

Expression and Secretion of Cathelicidin LL-37 in Human Epithelial Cells after Infection by *Mycobacterium bovis* Bacillus Calmette-Guérin[▽]

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The antimicrobial cathelicidin LL-37 is considered to play an important role in the innate immune response to tuberculosis infection. However, little is known about the induction and secretion of this antimicrobial peptide in A549 epithelial cells after infection with *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), the world's most widely used tuberculosis vaccine. In this study, we investigated the effect of *M. bovis* BCG on LL-37 mRNA levels in A549 cells by real-time PCR and on protein levels by Western blotting. Treatment of cells with *M. bovis* BCG upregulates LL-37 mRNA expression in a dose- and time-dependent manner. The quantitative analysis of LL-37 gene expression correlated with our Western blotting results. Moreover, our results demonstrated that treatment of cells with the transcriptional inhibitor actinomycin D effectively inhibited in a concentration-dependent manner the ability of *M. bovis* BCG to induce LL-37 mRNA expression. Finally, inhibition of the MEK1/2 and p38 mitogen-activated protein kinase (MAPK) signaling pathways reduced *M. bovis* BCG-mediated LL-37 mRNA expression, a reduction that correlated with the observed high level of downregulation of LL-37 protein induction. Thus, these results indicate that the MEK1/2 and p38 MAPK signaling pathways play a critical role in the regulation of inducible LL-37 gene expression in A549 cells infected with *M. bovis* BCG.

Tuberculosis is a leading global cause of morbidity and an important cause of death (8). The emergence of multidrug-resistant *Mycobacterium tuberculosis* strains is alarming and represents a worldwide health care problem (33). This situation underscores the need for more-efficient therapies against human tuberculosis. Recently, it has been demonstrated that the infection of epithelial cells with mycobacteria triggered the induction of antimicrobial peptides (17, 38). Mammalian cells produce different kinds of antimicrobial peptides, such as α -defensin in neutrophils, β -defensins in epithelia, histatins in saliva, and cathelicidin (CAP18 or LL-37) in neutrophils and epithelia (13, 26, 35). The antimicrobial peptides have the property of folding into amphipathic structures that have a positively charged hydrophilic face and a hydrophobic face. The main role of antimicrobial peptides is the direct lysis of mycobacteria through the permeabilization of cellular membranes (14). In addition, these peptides exhibit broad-spectrum antimicrobial activity against microbes, including chemotactic activity for neutrophils, monocytes, and some T cells (6, 7) and induction of interleukin-8 secretion from epithelial cell lines (28). The cationic antimicrobial peptide demonstrating the most significant immunoregulatory potential to date is LL-37 (22, 36). LL-37 is the sole human cathelicidin characterized to date (37). LL-37 is an 18-kDa protein which is the proteolytically processed extracellular form of CAP18, and the last 37 amino acid residues at the C terminus are active against bacteria (3, 12). LL-37 was initially identified in the specific granules of neutrophils, and expression was subsequently identified in various epithelia (1). Its expression is induced during the

course of bacterial infection or inflammation in a variety of tissues (5, 10, 32). LL-37 is produced at mucosal surfaces by epithelial cells and is upregulated in response to mycobacterial antigens (16). Although the synthesis and secretion of cationic peptides by epithelia have become recognized as an important mechanism for host defense, whether cathelicidin LL-37 is induced by *Mycobacterium bovis* BCG in human epithelial cells has not been determined. To our knowledge, this is the first report showing gene induction and secretion of LL-37 in epithelial cells after infection with *M. bovis* BCG. Elucidation of the effect of *M. bovis* BCG on LL-37 expression and induction in epithelial cells will aid in the development of novel therapeutic agents for treatment of mycobacterial infections.

MATERIALS AND METHODS

Specific reagents. U0126, PD98059, and SB203580 were purchased from Calbiochem (La Jolla, CA) and resuspended in sterile dimethyl sulfoxide (DMSO; Sigma). The inhibitors were added 30 min before the infection. Actinomycin D was purchased from Sigma-Aldrich (St. Louis, MO). Anti-human LL-37 antibody was purchased from Santa Cruz Biotechnology, Santa Cruz, CA.

Bacteria. *M. bovis* BCG (ATCC 35733) was obtained from the American Type Culture Collection (Manassas, VA). BCG was grown for 15 days in Sauton medium at 37°C. Cultures were harvested by centrifugation and then washed three times in medium. Aliquots of the stock were stored at -80°C until use.

Cell culture. The human lung epithelial cell line A549 (ATCC CCL 185) was cultured in 75-cm² culture flasks (Costar) to semiconfluence and maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂ in a Dulbecco's modified Eagle medium (Gibco-BRL, Grand Island, NY) nutrient mixture containing 10% fetal calf serum (HyClone Laboratories, Logan, UT), 100 U/ml penicillin G, and 100 μ g/ml streptomycin. Fetal calf serum contained <5 μ g lipopolysaccharide/100 ml, as certified by the manufacturer. After reaching confluence, cells were plated in 24-well dishes at a concentration of 10⁶ cells per well. The cells were infected with viable *M. bovis* BCG at multiplicities of infection (MOIs) of 1:1, 5:1, and 10:1. For all experiments, mycobacteria were added to cells on ice and incubated for 10 min, allowing mycobacteria to settle onto the cells, and then incubated for 18 h at 37°C in 5% CO₂. Control cultures with no mycobacteria were always included. Cell viability was assessed by replacing medium and adding 500 μ g/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tet-

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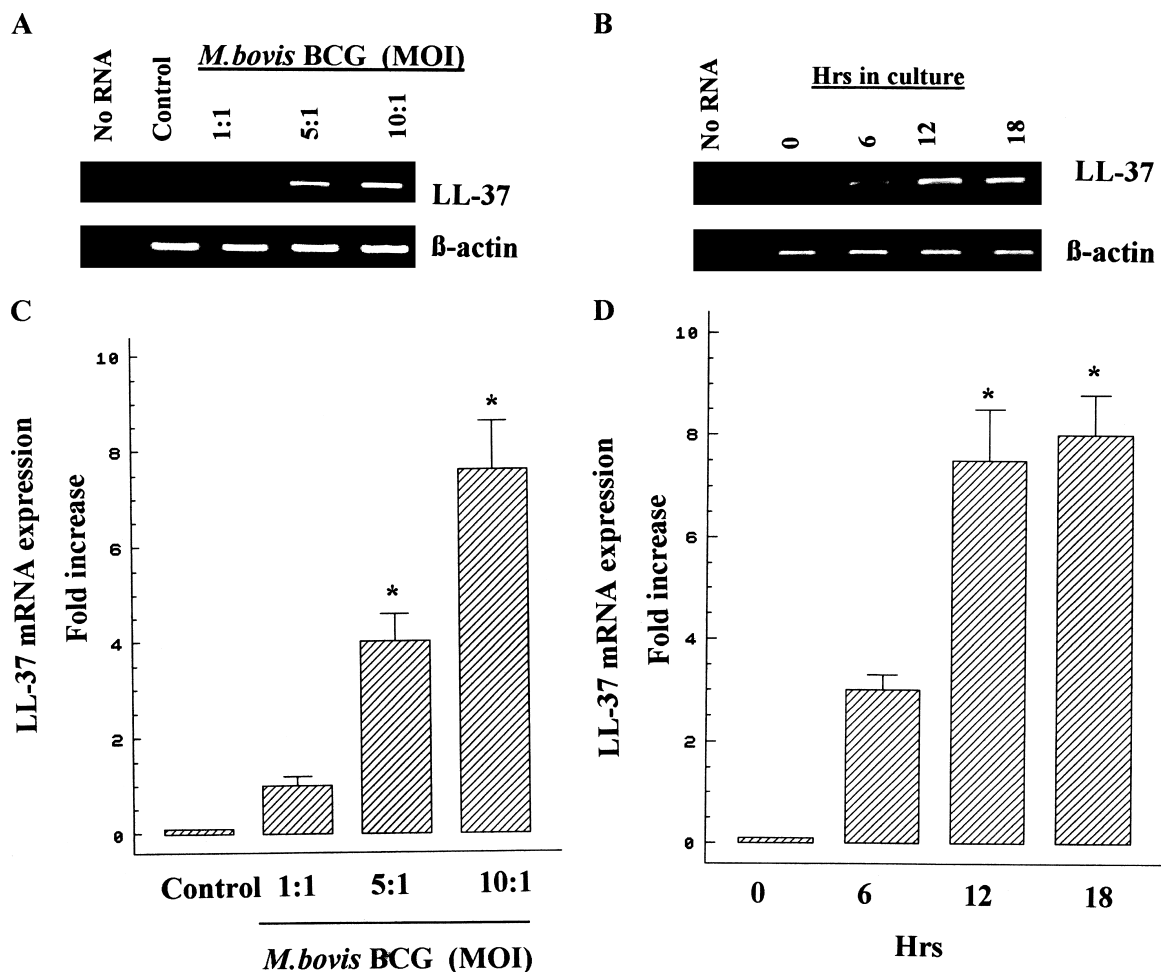


FIG. 1. Induction of LL-37 gene expression by *M. bovis* BCG in A549 cells. A549 cells were left unstimulated (control) or were stimulated with *M. bovis* BCG at MOIs of 1:1, 5:1, and 10:1 for 18 h. The kinetics of LL-37 mRNA induction was determined as described above. Total RNA was extracted, and RT-PCR (A and B) or real-time PCR (C and D) was conducted to quantify LL-37 mRNA levels. As a negative control, RNA was omitted from reverse transcription and PCR amplification (no RNA). The β -actin gene was used as a housekeeping gene. In RT-PCR analysis, data shown are representative of four separate experiments. In real-time PCR analysis, results are represented as the means \pm SD from at least three independent experiments. *, $P < 0.05$.

razolium bromide (MTT) (Sigma). After incubation for 3 h at 37°C, water-insoluble dark blue formazan crystals that formed from MTT cleaved in actively metabolizing cells were dissolved in lysis buffer containing 20% sodium dodecyl sulfate and 50% dimethylformamide, and the absorbance at 570 nm was determined. To examine the signaling pathway for LL-37 induction, cells were treated with extracellular signal-regulated kinase (ERK)/MEK inhibitors (U0196, and PD98059) and a p38 mitogen-activated protein kinase (MAPK) inhibitor (SB203580). An MTT assay was used to test the viability of cultured cells treated with each inhibitor. No obvious cytotoxicity (<0.5%) was found in cultures treated with these inhibitors at the doses used.

RT-PCR and real-time PCR. LL-37 gene expression was determined by semi-quantitative reverse transcriptase PCR (RT-PCR) analysis or quantitative (real-time) PCR using the β -actin gene as a housekeeping gene. Total RNA was isolated from cells, and samples of total RNA were quantified by measuring the optical density at 260 nm. Reverse transcription of LL-37 mRNA was performed using Superscript III reverse transcriptase with oligo(dT) primers in 20 μ l as described by the manufacturer (Invitrogen, Carlsbad, CA). Controls without reverse transcriptase were included in each experiment. First-strand cDNA was amplified by PCR. The following LL-37-specific primers were used: sense, 5'-A GGATTGTGACTTCAAGAAGGACG-3'; antisense, 5'-GTTTATTCTCAG AGCCAGAAAGC-3'. After an initial denaturing step (90°C for 1 min), the amplification profile was 35 cycles of 1 min of denaturation at 94°C and 2.5 min of annealing and extension at 66°C. The PCR products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining.

Quantitative PCR was performed using the 5700 sequence detection system (Applied Biosystems) according to the manufacturer's instructions. Briefly, a total of 1 μ l of cDNA (described above) was analyzed using the final concentration of 100 nM of primers and 2 \times Sybr green PCR master mix (Applied Biosystems, Foster City, CA) in a volume of 20 μ l. The sequences of forward and reverse primers, as designed by Primer Express (Applied Biosystems) for quantification of LL-37 mRNA were 5'-GAAGACCCAAAGGAATGCC-3' and 5'-CAGAGCCCAGAAGCCTGAGC-3'. A standard curve was constructed from serial dilutions of cDNA synthesized from a known quantity of total RNA. Negative controls were included in each real-time PCR run. The results are expressed as means \pm standard deviations (SD).

Western blot analysis. For LL-37 expression analysis, cells were washed with ice-cold phosphate-buffered saline containing 1 mM pervanadate and lysed (in phosphate-buffered saline with 1% Triton X-100, 1 mM EDTA, 1 mM NaVO₄, 1 mM NaF) and the cell lysates were clarified by centrifugation. The protein concentration was measured using the Bio-Rad assay kit. Equal amounts of protein from each sample were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. The material in gels was blotted onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, FL). The membranes were blocked with 3% bovine serum albumin in Tris-buffered saline buffer, pH 7.5. After washes (0.02% Tween 20, pH 7.5, Tris-buffered saline), immunolabeling was performed using polyclonal rabbit antibody against LL-37 or β -actin overnight. Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (LL-37) or goat anti-mouse immunoglobulin G (β -actin) was applied

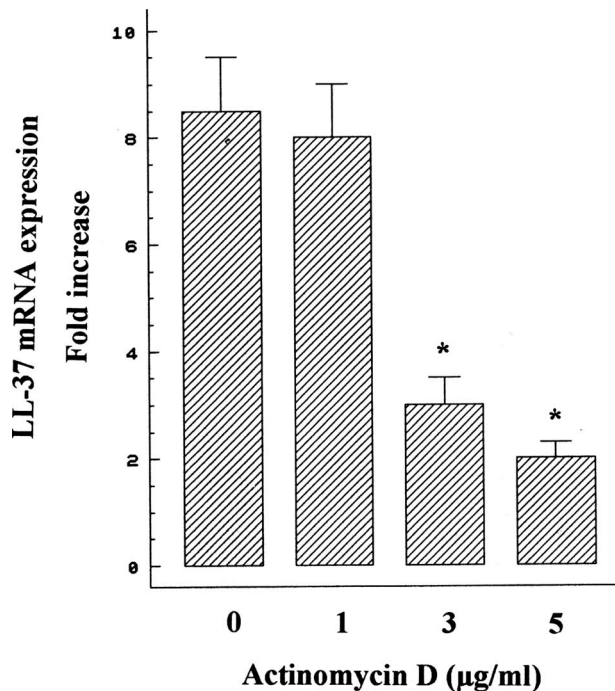


FIG. 2. Effect of actinomycin D on *M. bovis* BCG-induced LL-37 mRNA expression. A549 cells were infected with *M. bovis* BCG at an MOI of 10:1 for 18 h after treatment with or without various concentrations of actinomycin D. Total RNA was extracted, and real-time PCR was conducted to quantify LL-37 mRNA levels, normalized to β -actin. Graphs show means \pm SD from at least five independent experiments. *, $P < 0.05$, for comparison with *M. bovis* BCG cultures that did not receive actinomycin D.

to the membrane. The peroxidase-positive bands were detected by immersing the blots in a developing solution (73 mM sodium acetate, pH 6.2) containing 0.3% diaminobenzidine tetrahydrochloride and 0.04% H_2O_2 at room temperature for 5 min. The enzyme reaction was terminated by washing the blots in 0.1 M H_2SO_4 .

Statistical evaluation. Statistical analysis was performed using Student's *t* test with Microsoft Excel software. Significance was accepted at P values of <0.05 .

RESULTS

Induction of both LL-37 mRNA and protein expression by *M. bovis* BCG in A549 cells. Initially, we examined whether *M. bovis* BCG infection induces the augmentation of LL-37 mRNA expression in A549 cells. Cells were infected at MOIs of 1:1, 5:1, and 10:1. After 18 h of incubation, RNA was extracted for RT-PCR as described above. The β -actin gene was used as a housekeeping gene control. Figure 1A shows that unstimulated cells hardly showed LL-37 mRNA expression and that LL-37 mRNA levels were upregulated in a dose-dependent manner, indicating that LL-37 mRNA expression is augmented in A549 cells in response to stimulation with *M. bovis* BCG. To define the kinetics of LL-37 mRNA induction in response to *M. bovis* BCG, cells were infected with *M. bovis* BCG for 0, 6, 12, and 18 h. Total RNA was collected and used separately for cDNA synthesis. The cDNA was utilized as templates in the RT-PCRs for LL-37. RT-PCR results showed that the levels of LL-37 mRNA were upregulated in a time-dependent manner (Fig. 1B). To confirm the RT-PCR results, real-time PCR was conducted for LL-37. As indicated in Fig.

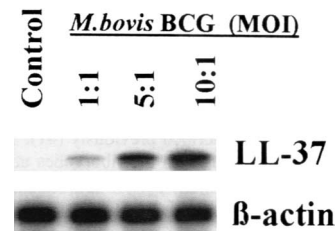


FIG. 3. Detection of LL-37 in cell extracts of A549 cells infected with *M. bovis* BCG by Western blot analysis. A549 cells were left unstimulated (control) or were stimulated with *M. bovis* BCG at MOIs of 1:1, 5:1, and 10:1 for 18 h. Total cell lysates were analyzed by Western blotting to observe LL-37 expression. The amount of protein from the lysates was determined by using the Bio-Rad assay kit, and equal amounts of protein were loaded in all wells. Parallel blotting was performed with an antibody to β -actin. Data from a representative experiment are presented, and similar results were obtained in four independent experiments.

1C, the levels of LL-37 mRNA expression were enhanced in response to *M. bovis* BCG in a dose-dependent manner and LL-37 gene expression in cells stimulated with *M. bovis* BCG at an MOI of 10 was eightfold higher than that in unstimulated cells ($P < 0.05$). Figure 1D shows that the levels of LL-37 mRNA were upregulated within 6 h of exposure, with a maximum induction at 12 to 18 h of incubation. In order to determine whether or not transcriptional upregulation of the LL-37 gene is the predominant mechanism through which *M. bovis* BCG induces LL-37 expression, A549 cells were treated with or without various concentrations of the transcriptional inhibitor actinomycin D and infected with *M. bovis* BCG at an MOI of 10 for 18 h. Results from all five experiments showed that addition of actinomycin D significantly ($P < 0.05$) reduced in a concentration-dependent manner the ability of *M. bovis* BCG to induce LL-37 mRNA expression, indicating that *M. bovis* BCG upregulates LL-37 expression primarily through transcriptional upregulation of the LL-37 gene in A549 cells (Fig. 2).

To determine whether the induction of LL-37 mRNA levels correlated with an increase in protein expression, Western blot analysis was performed. A549 cells were infected with *M. bovis* BCG at MOIs of 1:1, 5:1, and 10:1. After 18 h of incubation, protein expression was analyzed by Western blotting. As shown in Fig. 3, LL-37 was detected in whole-cell extracts of A549 cells stimulated with *M. bovis* BCG and was enhanced in response to *M. bovis* BCG in a dose-dependent manner. In addition, the protein was not observed in unstimulated cells. Taken together, these results suggest that *M. bovis* BCG induces both LL-37 mRNA and protein expression in A549 cells.

Mechanism of LL-37 mRNA induction by *M. bovis* BCG in A549 cells. Since it has been demonstrated that the MEK-ERK signaling pathway is involved in butyrate-mediated cathelicidin induction in colon epithelial cells (25), we examined whether this pathway is also required for *M. bovis* BCG activity. To do this, cells were treated with U0126, a specific inhibitor of ERK1/2 kinase (MEK1/2). The results demonstrated that *M. bovis* BCG-induced LL-37 mRNA expression was significantly reduced by the MEK-ERK inhibitor (U0126) in a dose-dependent manner (Fig. 4A). It is important to note that the vehicle DMSO, in an amount equivalent to that contained in 20 μ M

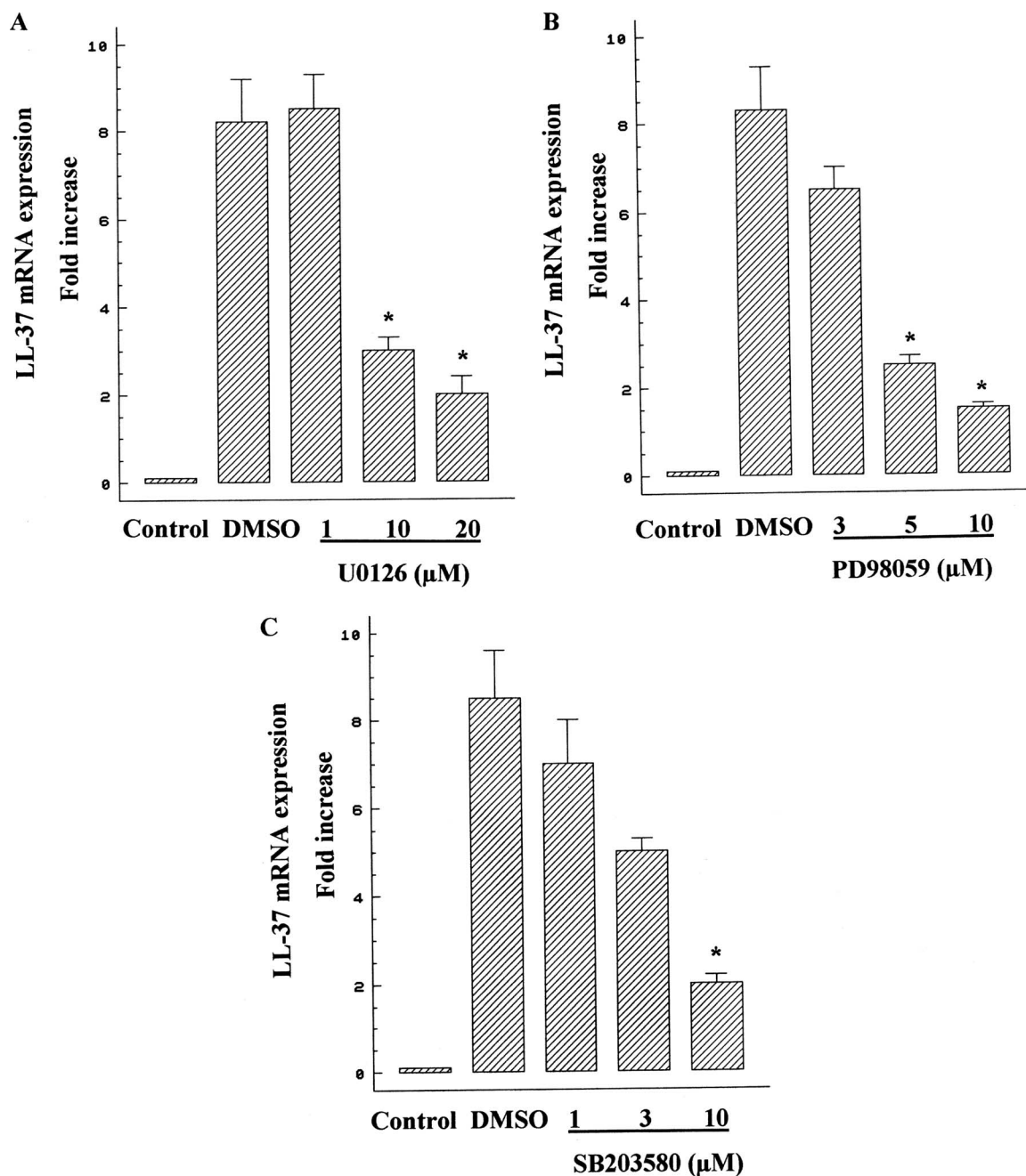


FIG. 4. Effect of specific MAPK inhibitors on *M. bovis* BCG-induced LL-37 mRNA expression. A549 cells were left unstimulated (control) or were stimulated with *M. bovis* BCG at an MOI of 10:1 for 18 h after preincubation with or without different concentrations of the MEK inhibitor U0126 (A) or PD98059 (B) or the p38 MAPK inhibitor SB203580 (C), and real-time PCR was conducted to quantify LL-37 mRNA levels, normalized to β -actin. Graphs show means \pm SD from at least three independent experiments. *, $P < 0.05$, for comparison with *M. bovis* BCG cultures that did not receive an inhibitor.

U0126, had no effect on LL-37 induction (Fig. 4A). To confirm the regulatory role of the MEK-ERK signaling pathway, we also evaluated the effect of a second MEK inhibitor (PD98059, an inhibitor of MEK via upstream-activator-dependent phosphorylation) before *M. bovis* BCG infection. Results were that *M. bovis* BCG-mediated LL-37 induction was also significantly reduced by PD98059 in a dose-dependent manner, beginning at 5 μ M PD98059 ($P < 0.05$; Fig. 4B).

To further investigate the mechanism underlying LL-37 gene activation in response to *M. bovis* BCG, we tested a specific p38 kinase inhibitor (SB203580). As shown in Fig. 4C, pretreatment with SB203580 inhibited ongoing LL-37 mRNA induction by *M. bovis* BCG. Taken together, these results indicate that these two MAPK pathways could be involved in *M. bovis* BCG-mediated LL-37 induction in A549 cells. The regulation of the LL-37 mRNA by the MEK-ERK and the p38

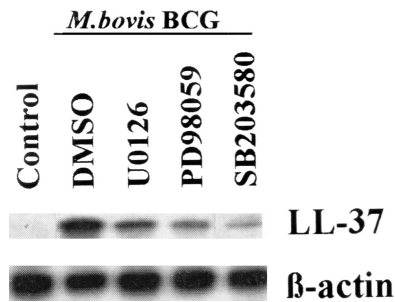


FIG. 5. Inhibitors of MAPK block *M. bovis* BCG-induced LL-37 protein expression. Cells were unstimulated (control) or stimulated with *M. bovis* BCG at an MOI of 10:1 after preincubation with or without the MEK inhibitor U0126 (20 μ M) or PD98059 (10 μ M) or the p38 MAPK inhibitor SB203580 (10 μ M) or with vehicle (DMSO), and total cell lysates were analyzed by Western blotting to observe LL-37 protein expression. Parallel blotting was performed with an antibody to β -actin. Data from a representative experiment are presented, and similar results were obtained in three independent experiments.

MAPK pathways correlated with reduced expression of the LL-37 protein, as determined by Western blotting (Fig. 5). Our previous results demonstrated that *M. bovis* BCG activates both ERK1/2 and p38 MAPK signaling pathways in A549 cells (17). In this study, phosphorylation of ERK1/2 by *M. bovis* BCG in A549 cells was inhibited by the MEK-ERK inhibitors U0126 and PD98059, whereas phosphorylation of p38 MAPK was not affected, showing the specificity and efficacy of U0126 and PD98059 (data not shown).

DISCUSSION

The prevalence of tuberculosis is increasing in the world. Consequently, there is an increased need for the development of new antituberculosis treatments, especially for patients who respond poorly to conventional therapy or present resistance to antimycobacterial drugs (9, 34). Since their antimycobacterial activities have demonstrated some therapeutic benefit during tuberculosis infection, antimicrobial peptides have gained the attention of researchers as novel therapeutic agents against human tuberculosis (2, 27). In particular, LL-37 displays broad-spectrum microbicidal activities (3). LL-37 has been recognized as an antimicrobial peptide which protects the skin from bacterial infection (20). Although LL-37 has been identified as an important peptide with a multifunctional role in host defense (4), its particular role in the control of the innate epithelial immune response prompted us to examine the expression of LL-37 by *M. bovis* BCG. Our results demonstrate both gene expression and protein secretion of LL-37 in epithelial cells after stimulation with *M. bovis* BCG. We chose to work with epithelial cells, because mycobacteria are transmitted primarily by the respiratory route and alveolar epithelial cells are among the first host cells to encounter mycobacteria (30, 31). We demonstrated that *M. bovis* BCG induced high levels of LL-37 mRNA in a dose-dependent manner. This observation was extended by using actinomycin D, which showed significantly suppressed *M. bovis* BCG-induced LL-37 mRNA expression in a dose-dependent manner, suggesting that this expression is at the level of transcription. Moreover,

our data were corroborated by Western blotting, which showed that *M. bovis* BCG induced significant levels of LL-37 protein secretion in a concentration-dependent manner. The augmented expression levels of LL-37 mRNA were almost constant for 12 to 18 h after stimulation with *M. bovis* BCG. This expression is coincident with the presence of HBD-2, a previously described distinct mammalian antimicrobial peptide produced by the same epithelial cell line after stimulation with *M. bovis* BCG (18). Since cathelicidins and defensins have synergistic action as antimicrobials (19), it is possible that LL-37 and HBD-2 may act synergistically against mycobacteria.

The underlying mechanisms of cathelicidin expression are only beginning to be understood (24), but recent studies have revealed that cathelicidin expression is regulated via the MAPK signaling pathway. Interestingly, it has been shown that the inhibition of p38 kinase pathway significantly decreased cathelicidin gene expression in human keratinocytes (23). However, Schaubert et al. demonstrated that inhibition of the ERK1/2 pathway but not the p38 MAPK pathway blocked sodium butyrate-induced cathelicidin gene expression in colonic, gastric, and hepatic cells (24). In contrast, our present data show that *M. bovis* BCG-stimulated LL-37 production can be regulated by both the ERK1/2 and p38 MAPK signaling pathways. This apparent conflict may arise because LL-37 expression appears to be differentially regulated among different cell types and dependent on stimuli.

There is now clear evidence that the LL-37 gene has potential binding sites for several transcription factors, including NF- κ B, NF-interleukin-6, and AP-1 (29). In addition, it has been reported that activation of AP-1 is regulated via MAPK signaling pathways (11). Thus, we could not exclude the participation of AP-1 in *M. bovis* BCG-induced LL-37 expression in A549 cells. This interesting point will be explored in future experiments.

It has been demonstrated that Toll-like receptor (TLR) pathways are important for optimal innate immune response against *M. tuberculosis* (21). Moreover, Liu et al. showed that TLR activation of cells induced killing of *M. tuberculosis* through LL-37 participation (15). Therefore, it should be possible in future studies to address whether such TLR pathways regulate *M. bovis* BCG-induced LL-37 expression in epithelial cells.

In summary, we investigated the mechanisms involved in *M. bovis* BCG-induced LL-37 gene expression in human lung epithelial cell line A549. Our data indicate that *M. bovis* BCG can induce both LL-37 mRNA and protein expression. Furthermore, we demonstrated that the ERK1/2 and p38 MAPK signaling pathways participate in the regulation of inducible LL-37 gene expression. It remains to be elucidated if *M. bovis* BCG can strengthen the epithelial defense barrier by upregulating LL-37 mRNA expression in vivo. However, studies on the induction of LL-37 by *M. bovis* BCG may result in therapeutic approaches that enhance the host immune defense against tuberculosis.

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