrophages were treated with this drug after phagocytosis had already taken place. However, for metabolic labeling at the two earliest time intervals of 0-1/2 h and 1/2-1 h cycloheximide was present 1/2 h before or at the time of phagocytosis. Therefore, the effect of this drug on phagocytosis itself was investigated. Macrophages, treated with cycloheximide, were incubated with mycobacteria and after 1/2 h the number of mycobacterial CFU phagocytized was determined by plating the contents of the infected cells (Fig. 2B). The results demonstrated that macrophages treated with cycloheximide phagocytized as many mycobacteria as nontreated macrophages (Table 1). Thus, treatment of macrophages with this drug did not affect uptake of mycobacteria.

<table>
<thead>
<tr>
<th>Original number of bacteria</th>
<th>Total number of bacteria recovered</th>
<th>Number phagocytized</th>
<th>Number in supernatant after phagocytosis</th>
<th>Percent phagocytized</th>
<th>Percent recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.0 x 10^4</td>
<td>5.6 x 10^4</td>
<td>6.0 x 10^7</td>
<td>5.0 x 10^7</td>
<td>10.7</td>
</tr>
<tr>
<td>Treated with cycloheximide</td>
<td>6.0 x 10^4</td>
<td>6.0 x 10^4</td>
<td>5.6 x 10^7</td>
<td>5.5 x 10^7</td>
<td>9.2</td>
</tr>
</tbody>
</table>

*Each experiment was repeated six times and the values are averages. The values were calculated by multiplying the number of CFU on BHI agar by the appropriate dilution factors.

**Effect(s) of RPMI 1640 culture medium and gentamicin on protein synthesis of *M. tuberculosis***

The culture medium used during labeling of intracellular mycobacteria was RPMI 1640 deficient in methionine/cysteine and supplemented with 5% dialyzed FBS. It has been reported that RPMI 1640 does not support the growth of mycobacteria [12], but it has not been determined whether this medium inhibits mycobacterial protein synthesis. Therefore, the effect of RPMI 1640 on protein synthesis of mycobacteria was examined. As indicated in Figure 3, this medium had no inhibitory effect and mycobacterial protein synthesis was similar to that during growth in Proskauer-Beck medium. On the other hand, when gentamicin was added to the culture medium protein synthesis was inhibited (Fig. 3, lane 4). Thus, gentamicin was used during labeling of intracellular mycobacteria to prevent protein synthesis by any mycobacteria that remained outside macrophages.

It was assumed that the majority of mycobacteria retained by macrophages after 1/2 h of incubation were phagocytized, even though mycobacteria attached to the external surface of macrophages could not be differentiated from those that were phagocytized. However, to ensure that protein synthesis of extracellular mycobacteria was inhibited during phagocytosis, the effect of gentamicin was also tested on the same number of mycobacteria. This was regarded as the maximum population of mycobacteria that could remain associated with macrophages without being internalized.

The number of mycobacterial CFU retained by macrophages after 1/2 h of incubation was calculated from the number of CFU on the agar after the contents of infected...
Therefore, tested the effects of different concentrations of cycloheximide on macrophage cell line IC-21 protein synthesis. As Figure 5 shows, a concentration of 200 μg/ml completely inhibited protein synthesis of these cells. This concentration of cycloheximide was subsequently used during labeling of intracellular mycobacteria to prevent macrophage protein synthesis.

A time interval comparison of mycobacterial protein synthesis during three stress conditions—heat shock at 45 and at 48°C and phagocytosis—is depicted in Figures 6A–C. by 7 h after exposure to each of the stress stimuli a 25-kDa protein, which was barely present under control conditions (37°C), was up-regulated. At the same time many other protein species, notably a 22-kDa protein, which was prominently expressed at 37°C, were down-regulated. Aside from this apparent similarity, each of the stress conditions triggered a unique pattern of protein synthesis. As Figure 6A indicates, mycobacterial response to heat shock at 45°C began with an immediate increase in the synthesis of a 65-kDa protein. After 1 h the 65-kDa protein, the 22-kDa protein, and several high-molecular-weight proteins were abruptly down-regulated and this event coincided with rapid up-regulation of macrophage protein synthesis.

Fig. 3. Effects of different culture media on mycobacterial protein synthesis. Mycobacteria (6 x 10^9 bacteria) were incubated for 1/2 h in 1 ml of one of the following media: RPMI 1640 (lane 1), methionine/cysteine-deficient RPMI 1640 supplemented with 5% dialyzed FBS (lane 2), Proskaur-Beck (lane 3), and Proskaur-Beck containing 5 μg gentamicin/ml (lane 4). Mycobacterial proteins were labeled in different media for 2 h in the presence of 50 μCi of [35S]methionine/cysteine. After labeling, mycobacteria were lysed and equal protein concentrations from each lysate were separated by SDS-PAGE. The resolved bands were stained with Coomassie Blue (A), after which the gel was dried and exposed to an X-ray film (B). As demonstrated, mycobacterial protein synthesis is similar in the presence of different media tested and is inhibited only when gentamicin is present (lane 4). Numbers indicate molecular mass standards.

Fig. 4. Effects of different gentamicin concentrations on mycobacterial protein synthesis. Inhibitory effects of gentamicin were tested on 6 x 10^7 mycobacteria, the estimated number of the bacteria retained by 1.4 x 10^7 macrophages after 1/2 h of infection with 6 x 10^8 mycobacteria. Mycobacteria were incubated with different concentration of gentamicin in 1 ml of P-B medium. After 1/2 h of incubation, mycobacteria were labeled with 50 μCi of radioactive amino acids for 2 h. Gentamicin concentrations used were 35 μg/ml (lane 1), 50 μg/ml (lane 2), 25 μg/ml (lane 3), 10 μg/ml (lane 4), 15 μg/ml (lane 5), 10 μg/ml (lane 6), 5 μg/ml (lane 7), 2.5 μg/ml (lane 8), and none (lane 9). After labeling, mycobacteria were lysed and equal protein concentrations from each lysate were subjected to SDS-PAGE. As demonstrated in the autoradiograph of the gel (A), the smallest gentamicin concentration tested (2.5 μg/ml) effectively inhibited mycobacteria protein synthesis. The gel stained with Coomassie Blue is included (B) to demonstrate the presence of equal protein concentrations in each lane.

Time interval analysis of mycobacterial protein synthesis during heat shock and phagocytosis

To monitor mycobacterial protein synthesis during phagocytosis it was critical to prevent protein synthesis of the macrophages themselves. Cycloheximide has often been used to inhibit specifically protein synthesis of macrophages [1, 14]. We therefore tested the effects of different concentrations of cycloheximide on macrophage cell line IC-21 protein synthesis. As Figure 5 shows, a concentration of 200 μg/ml completely inhibited protein synthesis of these cells. This concentration of cycloheximide was subsequently used during labeling of intracellular mycobacteria to prevent macrophage protein synthesis.

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of a 25-kDa protein to near its maximal level. Other noticeable events included the up-regulation of a 35-kDa and a 33-kDa protein. Both of these proteins were induced after 1 h of heat shock, but after 7 h of heat shock the 35-kDa protein disappeared.

Figure 6B demonstrates that the response of mycobacteria to heat shock at 48°C started with an immediate increase in the synthesis of 70-, 60-, 45-, and 37-kDa proteins. The 60- and 37-kDa proteins were repressed after 1 h, whereas the 70- and 45-kDa proteins persisted. The 70-kDa protein eventually disappeared after 7 h, but the 45-kDa protein was still present even during the 7–9-h time interval. A 65-kDa protein was also up-regulated after 2 h of heat shock at 48°C. This protein along with the 22-kDa protein continued to be prominently synthesized through 7 h of heat shock at 48°C. It is, however, interesting to note that during both heat shock conditions total repression of the 70- and 65-kDa proteins coincided with the disappearance of the 22-kDa protein.

**Fig. 5.** Concentration of cycloheximide needed to prevent macrophage protein synthesis. Mouse macrophage cell line IC-21, grown to confluence in 175-cm² tissue culture flasks, was incubated with 6 ml of methionine/cysteine-deficient RPMI 1640 medium supplemented with 5% dialyzed FBS and containing either no cycloheximide (lane 1) or one of the following concentrations of cycloheximide: 25 µg/ml (lane 2), 50 µg/ml (lane 3), 100 µg/ml (lane 4), 150 µg/ml (lane 5), and 200 µg/ml (lane 6). After 1/2 h of incubation, 100 µCi of radioactive label was added to each flask and labeling was allowed for 2 h. Labeled macrophages were lysed and equal protein concentrations from each lysate were resolved by SDS-PAGE. As indicated, 200 µg/ml cycloheximide efficiently inhibited macrophage protein synthesis (A). The gel stained with Coomassie Blue (B) demonstrates the presence of equal protein concentration (35 µg) in each lane.

**Fig. 6.** Time interval analysis of mycobacterial proteins synthesized during heat shock and phagocytosis. Mycobacterial proteins were labeled at different time intervals during heat shock at 45°C (A), heat shock at 48°C (B), and phagocytosis (C). After heat shock, the lysate of mycobacteria was pooled with its corresponding extracellular medium and equal amounts of radioactively labeled proteins (200,000 cpm) from each lysate were resolved by 10–20% gradient SDS-PAGE (A and B). Time intervals studied were 0–1/2 h (lane 1), 1/2–1 h (lane 2), 1–2 h (lane 3), 2–4 h (lane 4), 4–7 h (lane 5), and 7–9 h (lane 6) after exposure to heat shock. The lysate of mycobacteria labeled for 2 h at 37°C is included as the control (lane 7). Lysates of mycobacteria during phagocytosis were prepared by lysing the infected macrophages with 0.5% NP-40 and pooling this fraction with the bacterial lysate prepared from the NP-40-insoluble fraction. Equal amounts of radioactivity from each preparation (100,000 cpm) were separated by 10–20% SDS-PAGE (C). Macrophage proteins (26.6 µg) labeled in the presence of 200 µg/ml cycloheximide are included as the negative control (lane 1). Mycobacterial proteins synthesized at different time intervals during phagocytosis are: 0–1/2 h (lane 2), protein concentration 26.6 µg), 1/2–1 h (lane 3), 1–2 h (lane 4), 2–4 h (lane 5), 4–7 h (lane 6), and 7–9 h (lane 7). Mycobacterial proteins synthesized at 37°C for 2 h are also shown (lane 8). Figures on the right represent molecular size standards.
During heat shock at 48°C the synthesis of a 25-kDa protein was gradually increased from one time interval to the next, whereas the 25-kDa protein up-regulated during heat shock at 45°C reached its maximum level rapidly after exposure to the heat shock. The synthesis of a 35-kDa protein appeared to follow this gradual increase, reaching its peak at the 4-7 h time interval after exposure to 48°C.

During the 7-9 h time interval many of the components of mycobacterial response to heat shock at 45 and 48°C seemed identical. Under both stress conditions the 25-kDa protein and a 10.5-kDa protein were up-regulated, while several other proteins including the 70-, 65-, 60-, and 22-kDa proteins were down-regulated.

The response of mycobacteria to phagocytosis is indicated in Figure 6C. It must be pointed out that samples prepared for SDS-PAGE contained a small quantity of mycobacterial proteins, metabolically labeled during phagocytosis, and a large amount of unlabeled macrophage proteins. This combination limited the resolving power of SDS-PAGE analysis, but as Figure 6C demonstrates, some conclusions could still be drawn. Mycobacterial responses to phagocytosis and heat shock displayed some common features. Most noteworthy was the up-regulation of a 25-kDa band during each stress condition. During phagocytosis this up-regulation appeared to be gradual, as noticed during heat shock at 48°C. On the other hand, the rapid up-regulation of a 65-kDa protein and the lack of up-regulation of a 70-kDa protein were similar to the pattern observed during heat shock at 45°C. A protein of apparent molecular size 37 kDa was also up-regulated at least through the first 7 h of phagocytosis. Finally, the 22-kDa protein prominently present under control conditions (37°C) was gradually repressed during phagocytosis, although it could still be detected after 7 h of phagocytosis.

![Fig. 7. Mycobacterial protein synthesis during long-term exposure to heat shock and long-term infection of macrophages. Mycobacterial proteins were labeled at during a 20-23 h and a 45-48 h time interval after heat shock and after infection. Lysates were prepared as previously described and equal radioactivity counts from each preparation (200,000 cpm) were resolved by 10-20% gradient SDS-PAGE. The protein profiles are shown during heat shock at 45°C for time intervals of 20-23 h (lane 1) and 45-48 h (lane 2), during heat shock at 48°C for time intervals of 20-23 h (lane 3) and 45-48 h (lane 4), and during phagocytosis at time intervals of 20-23 h (lane 5) and 45-48 h (lane 6). Mycobacteria grown at 37°C are included as the control (lane 7). The proteins up-regulated during the 20-23 h time interval after heat shock are the 65-kDa (45°C) and 70-kDa (48°C) proteins. The protein profile at the 45-48 h time interval after heat shock resembles that observed during the 7-9 h time interval with the characteristic up-regulation of a 25-kDa protein and down-regulation of a 22-kDa protein. Mycobacterial protein synthesis during long-term infection does not appear to follow a cyclic pattern but the up-regulation of a 25-kDa protein may indicate a stress response.](image-url)

The long-term response of mycobacteria to heat shock and phagocytosis is presented in Figure 7. Surprisingly, some of the proteins that were immediately up-regulated after exposure to heat shock but disappeared shortly after—such as the 70- and 65-kDa proteins—were again up-regulated during the 20-23 h time interval after heat shock. On the other hand, during the 45-48 h time interval the pattern of protein synthesis was similar to that observed at the 7-9 h time interval, which was characterized by repression of the 70-, 65-, and 22-kDa proteins and induction of the 25-kDa band. This cyclic pattern did not seem to occur during phagocytosis at the time intervals studied.

**Localization of mycobacterial proteins induced during phagocytosis**

Several mycobacterial proteins have been reported to be either secreted by the bacterium or associated with its cell surface [10, 15-17]. In view of this, we asked whether any of the mycobacterial proteins synthesized during phagocytosis could be identified as an extracellular protein.

Two observations suggested that when infected macrophages were lysed with 0.5% NP-40, the detergent did not appear to lyse the intracellular mycobacteria: (1) after infected cells were lysed with 0.5% NP-40, the intracellular mycobacteria were viable, as shown by plating the insoluble material on BHI agar (Fig. 2A), and (2) the total number of mycobacterial CFU recovered from the in vitro infection assay—mycobacteria phagocytized plus those remaining in the extracellular medium after 1/2 h of incubation—was comparable to the number of mycobacterial CFU originally used to infect the macrophages (Table 1). Thus, very few mycobacteria were lost when 0.5% NP-40 was used to recover the intracellular bacilli. Consequently, when infected macrophages were lysed with 0.5% NP-40, the soluble fraction probably contained extracellular proteins of mycobacteria.

The infected macrophages, labeled for different time intervals in the presence of cycloheximide, were lysed with 0.5% NP-40 and divided into NP-40-soluble and -insoluble fractions. Measurement of total radioactivity counts in each fraction indicated that by 4-7 h after infection as much as 22% of the total radioactivity was partitioned in the NP-40-soluble fraction. SDS-PAGE analysis demonstrated that a 37-kDa and a 25-kDa protein were solubilized by 0.5% NP-40 (Fig. 8A), whereas a majority of mycobacterial proteins synthesized during phagocytosis were not solubilized by 0.5% NP-40 (Fig. 8B). This observation further suggested that 0.5% NP-40 did not lyse the intracellular mycobacteria, because more proteins would most likely have been released and solubilized if the intracellular mycobacteria had been lysed.

Of the two proteins solubilized by 0.5% NP-40, the 37-kDa protein reached its maximum level in the NP-40-soluble fraction at the 1-2 h time interval, whereas the 25-kDa protein was present prominently during 2-7 h after infection. Furthermore, the 25-kDa protein up-regulated during phagocytosis was entirely solubilized by 0.5% NP-40 (Fig. 8A), because none of the NP-40-insoluble fractions contained any noticeable 25-kDa protein species (Fig. 8B).

Comparison of the protein profiles of intracellular mycobacteria before (Fig. 6C) and after (Fig. 8B) removal of the NP-40-soluble fraction of infected macrophages indicated that removal of the NP-40-soluble fraction led to a much better resolution. As a result of this increased resolution, a more definitive observation could be made. Figure 8B, for example, clearly demonstrates that a 65-kDa protein, rather than a 70-kDa protein, was up-regulated during...
phagocytosis. Moreover, a 45-kDa protein, similar in size to a protein that was up-regulated during heat shock at 48°C, was also up-regulated during phagocytosis, reaching its peak by 1-2 h after infection.

When mycobacteria grown at 37°C were directly treated with 0.5% NP-40, several proteins were solubilized (Fig. 8A, lane 2). But when infected macrophages were lysed with the same detergent, these proteins were absent in the soluble fractions (Fig. 8A, lanes 3-8). This could be explained either by down-regulation of these proteins during phagocytosis or by sequestering of the intracellular mycobacteria from direct contact with 0.5% NP-40. An observation suggested that these mycobacterial proteins might be synthesized during phagocytosis: even though the NP-40-soluble fractions of infected macrophages did not contain any of these proteins, proteins of similar molecular size, for example, a 22-kDa protein, were present in the NP-40-insoluble fractions (Fig. 8B, lanes 2-7). However, down-regulation cannot be ruled out because proteins of similar size in these two experiments may not be the same.

**DISCUSSION**

The pathogenesis of *M. tuberculosis* infection is the outcome of its ability to survive and multiply within macrophages and to trigger a damaging immune response by the host [18, 19]. By mechanisms that are yet to be explained, *M. tuberculosis* is capable of evading the bactericidal activities of macrophages, which include production of oxygen free radicals, acidification of the phagosome, and lysosome-phagosome fusion [20]. Mycobacteria may adapt to this hostile environment by changing the pattern of its gene expression to express preferentially proteins that are essential for its survival. Some of these proteins may be identical to mycobacterial stress proteins induced in response to such stresses as heat shock [10, 21] and nutrient starvation [22].

In this study, mycobacterial protein synthesis during phagocytosis was monitored and compared with that at two different heat shock temperatures of 45 and 48°C.

Taken together, the results suggest that a common process may underlie the response of mycobacteria to the three stress conditions. This process is characterized by the eventual up-regulation of a 25-kDa protein and down-regulation of a large number of other protein species. If the 25-kDa protein up-regulated during phagocytosis is the same as that up-regulated during heat shock, the expression of this protein may be part of a common and fundamental mycobacterial response to any of a variety of stress stimuli. The present study also indicates that the mycobacterial response to each stress condition varies at least in terms of temporal pattern of protein synthesis and suggests an important role for a 65-kDa and a 70-kDa protein during the very early stages after exposure to stress.

A 65-kDa protein is prominently up-regulated shortly after heat shock at 45°C and phagocytosis and may be the mycobacterial 65-kDa heat shock protein [23]. On the other hand, a 70-kDa protein is up-regulated immediately after exposure to 48°C. It is, however, important to note that a 65-kDa protein is eventually up-regulated at 48°C as well. Thus, if the 65-kDa protein up-regulated during these different stress conditions is the same protein, it may also be part of the common mycobacterial stress response that is triggered by a wide range of stress stimuli.

Aside from the apparent common features, the patterns of mycobacterial protein synthesis during the first 9 h of phagocytosis and heat shock at 45 and 48°C display limited overlap. The kinetics of up-regulation of the 25-kDa protein during phagocytosis is similar to that of the 25-kDa protein up-regulated during heat shock at 48°C. During both phagocytosis and heat shock at 48°C, the 25-kDa protein is up-regulated in a gradual and progressive manner, reaching its maximal level of expression about 7 h after exposure to each stress.

An interesting observation made during this study was that the protein synthesis of mycobacteria after exposure to heat shock followed a cyclic pattern over a 48-h time period. Some of the stress proteins that were up-regulated transiently during early stages of heat shock were up-regulated again during 20-23-h time intervals. It is possible that this cyclic pattern is an indication of mycobacterial cell division under stress. The event of cell division may dilute the concentration of the stress proteins needed for the survival of the daughter cells under stressful conditions. This may, in turn,
force the newly divided bacteria to synthesize more of these stress proteins in order to bring their concentration back to their previous level.

As suggested earlier, the 65-kDa protein up-regulated during phagocytosis may be the mycobacterial 65-kDa heat shock protein. The identity of the remaining proteins cannot be inferred solely on the basis of their electrophoretic mobility. However, it is noteworthy that a 41-kDa protein that is a homologue of DnaJ of Escherichia coli has been reported to be induced when mycobacteria are exposed to 48°C [10]. In the present study the only proteins comparable in molecular size to the DnaJ homologue that are up-regulated during heat shock at 48°C are a 43- and a 37-kDa protein. Two proteins of apparently similar molecular sizes are also up-regulated during phagocytosis. Whether any of these mycobacterial proteins is identical to the reported DnaJ homologue remains to be determined.

It was noted that after infected macrophages were lysed with 0.5% NP-40, a 37- and a 25-kDa protein cofractionated with the soluble material. The viable intracellular bacilli and almost all the other mycobacterial proteins synthesized during phagocytosis remained insoluble. Interestingly, when mycobacteria were directly treated with 0.5% NP-40, several proteins were solubilized which had molecular sizes similar to those present in the NP-40-insoluble fractions of infected macrophages. If these proteins of similar size are in fact the same, it suggests that during lysis of infected cells these otherwise NP-40-insoluble proteins were not accessible to 0.5% NP-40. This could be possible if the intracellular mycobacteria were sheltered from the detergent, perhaps by residing within compartments such as phagosomes. These 37- and 25-kDa proteins that were almost entirely solubilized were accessible to 0.5% NP-40 and might have been released and transported out of the phagosomes.

On the other hand, these proteins of similar molecular size may not be the same. Therefore, the absence of NP-40-soluble proteins of mycobacteria in the NP-40-insoluble fraction of infected macrophages could be explained by their down-regulation during phagocytosis. Since 0.5% NP-40 appeared to solubilize the 37- and 25-kDa proteins without lysing the intracellular mycobacteria, these two proteins might still be regarded as extracellular but not necessarily released.

The functions of the 37- and 25-kDa proteins may be important for the adaptation of mycobacteria to stressful environments. Several mycobacterial proteins comparable in molecular size to these two proteins have been characterized. For example, a well-characterized mycobacterial lipoprotein has a molecular size of 37 kDa and is the homologue of E. coli phosphate transporter [15], which is induced and released during phosphate starvation [22]. It is likely that expression of such a protein is advantageous for the survival of mycobacteria in the nutrient-poor environment of phagosomes. Also, several partially characterized mycobacterial proteins ranging in size from 23 to 27 kDa have been identified as secretory proteins [24]. It would be interesting to determine whether any of them is identical to the 25-kDa protein reported in this study.

Mycobacterial proteins that are up-regulated during phagocytosis are likely to play some roles in survival of this organism within macrophages. The finding that some of these proteins may also be up-regulated during heat shock strengthens this view. The results of this study suggest that a 25-kDa protein up-regulated during heat shock may also be up-regulated during early stages of infection. This protein and a 37-kDa protein appear to be extracellular, because they are preferentially solubilized when infected cells are lysed with 0.5% NP-40 while other mycobacterial proteins synthesized during phagocytosis remain insoluble.

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