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Host Defense Responses to Infection by *Mycobacterium tuberculosis*

INDUCTION OF IRF-1 AND A SERINE PROTEASE INHIBITOR*

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Alveolar macrophages and newly recruited monocytes are targets of infection by *Mycobacterium tuberculosis*. Therefore, we examined the expression of interferon regulatory factor 1 (IRF-1), which plays an important role in host defense against *M. tuberculosis*, in undifferentiated and differentiated cells. Infection induced IRF-1 in both. IRF-1 from undifferentiated, uninfected monocytic cell lines was modified during extraction to produce specific species that were apparently smaller than intact IRF-1. After infection by *M. tuberculosis* or differentiation, intact IRF-1 was recovered. Subcellular fractions were assayed for the ability to modify IRF-1 or inhibit its modification. A serine protease on the cytoplasmic surface of an organelle or vesicle in the "lysosomal/mitochondrial" fraction from undifferentiated cells was responsible for the modification of IRF-1. Thus, the simplest explanation of the modification is cleavage of IRF-1 by the serine protease. Recovery of intact IRF-1 correlated with induction of a serine protease inhibitor that was able to significantly reduce the modification of IRF-1. The inhibitor was present in the cytoplasm of *M. tuberculosis*-infected or -differentiated cells. It is likely that induction of both IRF-1 and the serine protease inhibitor in response to infection by *M. tuberculosis* represent host defense mechanisms.

Tuberculosis begins with inhalation of *Mycobacterium tuberculosis* and infection of resident alveolar macrophages. Inflammation induced by *M. tuberculosis* then recruits monocytes (1), which also face infection in alveoli. The response of monocytes to *M. tuberculosis* is likely to differ from that of macrophages due to changes that occur during monocyte to macrophage differentiation. To date, few studies have compared infection of monocytes and macrophages. The human THP-1 cell line is a well established model system for such studies (2–4). Growing THP-1 cells are monocyte-like, but they stop proliferating and differentiate to a macrophage-like state when treated with 12-*O*-tetradecanoylphorbol 13-acetate (TPA).[1] One useful way to compare the two cell types is by examining the expression of the transcription factor interferon regulatory factor 1 (IRF-1), since it plays an important role in host defense against mycobacteria. For example, mice having a null mutation in the IRF-1 gene are susceptible to the normally non-pathogenic *Mycobacterium bovis* Bacille de Calmette-Guérin (*M. bovis* BCG) (5) and are more susceptible than wild-type mice to infection by *M. tuberculosis* (6).

IRF-1 is induced by many cytokines. It was purified, and its gene was cloned in the course of studies on induction of type I interferons (IFNs) (IFNα and IFNβ) by virus infection (7) and on induction of gene expression in response to IFNα (8). However, type II IFN (IFNγ) is far more potent than IFNα as an inducer of IRF-1, and virus infection is a poor inducer (8). Thus, it is not surprising that disruption of the IRF-1 gene prevents induction of some IFNγ-regulated genes, including inducible nitric-oxide synthase (5), but has little effect on viral induction of type I IFN genes or induction of gene expression by IFNα (9–11). The susceptibility of IRF-1 null mutant mice to mycobacterial infection may be due to the resultant disruption of the normal pathway of response to IFNγ, since null mutations in IFNγ, its receptor, or inducible nitric-oxide synthase also increase susceptibility to mycobacteria (5, 12, 13). However, IRF-1 might also play a role independent of the IFNγ system.

In the present study, we examined changes in IRF-1 DNA binding activity and protein after mycobacterial infection. *M. tuberculosis* increased both and induced a serine protease inhibitor activity that affected extraction of IRF-1. We suggest that induction of IRF-1 and the protease inhibitor may be functionally related as host defense responses to *M. tuberculosis*.

EXPERIMENTAL PROCEDURES

M. tuberculosis and Eukaryotic Cell Culture—All manipulations with viable *M. tuberculosis* were performed under biosafety level 3 containment. A clinical isolate of *M. tuberculosis*, TN913, from the Public Health Research Institute Tuberculosis Center was grown in Middlebrook 7H9 broth as previously described (14).

THP-1 cells (3) obtained from the American Type Culture Collection were maintained between 0.6 and 6.0 $\times$ 10^5/ml in RPMI 1640 supplemented with penicillin/streptomycin (BioWhittaker) and 10% defined supplemented calf bovine serum (Hyclone). Before infection, as previously described (15), cells were untreated or treated with 20 nM TPA (Sigma) for 24 h. As indicated, cells were stimulated with recombinant human IFNγ (a gift from Amgen) at 1 ng/ml for the final 2 h before harvest for preparation of extracts.

Conditioned media (CM) were collected 3 days post-infection from undifferentiated or TPA-treated THP-1 cells infected at the indicated multiplicity of infection (m.o.i.) or from parallel, uninfected cultures. The CM were sterilely filtered and used the same day or stored at 4 °C

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[1] The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; CM, conditioned media; EMSA, electrophoretic mobility shift assay; IFN, interferon; IRF-1, interferon regulatory factor 1; *M. bovis* BCG, *M. bovis* Bacille de Calmette-Guérin; m.o.i., multiplicity of infection; PAI-2, plasminogen activator inhibitor 2; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; SLPI, secretory leukocyte protease inhibitor.

for use the next day. THP-1 cells maintained in complete media were collected by centrifugation, suspended as indicated in CM, then grown for 3 days before harvest for preparation of extracts.

Experiments with primary cells were performed in accordance with all applicable laws and regulations. Cells obtained from healthy volunteers by bronchoalveolar lavage were suspended in RPMI 1640 plus 10% fetal bovine serum (Hyclone), placed in cell culture flasks, and infected with *M. tuberculosis* TN913 as previously described (15) for ~16 h. Uninfected cells were cultured in parallel. Nonadherent cells then were removed with the media, and the adherent cells were washed gently with phosphate-buffered saline (PBS). The remaining alveolar macrophages were 90–95% pure based on microscopic examination of morphology. Peripheral blood monocytes were purified with anti-CD14 monoclonal antibody from buffy coats obtained from healthy volunteers, then cultured in RPMI 1640 plus 15% fetal bovine serum. Infection with a single cell suspension of *M. tuberculosis* H37Rv at a m.o.i. of ~1 was begun after 4 or 5 days of adherence-induced differentiation and continued for ~16 h. Uninfected cells were cultured in parallel.

Preparation of Lysates and Extracts—All steps were performed at 0–4 °C except as indicated. Media were removed from adherent cells. The cells were washed once with PBS and then were scraped into additional PBS. Undifferentiated, uninfected THP-1 cells growing in suspension were collected by centrifugation, suspended in PBS, collected again by centrifugation, and suspended again in PBS. Cells that had been adherent or in suspension were then collected by centrifugation. Lysates and extracts were prepared with non-ionic detergent or without detergent. Lysates and extracts from cells infected by *M. tuberculosis* were filter-sterilized before removal from bio-safety level 3 containment.

For preparations with non-ionic detergent, cell pellets were suspended in 4 volumes of buffer I (0.5% Nonidet P-40, 0.1 mM EDTA, 20 mM Hepes, pH 7.9, 10% glycerol, 1 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride (PMSF), 3 μg/ml aprotinin, 1 μg/ml leupeptin, and 2 μg/ml pepstatin) and incubated on ice for 10 min. As indicated, cells were lysed, and extracts were prepared without protease inhibitors. Nuclei were sedimented by centrifuging the lysates at 1,000 × *g* for 10 min. The supernatants were recovered and adjusted to 0.3 M NaCl to produce the cytoplasmic extracts. The nuclear pellets were suspended with buffer I, sedimented again by centrifuging, and suspended with 4 volumes of buffer I plus 0.4 M NaCl. The suspended nuclei were incubated for 30 min with occasional mixing. The suspensions were clarified by centrifuging at 15,000 × *g* for 10 min. The supernatants were recovered as the nuclear extracts. Extracts were frozen rapidly on crushed dry ice and stored at −80 °C.

For preparations without detergent, cells were suspended in 9 volumes of buffer II (10 mM Hepes, pH 7.9, 10 mM KCl, 3 mM $MgCl_2$, 1 mM dithiothreitol) and allowed to swell for ~20 min. A lysate was prepared by disruption in a Dounce homogenizer with 25–40 strokes of a loose-fitting pestle to achieve 80–90% lysis, as determined by microscopic examination. Nuclei were then sedimented as described above. The supernatant was recovered and adjusted by the addition of 1/3 volume of 40% glycerol, 0.4 M NaCl in buffer II to produce a cytoplasmic extract. Nuclear pellets were resuspended in 3 volumes of buffer II containing 0.4 M NaCl and 10% glycerol. Suspended nuclei were extracted as described above. Alternatively, the lysate was adjusted by the addition of 1/3 volume of 40% glycerol, 0.4 M NaCl in buffer II and used for subcellular fractionation.

Subcellular Fractionation—Subcellular fractions prepared by differential centrifugation are designated based on the well established distribution of the predominant organelle(s) and vesicles (16). Vesicles derived from plasma membrane trafficking compartments including early or late endosomes, Golgi apparatus, and endoplasmic reticulum are primarily in the "microsomal" fraction, whereas a small portion of plasma membrane often sediments with nuclei (16–19). All steps were performed at 0–4 °C. Adjusted lysate from Dounce homogenization was centrifuged at 1,000 × *g* for 10 min to sediment nuclei. The post-nuclear supernatant was removed thoroughly, and a portion was set aside. The remainder was centrifuged at 13,000 × *g* for 10 min to sediment lysosomes and mitochondria. The post-lysosomal/mitochondrial supernatant was removed thoroughly, and a portion was set aside. The remainder was centrifuged at 130,000 × *g* for 1 h to sediment microsomes and ribosomes. The post-microsomal/ribosomal supernatant, also called the cytosol, was removed thoroughly. Each pellet was resuspended in the same volume of the same buffer as the fraction that was its source. Thus, equal volumes of each fraction are derived from equal numbers of the initial cells.

Electrophoretic Mobility Shift Assay (EMSA)—An EMSA was carried out as previously described (20). The radiolabeled probe was an oligonucleotide from −117 to −89 of the IFNα/β-stimulated gene 15, which includes the IFN-stimulated response element (21). Cell lysates, extracts, subcellular fractions, immunodepleted extracts (see below), partially purified IRF-1 (prepared by phosphocellulose (Whatman P11) chromatography of nuclear extracts from IFNγ-stimulated HeLa cells), proteases, protease inhibitors, and control buffers were included as indicated for individual assays. Unlabeled hepatocyte nuclear factor 4 distal element (22) or IFN-stimulated response element oligonucleotides were included at 100-fold molar excess to provide nonspecific or specific competition, respectively, as indicated for individual assays. All the components for each assay except reaction buffer, nonspecific DNA, and oligonucleotides were assembled on ice. Incubation was started by the addition of a mixture of these remaining components and then carried out for 30 min at room temperature before electrophoresis on 6% polyacrylamide gels at 4 °C with 20 mM Tris-borate, pH 8.3, 0.4 mM EDTA buffer. Images were obtained, and results were quantified with a PhosphorImager (Molecular Dynamics).

Immunodepletion—All specific antibodies were directed against human antigens. Rabbit anti-neutrophil/monocyte elastase and anti-cathepsin G as well as purified human neutrophil/monocyte elastase and cathepsin G were obtained from Calbiochem. Goat anti-secretory leukocyte protease inhibitor (SLPI) and recombinant human SLPI were obtained from R&D Systems. Anti-plasminogen activator inhibitor 2 (PAI-2) and recombinant human PAI-2 were obtained from American Diagnostica. Normal rabbit or goat IgG obtained from Zymed Laboratories Inc. was used as a nonspecific, control antibody for the respective specific antibodies. Protein A- or protein G-conjugated-Sepharose 4B beads were obtained from Zymed Laboratories Inc. Immunodepletion was performed at 4 °C by mixing 5 μl (5 μg) of control or specific antibody with 50 μl of a cytoplasmic extract (~150 μg of protein) prepared in buffer I and adjusted to 150 mM NaCl without or with added target protein as an external standard for 2 h, adding 50 μl of a 50% slurry of protein A or protein G beads (for reactions with rabbit or goat antibodies, respectively) and mixing for 4 h more and then recovering the beads by centrifugation at 12,000 × *g* for 20 s. The immunodepleted supernatants were then removed. The recovered beads were washed 3 times with buffer I plus 300 mM NaCl, and then bound material was eluted by boiling in SDS-PAGE sample buffer for 3 min. Eluates were recovered after centrifugation at 12,000 × *g* for 20 s. The immunodepleted supernatants and the eluates were then frozen in crushed dry-ice and stored at −80 °C.

To control for the efficiency of immunodepletion, elastase (1.5 μg), cathepsin G (1 μg), SLPI (50 ng), or PAI-2 (750 ng) was added to an extract, and immunodepletion was performed with the respective specific antibody or the control antibody (data not shown). As judged by immunoblot of the recovered supernatants and of the eluted immunoprecipitates, the amount of each added protein that was specifically removed was far in excess of the amounts of the endogenous proteins, which were undetectable when extracts were directly assayed by immunoblot. The control antibodies did not reduce the amount of added target protein in the supernatants or recover any in the immunoprecipitates.

Immunoblotting—Cytoplasmic or nuclear extracts, immunodepleted extracts, eluted immunoprecipitates, partially purified IRF-1, and protein standards in cytoplasmic extract buffer all were adjusted to 1× SDS-PAGE loading buffer before analysis by immunoblotting. All samples were boiled for 3 min, electrophoresed on 10% SDS-polyacrylamide gels, and then transferred to nitrocellulose membranes (Bio-Rad) by electroblotting. Membranes were blocked with 0.5% nonfat dry milk in PBS for detection of IRF-1 or with 5% nonfat dry milk and 0.2% Tween 20 in PBS for detection of cathepsin G, elastase, PAI-2, or SLPI. Rabbit polyclonal antiserum against human IRF-1 (8) or the antibodies against the other antigens (described above) were added to the respective solution. Membranes were washed with PBS, then incubated with horseradish peroxidase-conjugated secondary antibodies. Goat anti-rabbit immunoglobulin G and rabbit anti-goat immunoglobulin G (Zymed Laboratories Inc.) were used to detect rabbit and goat primary antibodies, respectively. Membranes were washed with PBS, then incubated with LumiGLO chemiluminescent substrate (Kirkegaard and Perry Laboratories). Signals were detected with x-ray film.

RESULTS

Infection by M. tuberculosis Induces IRF-1—We first investigated whether infection of macrophages by *M. tuberculosis* would alter IRF-1 DNA binding activity as measured by EMSA (Fig 1*A*). Alveolar macrophages had clearly detectable constitutive IRF-1 DNA binding activity (*lane 1*). The activity was

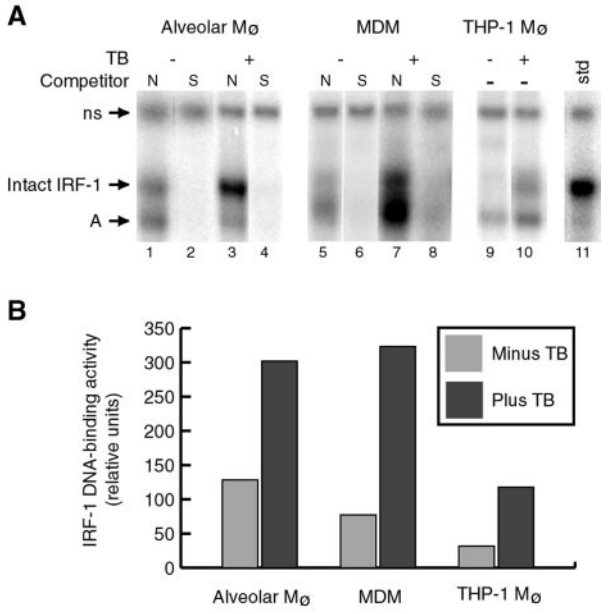

FIG. 1. ***M. tuberculosis* (*TB*) infection induces IRF-1 in macrophages.** *A*, alveolar macrophages (*Alveolar Mø*), peripheral blood monocyte-derived macrophages (*MDM*), or THP-1 cells differentiated by treatment with TPA (*THP-1 Mø*) were uninfected (−) or infected (+) as indicated. IRF-1 DNA binding activity was detected by EMSA of nuclear extracts from cells lysed with non-ionic detergent. An EMSA of partially purified full-length IRF-1 is shown as a standard for comparison (*std*). EMSA binding reactions included nonspecific (*N*) or specific (*S*) oligonucleotide competitor as indicated. The typical protein-DNA complex containing IRF-1 (*Intact IRF-1*), an additional complex containing a species of IRF-1 (*A*), and a nonspecific complex (*ns*) are indicated. *B*, specific IRF-1 DNA binding activity in the typical complex indicated in *panel A* was quantified relative to the indicated nonspecific complex, which serves as an internal standard for the amount of protein included in each assay and loaded in each lane.

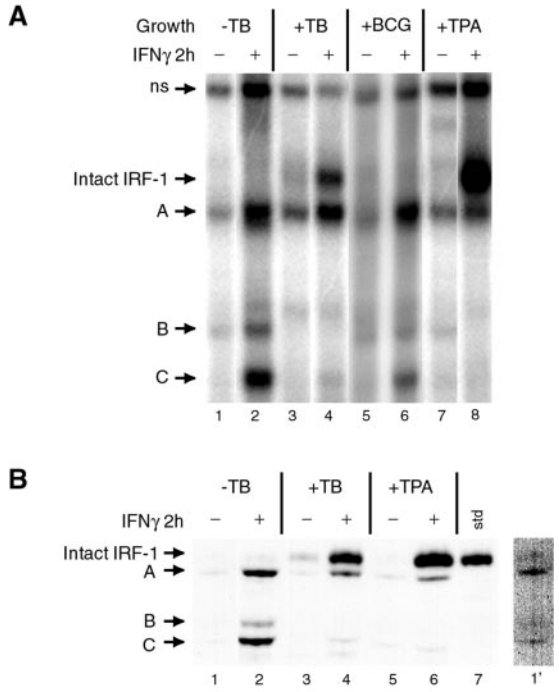

FIG. 2. **Recovery of IRF-1 from THP-1 monocytes is affected by *M. tuberculosis* infection or TPA-mediated differentiation.** THP-1 cells were uninfected (−*TB*), infected by *M. tuberculosis* (+*TB*), infected by *M. bovis* BCG (+*BCG*), or treated with TPA (+*TPA*), as indicated. Cells were not further treated (−) or were treated with IFNγ for the last 2 h before harvest (+), as indicated. Nuclear extracts were prepared from cells lysed with non-ionic detergent. *A*, the DNA binding activity of IRF-1 species (*intact IRF-1*, *A*, *B*, and *C*) was analyzed by EMSA. *B*, IRF-1 species (*intact IRF-1*, *A*, *B*, and *C*) were identified by immunoblot. Partially purified IRF-1 from HeLa cells (*std*) was included as a standard for intact IRF-1. *Lane 1′* is an overexposure of *lane 1*. *ns*, nonspecific complex.

near or below the lower limit of detection in peripheral blood monocyte-derived macrophages and THP-1 macrophages (*lanes 5* and *9*), as is typical of many cells (8, 20, 23–27). In each case, infection induced IRF-1 DNA binding activity (*lanes 3*, *7*, and *10*). The slower mobility induced complex comigrates with *in vitro* translated or partially purified full-length IRF-1 (*lane 11*) bound to the oligonucleotide probe (8). The identity of the complexes was confirmed by use of specific competitor oligonucleotide (for example, *lanes 2*, *4*, *6*, and *8*) and by reaction with anti-IRF-1 antibody (data not shown). The faster mobility-induced complex, labeled A, also contained a species of IRF-1. Altogether, total IRF-1 DNA binding activity clearly increased upon infection. Quantification of IRF-1 DNA binding activity relative to a nonspecific DNA-binding protein that served as an internal standard (Fig. 1*B*), showed that infection with *M. tuberculosis* caused full-length IRF-1 to increase ~4-fold in differentiated THP-1 cells and monocyte-derived macrophages. The increase was ~2-fold in alveolar macrophages compared with their unusually high constitutive level of IRF-1. Thus, in primary macrophages and THP-1 macrophages, infection by *M. tuberculosis* clearly resulted in induction of IRF-1 DNA binding activity. The induced level of IRF-1 was similar in the alveolar and monocyte-derived macrophages and somewhat lower in the THP-1 macrophages.

M. tuberculosis infection of THP-1 monocytes also induced IRF-1 DNA binding activity (Fig. 2*A*). Constitutive expression of IRF-1 was quite low (*lane 1*). The complexes that were detected (labeled *A*, *B*, and *C*) migrated more rapidly than the typical complex containing full-length IRF-1. Nuclear extracts from monocytes stimulated with IFNγ produced an increase in the rapidly migrating complexes, yet the typical complex was not induced (*lane 2*). Compared with extracts from uninfected, unstimulated cells, nuclear extracts from infected monocytes yielded a complex that had the mobility of full-length IRF-1 bound to the IFN-stimulated response element (*lane 3*). Furthermore, the abundance of complex A increased and that of complexes B and C decreased. As in differentiated THP-1 cells and primary macrophages, infection of undifferentiated THP-1 cells by *M. tuberculosis* led to an increase in total IRF-1 DNA binding activity. When nuclear extracts of IFNγ-stimulated infected monocytes were assayed (*lane 4*), the typical complex formed abundantly, whereas the complexes B and C were much less abundant than after IFNγ stimulation of uninfected cells. Recovery of full-length IRF-1 from THP-1 monocytes was a specific effect of infection by *M. tuberculosis*. After infection by *M. bovis* BCG at the same m.o.i. (*lanes 5* and *6*) or after phagocytosis of latex beads (data not shown), predominantly faster mobility IRF-1 complexes were detected. The complexes formed with extracts from untreated or IFNγ-treated infected monocytes had essentially the same mobility as the complexes formed with extracts from untreated or IFNγ-treated THP-1 macrophages (compare *lanes 3* and *4* with *lanes 7* and *8*). Thus, infection of THP-1 monocytes by *M. tuberculosis* and differentiation increased formation of typical complexes and decreased formation of rapidly migrating complexes detected by EMSA of the respective nuclear extracts compared with extracts from uninfected, undifferentiated cells.

Immunoblots were performed (Fig. 2*B*) to identify IRF-1 species present in the extracts that had been analyzed by EMSA. Anti-IRF-1 antiserum detected a pattern of proteins that corresponded precisely with the protein-DNA complexes

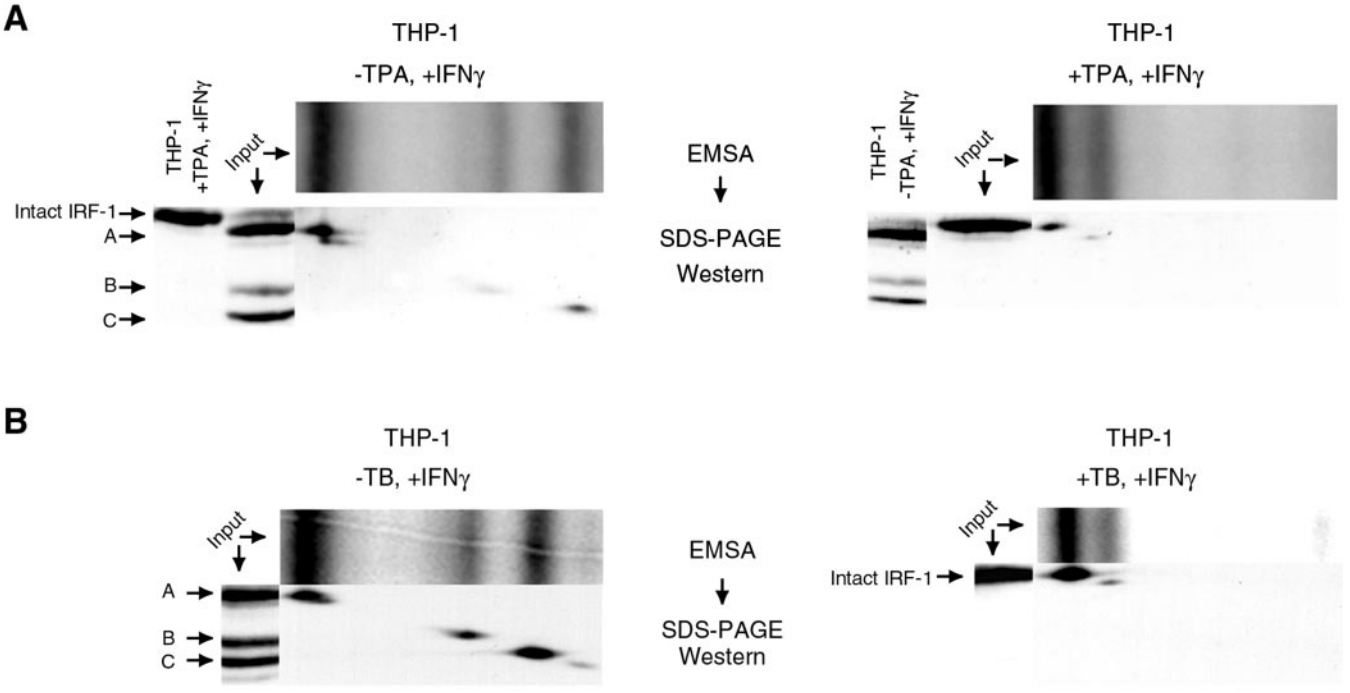

FIG. 3. **IRF-1/DNA complexes with differing mobility contain IRF-1 species of differing size.** *A*, monocytes (untreated THP-1 cells, *left panel*) or macrophages (THP-1 cells treated for 3 days with TPA, *right panel*) were stimulated for 2 h with IFNγ. Nuclear extracts were prepared from cells lysed with non-ionic detergent. For each extract, IRF-1·DNA complexes were resolved by EMSA in a pair of lanes, and the lanes were cut apart. The complexes resolved in one lane of each pair were detected with a PhosphorImager, and the other lane of the pair was equilibrated with SDS-PAGE loading buffer then applied horizontally as a sample for SDS-PAGE, with the top of the EMSA lane at the left. The extract used for the EMSA applied to each gel was also loaded directly for SDS-PAGE on the same gel (*Input*). Additionally, a sample of macrophage extract (*THP-1/+TPA +IFNγ*) was loaded next to the monocyte extract on the SDS-PAGE used to resolve the proteins bound in the EMSA of the monocyte extract (*left panel*) and vice versa (*THP-1/−TPA +IFNγ*; *right panel*). IRF-1 species (*Intact IRF-1*, *A*, *B*, and *C*) were separated by SDS-PAGE and detected by immunoblot. The IRF-1·DNA complexes resolved by EMSA are shown aligned above each immunoblot result in the same position as the lane used for the SDS-PAGE sample. *B*, THP-1 cells that were uninfected (*left panel*) or infected with *M. tuberculosis* for 3 days (*right panel*) were treated for 2 h with IFNγ. Extracts were prepared and analyzed as described for *panel A*, except that the extract from infected cells was not included on the SDS-PAGE used for extracts from uninfected cells, and vice versa.

detected by EMSA. Constitutively expressed IRF-1 recovered from monocytes appeared as three species, labeled *A*, *B*, and *C* (*lanes 1* and *1′*). Each of those was induced by IFNγ (*lane 2*). Extracts of infected monocytes contained an additional, slower mobility IRF-1 species, and total recovery of IRF-1 increased (*lane 3*). IFNγ treatment of infected monocytes strongly induced the additional IRF-1 species, and to a lesser extent, higher mobility species were recovered. IRF-1 species A was constitutively present in macrophage extracts (*lane 5*). Recovery of this species increased slightly, and the slowest mobility IRF-1 species was abundant in extracts from IFNγ-treated macrophages (*lane 6*). Consistent with the EMSA results, the slower mobility-induced species comigrated with *in vitro* translated or partially purified full-length IRF-1 (*lane 7*) (8). Comparison of the EMSA and immunoblot results indicated that the ratio of IRF-1 DNA binding activity and protein were similar under all conditions and for all species of IRF-1. There was also a close correlation between the mobility of the protein-DNA complexes detected by EMSA and the mobility of the IRF-1 protein species. Furthermore, infection or differentiation led to decreased recovery of higher mobility IRF-1 species and increased recovery of full-length IRF-1.

To unambiguously demonstrate which protein species was contained in which protein-DNA complexes, we next performed two-dimensional analyses. To increase sensitivity, the extracts were prepared from IFNγ-treated cells. Fig. 3*A* shows the results obtained with extracts from monocytes (*left panel*) and macrophages (*right panel*). The initial separation by EMSA (in a *parallel lane* not used for a SDS-PAGE sample) is shown aligned in the same position as the lane used for a SDS-PAGE sample. The immunoblot result from the SDS-PAGE separations of the initial protein samples and the EMSA complexes formed by those samples is shown beneath the EMSA separations.

Each complex resolved by EMSA contained one species of IRF-1 that precisely comigrated with a species resolved by SDS-PAGE alone. Thus, each species of IRF-1 protein detected in extracts from monocytes was found in only one of the complexes detected by EMSA, and the slower mobility complex detected by EMSA of extracts from macrophages contained full-length IRF-1. The two-dimensional analyses of extracts from uninfected and infected monocytes (Fig. 3*B*) also showed that one characteristic higher mobility IRF-1 species was present in each protein-DNA complex resolved by EMSA of monocyte extract (*left panel*) and that the slower mobility complex resolved by EMSA of the extract from infected monocytes contained only full-length IRF-1 (*right panel*). Thus, the complexes resolved by EMSA reflect the presence of the corresponding distinct species of IRF-1 and can be used as an assay for their abundance.

A Monocyte Membrane-bound Serine Protease Is Responsible for Production of High Mobility IRF-1 Species—We hypothesized that the presence of higher mobility IRF-1 species was the result of protease activity and found that full-length IRF-1 was recovered in nuclear extracts when THP-1 monocytes were lysed with non-ionic detergent and sufficient (2 mM) PMSF (Fig. 4*A*, *lane 2*). This result suggested that higher mobility IRF-1 species were produced during extraction, in which case they might also be produced *in vitro*. A cytoplasmic extract from untreated THP-1 cells was prepared after lysis with non-

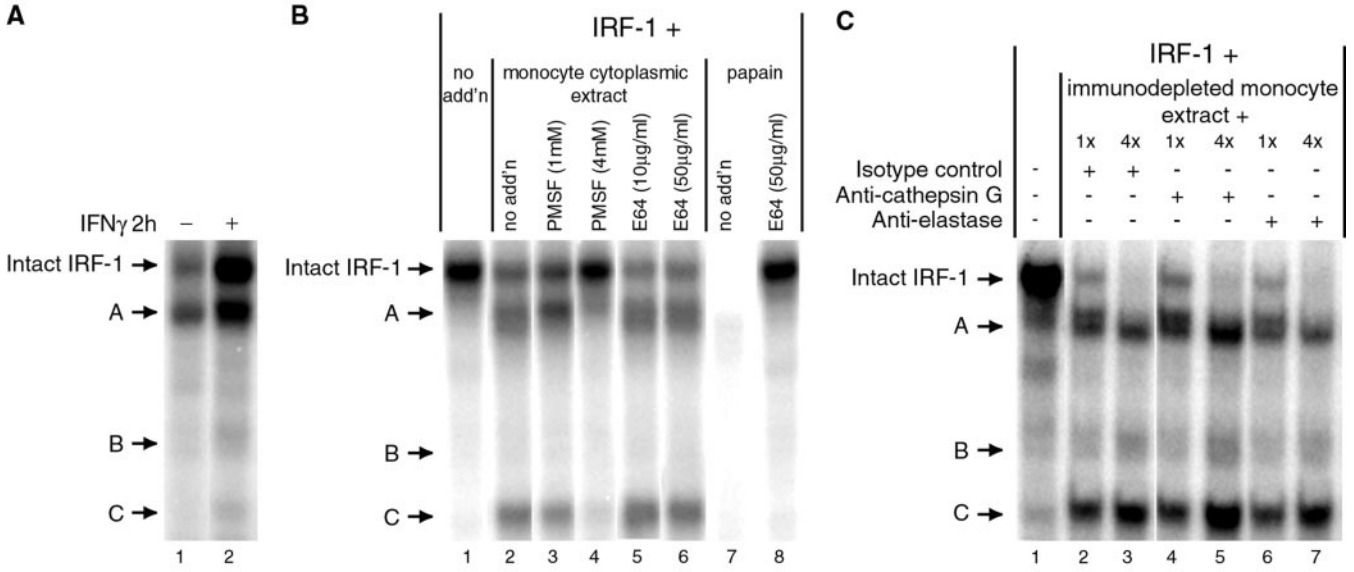

FIG. 4. **A serine protease in THP-1 monocytes is responsible for production of higher mobility IRF-1 species.** *A*, nuclear extract was prepared from untreated (−) or IFNγ-treated (+) THP-1 monocytes that had been lysed with non-ionic detergent in the presence of 2 mM PMSF. The DNA binding activity of IRF-1 species (*Intact IRF-1*, *A*, *B*, and *C*) was analyzed by EMSA. *B*, partially purified IRF-1 was mixed with control buffer (*no add'n*), with cytoplasmic extract prepared from THP-1 monocytes lysed by Dounce homogenization, or with papain, a nonspecific cysteine protease, as indicated. Protease inhibitors PMSF and E64 were included as indicated. The DNA binding activity of IRF-1 species (*Intact IRF-1*, *A*, *B*, and *C*) was analyzed by EMSA. *C*, cytoplasmic extract from THP-1 monocytes lysed with non-ionic detergent was immunodepleted with the indicated antibodies. The indicated relative amount of each immunodepleted extract (1× or 4×, equivalent to 0.5 and 2 μl of the starting extract) was mixed with partially purified IRF-1. The DNA binding activity of IRF-1 species (*Intact IRF-1*, *A*, *B*, and *C*) was analyzed by EMSA.

ionic detergent in the absence of protease inhibitors. The extract was mixed with partially purified IRF-1 in the absence or presence of protease inhibitors, and whether high mobility IRF-1 species were produced was determined in an EMSA (Fig. 4*B*). IRF-1 assayed with no extract produced the typical protein-DNA complex expected for intact IRF-1 (*lane 1*). Addition of the extract (*lane 2*) produced the specific species previously observed for endogenous IRF-1 recovered from THP-1 monocytes. PMSF, which reversibly inhibits cysteine proteases and irreversibly inhibits serine proteases, substantially reduced production of higher mobility IRF-1 species (*lanes 3* and *4*). In contrast, E64, a specific cysteine protease inhibitor, did not (*lanes 5* and *6*). To confirm that the E64 was active, its ability to inhibit papain was tested. Papain completely degraded IRF-1 (*lane 7*), but inclusion of E64 at 50 μg/ml completely protected the IRF-1 against proteolysis (*lane 8*). Thus, monocytes contain a serine protease that can lead to production of higher mobility IRF-1 species *in vitro*.

We tested whether either of two major monocyte serine proteases, the lysosomal enzymes neutrophil/monocyte elastase and cathepsin G, was responsible for the appearance of high mobility IRF-1 species. Antibodies against them were used for immunodepletion of monocyte cytoplasmic extract prepared after lysis with non-ionic detergent in the absence of protease inhibitors (Fig. 4*C*). Partially purified IRF-1 (*lane 1*) yielded higher mobility species when mixed with the extract after it had been immunodepleted with control antibody (*lanes 2* and *3*), anti-cathepsin G (*lanes 4* and *5*), or anti-elastase (*lanes 6* and *7*). The comparable result obtained with the nonspecific and specific antibodies together with the confirmed ability of the specific antibodies to immunodeplete their target proteins (see "Experimental Procedures") indicate that these proteases did not contribute to production of higher mobility IRF-1 species.

To further address the source of the protease, we performed EMSA in which partially purified IRF-1 was mixed with cell lysate or classical subcellular fractions (Ref. 16; see "Experimental Procedures"). For these experiments, cells were lysed by Dounce homogenization without detergent or protease inhibitors. Equal proportions of the lysate or subcellular fractions were assayed on a per cell basis, and the samples were in the same buffer. In one experiment, lysate was compared with the nuclear pellet, and post-nuclear supernatant was prepared from that lysate (Fig. 5*A*). The lysate yielded high mobility IRF-1 species, and as expected (*lane 1*), the nuclear pellet did not (*lane 2*) and the post-nuclear supernatant did (*lane 3*), as judged by comparison to the complexes formed by the added partially purified IRF-1 (*lane 4*). In a separate experiment (Fig. 5*B*), further differential centrifugation was performed to fractionate the post-nuclear supernatant. The production of high mobility species of IRF-1 that occurred upon incubation with the post-nuclear supernatant (*lane 1*) was enhanced upon incubation with the lysosomal/mitochondrial pellet (*lane 2*). The post-lysosomal/mitochondrial supernatant (*lane 3*), the microsomal/ribosomal pellet (*lane 4*), and the cytosol (*lane 5*) each resulted in production of small amounts of the high mobility species, as judged by comparison to the input of partially purified IRF-1 for this assay (*lane 6*). This distribution of activity in fractions obtained from cells lysed gently without detergent indicates that the protease is located on the cytoplasmic surface of an organelle/vesicle in the lysosomal/mitochondrial pellet.

Regulation of Monocyte Protease Activity—We prepared extracts at various times after TPA treatment and performed EMSA to determine the kinetics for recovery of intact IRF-1 during differentiation of THP-1 cells (Fig. 6). The three characteristic complexes, A, B, and C, formed with an extract from IFNγ-treated monocytes (compare *lanes 1* and *2*). Extracts prepared from IFNγ-treated cells after 2 or 6 h of TPA treatment yielded some of the complex that contains intact IRF-1 (*lanes 3* and *4*). An increased amount of more rapidly migrating complex A and reduced amounts of complexes B and C were also observed with these extracts. Intact IRF-1 was the major species recovered after cells were grown in the presence of TPA for 16, 24, 48, or 72 h and stimulated with IFNγ for the final 2 h before extraction (*lanes 5–10*). Complex A, the IFNγ-induced

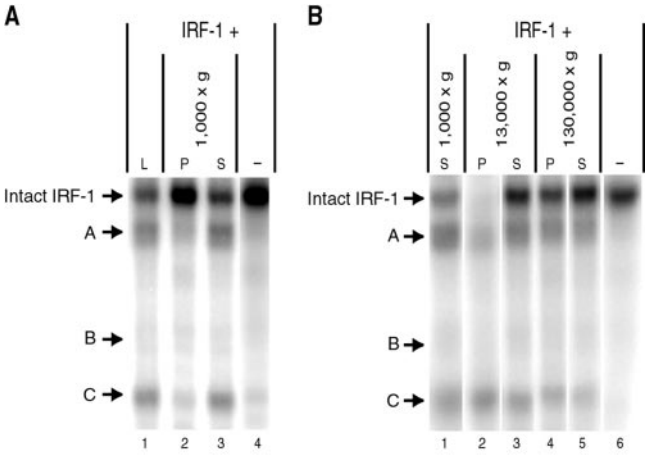

FIG. 5. **The serine protease responsible for production of higher mobility IRF-1 species is located on the cytoplasmic face of an organelle or vesicle in the lysosomal/mitochondrial subcellular fraction of THP-1 monocytes.** THP-1 cells were lysed by Dounce homogenization, and subcellular fractions were prepared as described under "Experimental Procedures." *A* and *B*, partially purified IRF-1 was incubated with lysate (*L*), with the resuspended pellet (*P*), or the supernatant (*S*) obtained after the indicated centrifugation or with control buffer (−). The DNA binding activity of IRF-1 species (*Intact IRF-1*, *A*, *B*, and *C*) was analyzed by EMSA.

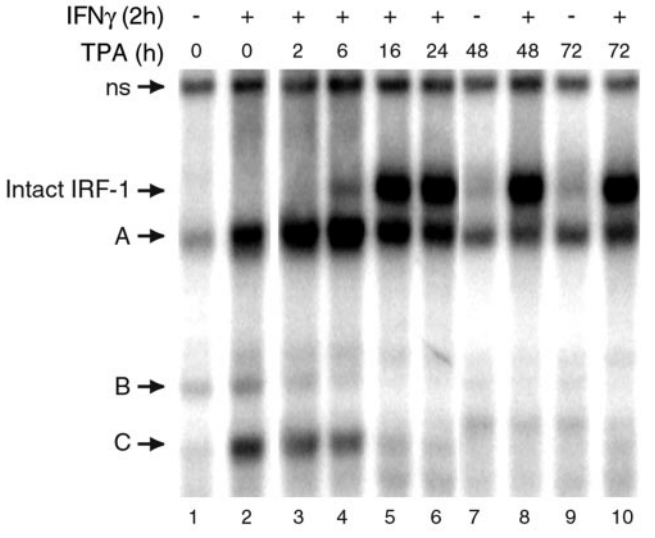

FIG. 6. **Kinetics of recovery of intact IRF-1 during TPA-induced differentiation of THP-1 cells.** THP-1 cells were treated with TPA for various times and either received no other treatment or were stimulated with IFNγ for the last 2 h before harvest, as indicated. Nuclear extracts were prepared from cells lysed with non-ionic detergent. The DNA binding activity of IRF-1 species (*Intact IRF-1*, *A*, *B*, and *C*) was analyzed by EMSA. *ns*, nonspecific complex.

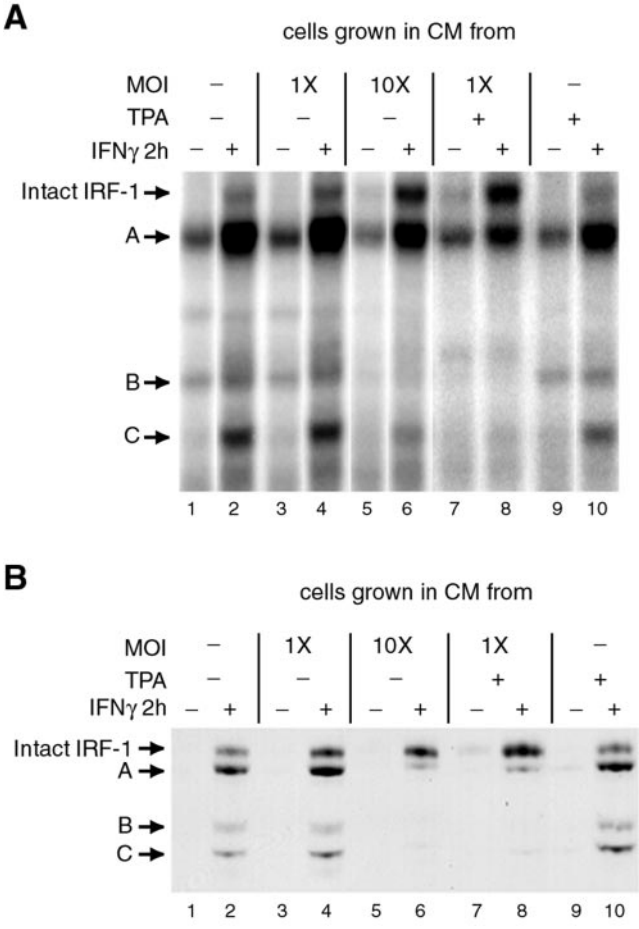

FIG. 7. **CM obtained after *M. tuberculosis* infection of THP-1 cells affects the species of IRF-1 recovered from THP-1 monocytes, but conditioned media obtained after TPA-mediated differentiation does not.** THP-1 cells were grown for 1 day in fresh media, then resuspended in fresh media or various CM as indicated and grown for 3 days. Cells received no other treatment (−) or were stimulated with IFNγ for the last 2 h before harvest (+), as indicated. Nuclear extracts were prepared from cells lysed with non-ionic detergent. *A*, the DNA binding activity of IRF-1 species (*Intact IRF-1*, *A*, *B*, and *C*) was analyzed by EMSA. *B*, IRF-1 species (*Intact IRF-1*, *A*, *B*, and *C*) were identified by immunoblot.

complex that was predominant at earlier times, was greatly reduced and only slightly inducible at 48 or 72 h after TPA treatment, and complexes B and C were no longer detectable. The concomitant loss of higher mobility IRF-1 species and appearance of lower mobility species, culminating in recovery of predominantly intact IRF-1, indicates that the protease activity responsible for production of higher mobility species diminishes as differentiation proceeds.

Autocrine or paracrine mechanisms could account for the effect of *M. tuberculosis* infection on induction of IRF-1 and regulation of the protease activity, since only 10–30% of monocytes or 30–50% of macrophages in a culture are actually infected upon incubation with *M. tuberculosis*. The effect of TPA might be entirely direct or in part mediated by secreted factors. To examine these possibilities, we grew THP-1 monocytes in CM obtained from THP-1 monocytes and macrophages that had been infected for 3 days or had been cultured in parallel without infection. We then performed EMSA and immunoblotting to examine the IRF-1 recovered by lysis and extraction with non-ionic detergent (Fig. 7). The constitutive and IFNγ-induced IRF-1 species recovered from monocytes grown in CM from uninfected monocytes (*lanes 1* and *2* in *panels A* and *B*) were similar to the species detected in extracts from monocytes grown in fresh media (Fig. 2). The same was true when monocytes were grown in media conditioned by growth of *M. tuberculosis* alone (data not shown). However, a higher mobility species of IRF-1 were reduced, and intact IRF-1 was detected in extracts from monocytes grown in CM from monocytes infected at high m.o.i. (*lane 5* in *panel A*). This effect of growth in CM from infected monocytes was also seen when the cells were treated with IFNγ (*lane 6* in *panels A* and *B*). Growth of monocytes in conditioned media from cells infected at low multiplicity after TPA treatment led to a similar reduction in higher mobility species of IRF-1 and an increase in intact IRF-1 as growth in CM from monocytes infected at high m.o.i., which was again seen in cells treated with IFNγ (*lanes 7* and *8* in *panels A* and *B*). Conditioned media from cells grown for 4 days in the presence of TPA had no effect on recovery of

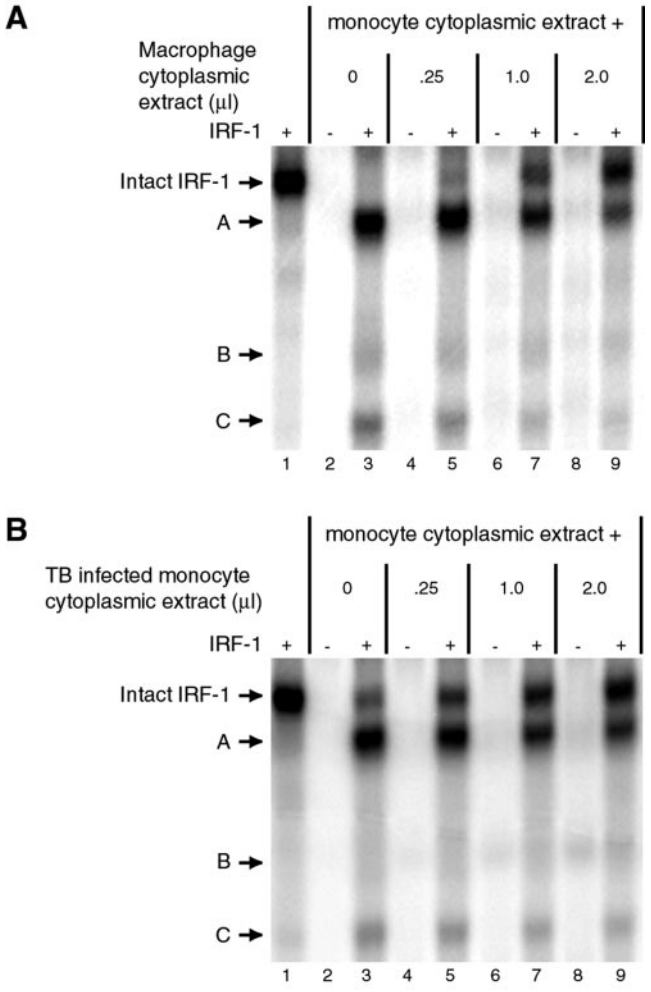

FIG. 8. **A serine protease inhibitor is induced by TPA treatment or *M. tuberculosis* infection of THP-1 cells. Partially purified IRF-1 (+) or buffer (−) was incubated without or with monocyte cytoplasmic extract, as indicated.** Additional buffer containing increasing amounts of cytoplasmic extract from TPA-treated THP-1 cells (*A*) or THP-1 monocytes infected by *M. tuberculosis* (*B*) was included in the incubation, as indicated. The DNA binding activity of IRF-1 species (*Intact IRF-1*, *A*, *B*, and *C*) was analyzed by EMSA.

constitutive or IFNγ-induced IRF-1 from monocytes (*lanes 9* and *10* in *panels A* and *B*). Thus, the effect of TPA on recovery of IRF-1 from uninfected cells is not likely to be mediated indirectly by factors secreted in response to TPA treatment. Furthermore, because conditioned media from infected, TPA-treated cells had a greater effect than conditioned media from monocytes infected at the same m.o.i. (compare *lanes 7* and *8* with *lanes 3* and *4*), infection after TPA treatment leads to greater production of the secreted factor(s) that modulates the protease activity which mediates production of high mobility IRF-1 species.

Infection of Monocytes by M. tuberculosis or TPA-mediated Differentiation Induces a Cytoplasmic Protease Inhibitor—Recovery of intact IRF-1 upon extraction of TPA-treated or infected monocytes (Figs. 1 and 2) could be due to a decrease in the monocyte protease, induction of a protease inhibitor, or both. To examine these possibilities, we first tested the effect of adding cytoplasmic extract from TPA-treated cells to cytoplasmic extract from untreated monocytes on production of high mobility IRF-1 species (Fig. 8*A*). The input of partially purified IRF-1 is shown in *lane 1*. Monocyte cytoplasmic extract alone had no DNA binding activity (*lane 2*). Incubation of this fraction with the partially purified IRF-1 eliminated the intact protein and produced higher mobility species, as expected (*lane 3*). Increasing amounts of cytoplasmic extract from TPA-treated cells added to monocyte cytoplasmic extract did not produce any specific protein-DNA complexes (*lanes 4*, *6*, and *8*). When IRF-1 was also present, the intact protein was increasingly recovered, and the higher mobility species decreased correspondingly (*lanes 5*, *7*, and *9*). Cytoplasmic extract from infected monocytes similarly inhibited the monocyte protease (Fig. 8*B*). In this experiment, most of the intact input IRF-1 (*lane 1*) was converted to higher mobility species upon the addition of monocyte cytoplasmic extract (*lane 3*). When the reactions included increasing amounts of cytoplasmic extract from infected monocytes, the protease in the cytoplasmic extract from uninfected monocytes was increasingly inhibited, as evidenced by the increasing amounts of intact IRF-1 and the decreasing amounts of higher mobility species that were detected (*lanes 5*, *7*, and *9*). As in the previous experiment, the monocyte extract alone had no DNA binding activity (*lane 2*), and increasing amounts of extract from infected monocytes did not produce any specific protein-DNA complexes (*lanes 4*, *6*, and *8*). These data demonstrate that a protease inhibitor was induced by TPA-mediated differentiation or by infection with *M. tuberculosis*.

To determine the subcellular localization of the protease inhibitor, lysate from TPA-treated THP-1 cells was prepared without detergent or protease inhibitors, then fractionated. EMSA was performed to examine the effect of the lysate or subcellular fractions on the ability of monocyte cytoplasmic extract to modify partially purified IRF-1 (Fig. 9*A*). Intact input IRF-1 (*lane 1*) was reduced by the monocyte cytoplasmic extract, and higher mobility species were increased, as expected (*lane 2*). When supernatants from 1,000 × *g* (*lane 3*), 13,000 × *g* (*lane 5*), or 130,000 × *g* (*lane 7*) centrifugation of lysates from TPA-treated cells were added to the assay, production of higher mobility IRF-1 species was reduced, and slightly more intact IRF-1 was recovered. In contrast, the addition to the assay of the lysosomal/mitochondrial (13,000 × *g*) pellet from TPA-treated cells resulted in a substantial loss of IRF-1 DNA binding activity (*lane 4*), and the microsomal/ribosomal (130,000 × *g*) pellet had only a slight inhibitory effect on the monocyte protease (*lane 6*). Thus, the particulate fractions contained little or none of the induced protease inhibitor; it was recovered in the cytosol after extensive centrifugation.

We attempted to determine whether SLPI or PAI-2 might be the inhibitor induced by differentiation or *M. tuberculosis* infection of THP-1 cells, because among cytoplasmic serine protease inhibitors, these two are known to be induced by monocyte differentiation or bacterial infection (28–31). Their possible role after differentiation was examined first (Fig. 9*B*). Partially purified IRF-1 (*lane 1*) yielded higher mobility complexes A, B, and C when mixed with monocyte extract (*lane 2*). Increasing amounts of extract from TPA-treated cells first led to an increase in complex A and then to the appearance of the intact IRF-1 complex and a decrease in complex C whether the extract had been immunodepleted with control antibody (*lanes 3* and *4*), antibody against SLPI (*lanes 5* and *6*), or antibody against PAI-2 (*lanes 7* and *8*). The possible role of SLPI and PAI-2 in the effect of infection by *M. tuberculosis* was examined next (Fig. 9*C*). The complex formed by partially purified IRF-1 (*lane 1*) and the complexes formed when monocyte extract was added (*lane 2*) are shown for comparison with the complexes formed after incubation of partially purified IRF-1 with extract immunodepleted by control antibody (*lane 3*), anti-SLPI (*lane 4*), or anti-PAI-2 (*lane 5*). In each case, upon the addition of the immunodepleted extract there was a slight decrease in production of higher mobility IRF-1 species B and C, and a small

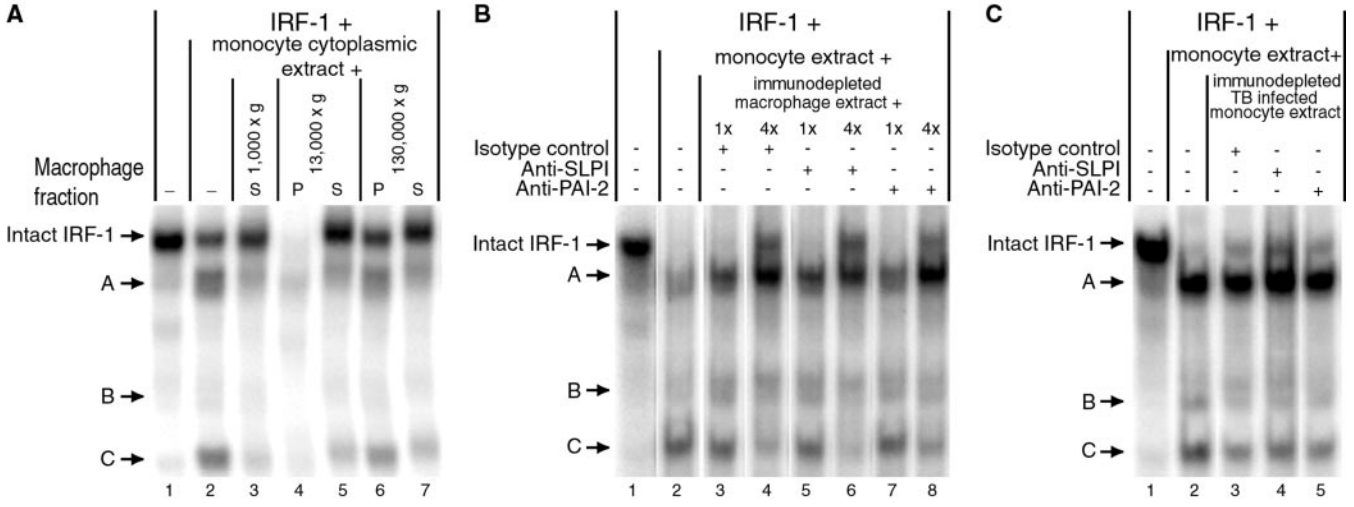

FIG. 9. **The induced serine protease inhibitor is cytosolic.** TPA-treated THP-1 cells were lysed by Dounce homogenization, and subcellular fractions were prepared as described under "Experimental Procedures." *A*, partially purified IRF-1 was incubated without or with THP-1 monocyte cytoplasmic extract, and as indicated, the supernatant (*S*) or the resuspended pellet (*P*) was obtained after the indicated sequential centrifugation of the lysate from the TPA-treated cells. Buffer (−) was included in place of cytoplasmic extract and/or subcellular fractions as indicated. The DNA binding activity of IRF-1 species (*Intact IRF-1*, *A*, *B*, and *C*) was analyzed by EMSA. *B*, cytoplasmic extract from THP-1 macrophages was immunodepleted with the indicated antibodies. The indicated relative amount of each immunodepleted extract (1× or 4×, equivalent to 1 and 4 μl of the starting extract) was mixed with cytoplasmic extract from THP-1 monocytes and with partially purified IRF-1. The DNA binding activity of IRF-1 species (*Intact IRF-1*, *A*, *B*, and *C*) was analyzed by EMSA. *C*, cytoplasmic extract was prepared from THP-1 monocytes that had been infected by *M. tuberculosis* (*TB*) and was immunodepleted with the indicated antibodies. An amount of each immunodepleted extract equivalent to 1 μl of the starting extract was mixed with cytoplasmic extract from THP-1 monocytes and with partially purified IRF-1. The DNA binding activity of IRF-1 species (*Intact IRF-1*, *A*, *B*, and *C*) was analyzed by EMSA.

amount of intact IRF-1 was recovered. These data demonstrate that the protease inhibitor was still present after immunodepletion. The comparable result obtained with the nonspecific and specific antibodies together with the confirmed ability of the specific antibodies to immunodeplete their target proteins (see "Experimental Procedures") indicate that neither SLPI nor PAI-2 was the inhibitor induced by differentiation or infection.

DISCUSSION

A framework that includes seven major points summarizes the results and conclusions of this study. First, infection of monocytes and macrophages by *M. tuberculosis* induces IRF-1, as judged by its DNA binding activity (Figs. 1 and 2). Second, IRF-1 species having greater electrophoretic mobility than full-length IRF-1 are obtained upon extraction of THP-1 monocytes that are uninfected or infected by *M. bovis* BCG, and full-length IRF-1 is recovered only after infection by *M. tuberculosis* or TPA-induced monocyte-to-macrophage differentiation (Fig. 2). Third, the IRF-1·DNA complexes detected by EMSA each contain one characteristic species of IRF-1 protein (Fig. 3). Fourth, a serine protease localized to the cytoplasmic surface of an organelle or vesicle in the lysosomal/mitochondrial fraction is apparently responsible for production of the higher mobility IRF-1 species (Figs. 4 and 5). Fifth, as judged by the recovered species of IRF-1, there are differences in regulation of the change in protease activity due to TPA-induced differentiation and due to infection. The response to TPA is clear within 6 h and is nearly complete within 16 h (Fig. 6). It occurs apparently independent of autocrine or paracrine pathways (Fig. 7). The effect of infection on the protease activity is minimal at 24 h post-infection[2] and involves factors secreted from cells during infection (Fig. 7). Sixth, infection by *M. tuberculosis* or differentiation induces a cytoplasmic serine protease inhibitor (Figs. 8 and 9). Thus, the protease and inhibitor would be expected to interact physiologically, and that interaction is likely to account for recovery of intact IRF-1. Seventh, induction of IRF-1 and a serine protease inhibitor by *M. tuberculosis* infection are likely to be host defense responses to infection, because it is independently known that IRF-1 contributes to host defense against mycobacteria and that serine protease inhibitors can be induced by and protect against inflammatory stimuli.

Induction of IRF-1 by M. tuberculosis Infection—Induction of IRF-1 by *M. tuberculosis* infection of monocytes and macrophages is likely to be a host-defense mechanism, because the pleiotropic functions of IRF-1 in the immune system, such as antiviral and antibacterial phenotypes, or involvement in production of NK cells and development of Th1 cell-mediated responses all contribute to host defense against infectious disease (reviewed in Ref. 32). Of particular note, targeted disruption of the IRF-1 gene greatly reduces expression of inducible nitric-oxide synthase in response to infection by *M. bovis* BCG and makes mice susceptible to infection by *M. bovis* BCG and *M. tuberculosis* (5, 6).

Cleavage of IRF-1 by a Monocyte Serine Protease—Three considerations strongly suggest, but do not prove, that cleavage of IRF-1 by a serine protease directly yields higher mobility species of IRF-1. First, truncated species of IRF-1 produce EMSA complexes of higher mobility and have higher mobility on SDS-PAGE than intact IRF-1 (33). Second, the IRF-1 species recovered from THP-1 monocytes correspond to those produced *in vitro* by incubation of monocyte extracts or fractions with partially purified IRF-1. Third, PMSF inhibits recovery of higher mobility IRF-1 species. Although the apparent cleavage of IRF-1 occurred during nuclear extract preparation, it clearly reflected physiological changes in a protease activity that occurred upon infection of monocytic THP-1 cells by *M. tuberculosis* or their TPA-induced differentiation. This regulation is likely to be a general characteristic of monocytic cells, since we obtained the same higher mobility IRF-1 species in extracts of undifferentiated U937 and NB4 monocytic cell lines and observed that they yielded intact IRF-1 after TPA treatment.[2]

Major monocyte serine proteases such as cathepsin G and elastase are localized within lysosomes or intracellular vesicles

[2] Y. Qiao, A. Canova, and R. Pine, unpublished observations.

(reviewed in Ref. 34), but it is unlikely that production of high mobility IRF-1 species was due to leakage of organelle or vesicle contents. Incubation of cathepsin G or elastase with IRF-1 *in vitro* produced patterns of cleavage products that differed from the characteristic species of IRF-1 recovered from cells and produced by incubation of IRF-1 with monocyte cytoplasmic extract *in vitro*.[3] Moreover, the cytosol of cells lysed without detergent had little or none of the activity that produced the characteristic IRF-1 species, and immunodepletion of elastase and cathepsin G from monocyte cytoplasmic extract did not change the pattern of species produced from partially purified IRF-1.

A change from recovery of cleaved proteins to recovery of intact proteins upon monocyte-to-macrophage differentiation has also been described for the transcription factors SP1 and p65. Cleavage of p65 occurs during extract preparation (35); whether the cleavage of SP1 occurs physiologically has not been addressed (36). In contrast to the recovery of intact IRF-1, it was not clear whether the increased recovery of intact SP1 or p65 in extracts of differentiated cells reflected induction of a proteinase inhibitor.

Induction of a Serine Protease Inhibitor—Even without knowing the identity of the protease inhibitor, since it is cytosolic, it is reasonable to conclude that it could physiologically inhibit the serine protease that we detected on the cytoplasmic surface of an organelle or vesicle. It seems that neither of two likely candidates, SLPI and PAI-2, mediate inhibition of the protease. Using antibodies against SLPI and PAI-2 to immunodeplete cytoplasmic extract from infected THP-1 monocytes or from THP-1 macrophages did not affect the ability of the extracts to inhibit cleavage of partially purified IRF-1 by cytoplasmic extract from THP-1 monocytes. Moreover, the addition of recombinant SLPI or recombinant PAI-2 to cytoplasmic extract from THP-1 monocytes had no effect, although they were able to strongly inhibit *in vitro* cleavage of partially purified IRF-1 by trypsin or urokinase plasminogen activator, respectively.[3] Nonetheless, several observations about SLPI and PAI-2 are relevant to the effects of *M. tuberculosis* infection on protease inhibitor expression. First, SLPI, apparently acting intracellularly (37), inhibits several responses to LPS (31, 38). Second, PAI-2 induction by tumor necrosis factor α or by *Mycobacterium avium* infection limits apoptosis evoked by those inflammatory stimuli (29, 39, 40). Thus, although *M. tuberculosis* infection causes apoptosis at least in part through induction of tumor necrosis factor α (41), tumor necrosis factor α induction of PAI-2 might also limit the extent of apoptosis. It is believed that limiting apoptosis may serve to keep bacteria intracellular and prevent dissemination within the host (29, 42). Third, *M. tuberculosis* infection and expression of PAI-2, both, induce type I IFN production (15, 43), and type I IFN contributes to host defense against *M. tuberculosis* through mechanisms that are yet unknown (6). These observations suggest that, like induction of IRF-1, induction of a serine protease inhibitor is likely to be a defensive host response to *M. tuberculosis* infection.

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[3] Y. Qiao and R. Pine, unpublished observations.

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Host Defense Responses to Infection by *Mycobacterium tuberculosis* : INDUCTION OF IRF-1 AND A SERINE PROTEASE INHIBITOR

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