Peptidoglycan (PGN), the major cell wall component of Gram-positive bacteria, induces secretion of cytokines in macrophages through CD14, the pattern recognition receptor that binds lipopolysaccharide and other microbial products. To begin to elucidate the mechanisms that regulate the transcription of cytokine genes, we wanted to determine which transcription factors are activated by PGN in mouse RAW264.7 and human THP-1 macrophage cells. Our results demonstrated that: (i) PGN induced phosphorylation of the transcription factors ATF-1 and CREB; (ii) ATF-1 and CREB bound DNA as a dimer and induced transcriptional activation of a CRE reporter plasmid, which was inhibited by dominant negative CREB and ATF-1; (iii) PGN induced phosphorylation of c-Jun, protein synthesis of JunB and c-Fos, and transcriptional activation of the AP-1 reporter plasmid, which was inhibited by dominant negative c-Fos; and (iv) PGN-induced activation of CREB/ATF and AP-1 was mediated through CD14. This is the first study to demonstrate activation of CREB/ATF and AP-1 transcription factors by PGN or by any other component of Gram-positive bacteria.

The CREB/ATF family of transcription factors are leucine zipper proteins that bind to the cAMP response element (CRE) with the consensus sequence, 5′-TGACGTC-A-3′ (17). CREB, the most extensively studied CRE-binding protein, is phosphorylated at serine 133 by protein kinase A in response to cAMP, and this leads to transcriptional activation (17) of genes whose promoters contain the CRE sequence. There are other signaling pathways that lead to phosphorylation and activation of CREB, such as calmodulin kinase, which phosphorylates CREB in response to increased intracellular Ca2+ (17), or RSK2 which is activated by mitogen-activated protein (MAP) kinases (18). ATF-1, another member of this family of transcription factors, has significant sequence similarity to CREB, including a phosphorylation site for CREB, but it is not known if PGN induces activation of any other transcription factors. It is also not known which transcription factors are required for PGN-induced activation of cytokine genes.

The AP-1 family of transcription factors consists of the Jun and Fos families of proteins that bind the 5′-TGACGTCA-3′ (17). ATF-1 forms heterodimers only with CREB; however, the other ATF proteins can also form heterodimers with specific members of the AP-1 family of transcription factors. In addition, different heterodimers may bind variant CRE sequences, thus increasing the number of potential regulatory mechanisms.

The AP-1 family of transcription factors consists of the Jun and Fos families of proteins that bind the 5′-TGACGTCA-3′-acetate response element (TPA-RE), 5′-TGACGTCA-3′ and induce transcription in response to many different stimuli, including phorbol esters (19). These proteins bind DNA as dimers, which are formed through leucine zippers. The Jun proteins can bind DNA as homodimers or as heterodimers with Fos proteins; however, the Fos proteins can only bind DNA as heterodimers (19). In addition to forming heterodimers within the AP-1 family, Jun proteins can heterodimerize with certain members of other transcription factor families, such as ATF and C/EBPβ (19). Different dimers can bind different sequences, e.g., Jun-Jun and Jun-Fos dimers preferentially bind TPA-RE, while Jun-ATF dimers prefer to bind the CRE sequence (19).

The objective of this study was to: (i) determine if PGN activates the transcription factors CREB/ATF and AP-1, (ii) identify the specific members of these two families of transcription factors activated by PGN, and (iii) determine the specific mechanisms by which PGN activates these transcription factors.

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† To whom correspondence should be addressed: Northwest Center for Medical Education, Indiana University School of Medicine, 3400 Broadway, Gary, Indiana 46408. Tel.: 219-980-6557; Fax: 219-980-6556, E-mail: dgupta@meded.iun.indiana.edu.

‡ The abbreviations used are: PGN, peptidoglycan; Ab, antibody; AP-1, activating protein-1; ATF, activating transcription factor; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; CRE, CAMP response element; CREB, CRE-binding protein; Egr, early growth response; ERK, extracellular signal-regulated kinase; IL-1, interleukin 1; IL-6, interleukin 6; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAP, mitogen-activated protein; NF, nuclear factor; PAGE, polyacrylamide gel electrophoresis; ReLPS, LPS from S. minnesota Re 595; sPGN, soluble PGN; TNF-α, tumor necrosis factor-α; TPA-RE, 12-O-tetradecanoylphorbol-13-acetate response element.
tion factors that are activated by PGN, and (iii) determine if this activation is CD14-dependent.

**EXPERIMENTAL PROCEDURES**

**Materials—**Soluble PGN (sPGN), a soluble polymeric form of PGN, was isolated from *Staphylococcus aureus* Rb grown in the presence of penicillin (20) and purified by vancomycin affinity chromatography, and its purity has been previously described (21). sPGN contained <24 pg of endotoxin/mg, as determined by the *Limulus* lysate assay (21). LPS from *Salmonella minnesota* Re595 (RelPS, a minimal naturally occurring endotoxin structure of LPS), obtained by phenol-chloroform-petroleum ether extraction, was purchased from Sigma. All other chemicals were from Sigma, unless otherwise indicated.

**Cells—**Murine macrophage RAW264.7 cell line, obtained from ATCC (Rockville, MD), was cultured in Dulbecco's modified Eagle's medium with 10% defined fetal calf serum (HyClone, Logan, UT; endotoxin content <6 pg/ml). Human monocytic THP-1 cell line, obtained from ATCC, was cultured in RPMI 1640 with 10% defined fetal calf serum. For each experiment, THP-1 cells were allowed to differentiate for 72 h in the presence of 100 nm 1a,25-dihydroxyvitamin D3 (Biomol, Plymouth Meeting, PA).

**Phosphorylation of ATF-1 and CREB and Western Blots—**RAW264.7 cells were cultured at 0.35–0.4 × 10^6^ 24-well plates (2 ml/well) and cultured for 16–20 h. THP-1 cells were seeded at 0.15 × 10^6^ ml in 24-well plates (2 ml/well) and allowed to differentiate as above for 72 h. All cells were activated with the stimulants indicated under “Results” and then washed and lysed as before (6). In some experiments, sPGN and RelPS were incubated with 5 μg/ml polymyxin B for 30 min and then added to cells. In other experiments, THP-1 cells were incubated with 10 μg/ml anti-CD14 monoclonal antibodies, MY4 (Coulter, Hialeah, FL) or MEM18 (Sanbio-Monosan, Uden, The Netherlands), or the isotype control IgG2b (clone MPO-11; Coulter) at 30 min at 37 °C before stimulation. Cell lysates were separated on 12% SDS-PAGE and transferred to Immobilon P (6). Phosphorylation of ATF-1 and CREB was determined by Western blotting with 0.5 μg/ml rabbit anti-pCREB antibody (Upstate Biotechnology, Inc., Lake Placid, NY), and detected against a c-Jun peptide containing phosphorylated serine 63 (Santa Cruz Biotechnology). Nonphosphorylated c-Jun, Jun B, Jun D, and jun Fos were also detected by Western blots using antibodies from Santa Cruz Biotechnology. Nonphosphorylated and nonphosphorylated c-Jun by Western blots. sPGN-induced phosphorylation of ATF-1 and CREB was not inhibited by polymyxin B (an antibiotic that binds LPS and inhibits its biologic effects), unlike the induction by ReLPS, which was almost completely inhibited (Fig. 1D). This confirms that the sPGN-induced effect was not due to endotoxin contamination. In control experiments, the amounts of total (phosphorylated and nonphosphorylated) CREB (Fig. 1B) and ATF-1 (Fig. 1C) showed equal amounts of CREB and ATF-1 protein present in all lanes.

**RESULTS**

**sPGN Induces Phosphorylation of ATF-1 and CREB—**Activation of the transcription factor CREB is regulated by phosphorylation at serine 133. ATF-1, also a CRE-binding protein, has extensive homology to CREB, including a conserved phosphorylation site. We tested if sPGN induces phosphorylation of CREB and ATF-1 in RAW264.7 cells, using an antibody that specifically recognizes both phosphorylated CREB and phosphorylated ATF-1. sPGN induced rapid and transient dose-dependent phosphorylation of both ATF-1 and CREB (Fig. 1, A and D) with similar kinetics. The control stimulant, ReLPS, also induced phosphorylation of ATF-1 and CREB with kinetics similar to those seen with sPGN. The phosphorylation of ATF-1 was stronger than phosphorylation of CREB for both stimulants. Identical samples analyzed with antibodies that recognize both phosphorylated and nonphosphorylated CREB (Fig. 1B) and ATF-1 (Fig. 1C) showed equal amounts of CREB and ATF-1 protein present in all lanes.

**Phosphatase Treatment—**Stimulated and control RAW264.7 cells were lysed in three different buffers depending on the phosphatase treatment (22). The first group of cells was lysed in the same buffer as above with 0.8% Nonidet P-40 for control samples. The second group of cells was lysed in 50 mM Tris, pH 7.5, 1 mM MgCl2, 0.8% Nonidet P-40, and protease inhibitors, and digested with 250 units/ml alkaline phosphatase for 15 min at 30 °C. The third group of cells was lysed in 50 mM Tris, pH 7.5, 1 mM MgCl2, 1 mM dithiothreitol, with 0.8% Nonidet P-40, 1.0 mM okadaic acid, and protease inhibitors, and digested with 2.9 units/ml protein phosphatase 2A (Calbiochem, San Diego, CA) for 20 min at 37 °C. The final Nonidet P-40 concentration in all samples was 0.6%. The samples were separated on 12% SDS-PAGE and analyzed for phosphorylated and nonphosphorylated c-Jun by Western blots.

**Composition of CREB/ATF Complexes That Bind CRE Consensus Sequence—**To determine if CREB and ATF-1 bind to the CRE sequence and to determine the composition of the CRE-binding complexes, supershift assays were performed with an oligonucleotide containing the CRE consensus sequence and antibodies to specific proteins in the CREB/ATF and AP-1 families of transcription factors. The AP-1 family was included because Jun proteins also bind CRE sequence.

**Data Analysis—**A densitometric scan of Western blots was performed using Scion Image software (Scion Corporation, Frederick, MD). Phosphorylation of ATF-1 and CREB and Western Blots—RAW264.7 cells were cultured at 0.35–0.4 × 10^6^ 24-well plates (2 ml/well) and allowed to differentiate as above for 72 h. All cells were activated with the stimulants indicated under “Results” and then washed and lysed as before (6). In some experiments, sPGN and RelPS were incubated with 5 μg/ml polymyxin B for 30 min and then added to cells. In other experiments, THP-1 cells were incubated with 10 μg/ml anti-CD14 monoclonal antibodies, MY4 (Coulter, Hialeah, FL) or MEM18 (Sanbio-Monosan, Uden, The Netherlands), or the isotype control IgG2b (clone MPO-11; Coulter) at 30 min at 37 °C before stimulation. Cell lysates were separated on 12% SDS-PAGE and transferred to Immobilon P (6). Phosphorylation of ATF-1 and CREB was determined by Western blotting with 0.5 μg/ml rabbit anti-pCREB (Upstate Biotechnology) or rabbit anti-ATF-1 monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), which recognize both phosphorylated and nonphosphorylated forms of CREB or ATF-1, respectively.

**Activation of AP-1 and Western Blots—**Cells were cultured, activated, and lysed as described for ATF-1/CREB. Phosphorylation of c-Jun protein was detected using a monoclonal antibody generated against a c-Jun peptide containing phosphorylated serine 63 (Santa Cruz Biotechnology). Nonphosphorylated c-Jun, Jun B, Jun D, and c-Fos were also detected by Western blots using antibodies from Santa Cruz Biotechnology.

**Enzyme Digestions—**30-μg aliquots of sPGN biosynthetically labeled with [3H]cAlanine (20) were digested for 72 h at 37 °C with 1 mg/ml affinity-purified lysostaphin or 1 mg/ml lysozyme (Grade I from chicken egg, from Sigma), or buffer alone (as a control), and were dialyzed four times (10–12-kDa cut-off) against Dulbecco's phosphate-buffered saline at 4 °C (10). The extent of digestion was determined by measuring the amount of [3H]cAlanine remaining in the samples after dialysis.

**Phosphatase Treatment—**Stimulated and control RAW264.7 cells were lysed in three different buffers depending on the phosphatase treatment (22). The first group of cells was lysed in the same buffer as above with 0.8% Nonidet P-40 for control samples. The second group of cells was lysed in 50 mM Tris, pH 7.5, 1 mM MgCl2, 0.8% Nonidet P-40, and protease inhibitors, and digested with 250 units/ml alkaline phosphatase for 15 min at 30 °C. The third group of cells was lysed in 50 mM Tris, pH 7.5, 1 mM MgCl2, 1 mM dithiothreitol, with 0.8% Nonidet P-40, 1.0 mM okadaic acid, and protease inhibitors, and digested with 2.9 units/ml protein phosphatase 2A (Calbiochem, San Diego, CA) for 20 min at 37 °C. The final Nonidet P-40 concentration in all samples was 0.6%. The samples were separated on 12% SDS-PAGE and analyzed for phosphorylated and nonphosphorylated c-Jun by Western blots.

**Composition of CREB/ATF Complexes That Bind CRE Consensus Sequence—**To determine if CREB and ATF-1 bind to the CRE sequence and to determine the composition of the CRE-binding complexes, supershift assays were performed with an oligonucleotide containing the CRE consensus sequence and antibodies to specific proteins in the CREB/ATF and AP-1 families of transcription factors. The AP-1 family was included because Jun proteins also bind CRE sequence.

**Nuclear extracts from sPGN- or ReLPS-stimulated RAW264.7 cells contained proteins that bind to the CRE consensus site (the protein-DNA complex ran higher than the free oligonucleotide) (Fig. 2A). There was no difference in the binding of proteins to the CRE site between stimulated and control cells (Fig. 2A, compare lanes 1 for Nil, sPGN, and ReLPS samples). This is characteristic of the protein complex that binds CRE sequence, where activation does not result in a change of the binding of the proteins, but induces phosphorylation of the already bound protein (17).

**The protein-DNA complexes with nuclear extracts from sPGN- or ReLPS-stimulated cells were supershifted by antibodies against CREB-1, pCREB, and ATF-1, but not against ATF-2, Jun, and c-Fos (Fig. 2A). This indicates that the protein dimer that binds CRE consensus sequence in sPGN- or ReLPS-stimulated RAW264.7 cells consists of ATF-1 and CREB.
were no qualitative differences in the specific transcription factors that bind the CRE site between stimulated and non-stimulated cells; however, there was a difference in the amount of CREB and pCREB in the bound complex between stimulated and nonstimulated cells (Fig. 2A). The amount of pCREB increased upon stimulation by sPGN or ReLPS, as the antibodies against pCREB caused a complete shift of the protein-oligonucleotide complex from the stimulated cells (compare the pCREB lanes between stimulated and control in Fig. 2A). These results confirm that upon stimulation DNA-bound CREB undergoes phosphorylation, which has been shown to activate CREB.

The specificity of binding to the CRE sequence was confirmed using excess unlabeled specific and nonspecific oligonucleotides (Fig. 2B). In nuclear extracts from both sPGN- or ReLPS-stimulated cells, the binding of proteins to the oligonucleotide carrying CRE sequence was inhibited by an excess of unlabeled specific oligonucleotide (CRE), but not by a nonspecific (NS) oligonucleotide with no CRE sequence (Fig. 2B). These data indicate that the proteins that bind the CRE oligonucleotide specifically recognize the CRE sequence.

**sPGN Induces Transactivation of a CREB-regulated Gene**

That Is Inhibited by Dominant Negative CREB and Dominant Negative ATF-1—To determine if sPGN- or ReLPS-induced phosphorylation of CREB and ATF-1 results in functional activation of these transcription factors, we transfected

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**Fig. 1.** sPGN and ReLPS induce phosphorylation of ATF-1 and CREB: time kinetics, dose response, and effect of polymyxin B.

RAW264.7 cells were stimulated with 10 μg/ml sPGN or 10 ng/ml ReLPS for the indicated times, and total cell lysates were subjected to SDS-polyacrylamide gel electrophoresis and analyzed by Western blots for: phosphorylation of ATF-1 and CREB using an anti-phosphorylated CREB Ab (A), total (phosphorylated and nonphosphorylated) CREB using an anti-CREB Ab (B), or total ATF-1 protein using an anti-ATF-1 Ab (C). Cells were stimulated with the indicated concentrations of sPGN or ReLPS for 30 min. Cells stimulated in the presence or absence of polymyxin B were stimulated with 10 μg/ml sPGN or 10 ng/ml ReLPS. Total cell lysates were analyzed for phosphorylation of ATF-1 and CREB (D) or total CREB (E). pCREB, pATF-1, CREB, and ATF-1 are indicated by arrows; all other bands are nonspecific. The results are from one of three similar experiments.
RAW264.7 cells with the plasmid Δ(−71)Som-CAT or empty vector (CMV) and tested for chloramphenicol acetyltransferase (CAT) activity in the transfected cells after stimulation with sPGN or ReLPS. The plasmid Δ(−71)Som-CAT contains −71 to +53 bp of somatostatin promoter fused to the CAT gene, and this plasmid has been shown previously to be regulated by CREB (23). Both sPGN and ReLPS induced 14.1±2.5-fold and 13.4±2.1-fold (means ± S.E., n=6) increase, respectively, in CAT activity in transfected macrophage cells (Fig. 3A). RAW264.7 cells transfected with the empty vector showed no induced CAT activity upon stimulation (Fig. 3A).

To determine if the induced CAT activity in sPGN- or ReLPS-stimulated cells was due to ATF-1 and/or CREB, we co-transfected RAW264.7 cells with Δ(−71)Som-CAT and A-ATF1 (a dominant negative inhibitor of ATF-1), A-CREB (a dominant negative inhibitor of CREB), or the empty CMV. These dominant negative proteins were constructed by fusing an acidic extension at the N terminus of the leucine zipper domain. This acidic region of the recombinant protein binds the basic region of the wild type protein, and the basic region is thus no longer available for binding to DNA. Both dominant negative ATF-1 and dominant negative CREB, individually or in combination, inhibited the sPGN- or ReLPS-induced activation of CAT reporter gene (Fig. 3B). This inhibition was specific for A-ATF1 and A-CREB, as the empty vector did not inhibit sPGN- or ReLPS-induced CAT activity. These results provide evidence that sPGN and ReLPS induce functional activation of the transcription factors ATF-1 and CREB.

sPGN and ReLPS Induce Phosphorylation of c-Jun and Protein Synthesis of JunB and c-Fos—We next determined if sPGN and ReLPS induce activation of the transcription factor AP-1.

The AP-1 transcription factor family consists of proteins which include c-Jun, JunB, JunD, and c-Fos. This transcription factor binds to the 12-O-tetradecanoylphorbol 13-acetate response element, usually as a Jun-Fos heterodimer. c-Jun is activated by phosphorylation of serine 63 and serine 73. To test if sPGN activates c-Jun, RAW264.7 cells were stimulated with sPGN or ReLPS and phosphorylation of c-Jun was measured using an antibody specific to phosphorylated serine 63 and the adjacent c-Jun sequence. sPGN consistently induced rapid and transient dose-dependent phosphorylation and hyperphosphorylation of c-Jun (Fig. 4, A and C). ReLPS induced phosphorylation of c-Jun similar to that seen with sPGN (Fig. 4, A and C). Stripping and rebinding of the blots with anti-c-Jun antibody, which recognizes both phosphorylated and nonphosphorylated protein, revealed that sPGN and ReLPS stimulation also causes a modest increase (1.5–2 times) in the total (phosphorylated and nonphosphorylated) amount of c-Jun protein (Fig. 4, B and D).

This is not unexpected as activated c-Jun protein induces transcription of the c-Jun gene (19). sPGN-induced phosphorylation of c-Jun was not inhibited by polymyxin B, in contrast to the phosphorylation induced by ReLPS, which was completely inhibited by polymyxin B (Fig. 4C). These data confirm that sPGN-induced activation of RAW264.7 cells and phosphorylation of c-Jun is due to PGN and not due to an endotoxin contaminant in our sPGN preparation. In control experiments, the amount of c-Jun protein did not change with polymyxin B treatment, for either stimulant (Fig. 4D).

To confirm that the anti-phosphorylated c-Jun antibody recognizes phosphorylated c-Jun, samples were treated with different phosphatases and then analyzed by Western blot. Cell lysates from both sPGN- and ReLPS-stimulated cells, treated with alkaline phosphatase, a nonspecific phosphatase, did not show any binding to the anti-phosphorylated c-Jun antibody (Fig. 4E). Furthermore, protein phosphatase 2A, a serine phosphatase, strongly diminished binding to the anti-phosphorylated c-Jun antibody (Fig. 4E). However, the binding to the anti-c-Jun antibody was not eliminated or reduced by either sPGN or ReLPS-stimulated cells.
phosphatase treatment (Fig. 4F). These results confirmed that the anti-phosphorylated c-Jun antibody was indeed specific for the phosphorylated c-Jun.

JunB, JunD, and c-Fos are other members of the AP-1 family of transcription factors. sPGN consistently induced a dose-dependent increase of JunB and c-Fos, but not of JunD protein synthesis in RAW264.7 cells (Fig. 5). ReLPS also induced increases in JunB and c-Fos proteins, but not of JunD protein (Fig. 5). The up-regulation of JunB by sPGN was not inhibited by polymyxin B, in contrast to ReLPS-induced increase in JunB protein, which was completely inhibited by polymyxin B (Fig. 5B).
c-Jun, JunB, and c-Fos Bind to the TPA-RE Consensus Sequence in sPGN-stimulated Cells—

To identify the specific members of the AP-1 family of transcription factors that bind the TPA-RE consensus sequence, gel shift assays were performed. The binding of proteins to an oligonucleotide with the TPA-RE sequence showed no differences between sPGN- or ReLPS-stimulated and unstimulated RAW264.7 cells (Fig. 6; gel shift assays with unstimulated lysates are not shown).

Using a supershift assay and a series of antibodies that recognize different members of the AP-1 and CREB family of transcription factors, we determined that in both sPGN- and ReLPS-stimulated cells (Fig. 6A) and in unstimulated cells (data not shown) c-Jun, JunB, and c-Fos bind to the TPA-RE sequence. The specificity of the oligonucleotide that was used in the supershift assays was confirmed by inhibition of binding of nuclear proteins by an excess of unlabeled specific oligonucleotide, but not by a nonspecific oligonucleotide with no TPA-RE sequence (Fig. 6B).

sPGN Induces Transactivation of an AP-1-regulated Gene, and this induction is inhibited by dominant negative c-Fos—

To determine if sPGN induced functional activation of AP-1, we transfected RAW264.7 cells with the plasmid pAP1-Luc, which has seven AP-1 binding sites upstream of the luciferase gene, or −60Col-Luc, which has no AP-1 binding sites. Cells transfected with pAP1-Luc showed an average 9.0 ± 0.6-fold and 9.4 ± 1.4-fold (means ± S.E., n = 3) increase in inducible luciferase activity when stimulated by sPGN or ReLPS, respectively (Fig. 7A). Cells transfected with the control plasmid −60Col-Luc showed no luciferase activity in the absence or presence of the stimulants (Fig. 7A). We also confirmed activation of AP-1 by sPGN or ReLPS using two additional plasmids: −73Col-Luc, which has −73 to +63 bp of the collagenase promoter fused to the luciferase gene, and 2xAP1-Luc, which has two AP-1 binding sites upstream of the luciferase gene (data not shown). These results demonstrate that sPGN and ReLPS induced functional activation of the transcription factor AP-1.
To confirm that the sPGN- or ReLPS-induced luciferase activity was due to AP-1, we co-transfected RAW264.7 cells with pAP1-Luc and A-Fos, a dominant negative mutant of c-Fos, or empty vector (CMV). A-Fos heterodimerizes with Jun proteins in an AP-1 complex and inactivates their ability to bind DNA. A-Fos, but not the empty vector, inhibited both sPGN- and ReLPS-induced increases in luciferase activity (Fig. 7B). These results confirm that sPGN and ReLPS induce a functionally active AP-1, and that c-Fos and Jun form the active AP-1 complex.

sPGN-induced Phosphorylation of CREB and c-Jun in Human Monocytes Is CD14-dependent—Since we have previously shown that sPGN-induced activation of NF-κB is mediated through the membrane receptor CD14 (8), we now determined if sPGN-induced phosphorylation of CREB and c-Jun also requires CD14. We first tested if sPGN induced phosphorylation of CREB and c-Jun in the human monocyte cell line THP-1. THP-1 cells activated with sPGN and ReLPS showed phosphorylation of CREB (Fig. 8A). THP-1 cells differed from the mouse RAW264.7 cells, in that they did not have detectable levels of ATF-1, which in RAW264.7 cells was present in high amounts (Fig. 1C) and was strongly phosphorylated upon stimulation of the cells (Fig. 1A). Total amount of CREB did not change upon cell activation by sPGN or ReLPS (Fig. 8B).

sPGN or ReLPS also induced phosphorylation of c-Jun in THP-1 cells (Fig. 8C) and an increase in the total amount of c-Jun protein (Fig. 8D). The increase in c-Jun protein may be due to the activated c-Jun itself, which is known to induce transcription of the c-Jun gene (19).

Anti-CD14 monoclonal antibodies, MY4 and MEM18, inhibited both sPGN- and ReLPS-induced phosphorylation of CREB (Fig. 8E) and c-Jun (Fig. 8F). These data confirm that sPGN and ReLPS activate cells through CD14 and that CD14 is required for both sPGN- and ReLPS-induced phosphorylation of CREB and c-Jun.

Lysostaphin and Lysozyme Reduce sPGN-induced Phosphorylation of ATF-1, CREB, and c-Jun—To confirm the identity of sPGN as the activating molecule that induces phosphorylation of ATF-1, CREB, and c-Jun, sPGN was digested with lysostaphin or lysozyme, enzymes that specifically degrade PGN. Digestion with both enzymes reduced sPGN-induced phosphorylation of ATF-1 and CREB (Fig. 9A) and c-Jun (Fig. 9C), and this reduction was proportional to the extent of digestion of sPGN (Fig. 9E). As expected, the total amount of CREB (Fig. 9B) and c-Jun (Fig. 9D) proteins remained the same in all treated and untreated groups.

**DISCUSSION**

Our results demonstrate that: (i) PGN induces phosphorylation and functional activation of the transcription factors
also that the AP-1 site is required for LPS-induced transcriptional activity of these genes. Also in agreement with our findings are the results showing that site-specific mutations in the CRE site in the IL-1β promoter result in a substantial loss in transcriptional induction following combined stimulation with LPS, PMA, and dibutyryl cAMP (33), and also that the AP-1 site is required for LPS-induced transcriptional activation of tissue factor (34) and heme oxygenase (35) genes.

However, the actual protein complex that binds the CRE or AP-1 site and regulates transcription may be different for different genes, e.g. the CRE site in the IL-1β promoter binds CREB and ATF-1 (33), while the c-Jun protein binds the CRE site in the human TNF-α promoter, and the amount of c-Jun bound to this site increases when cells are stimulated with LPS (30). In addition, different proteins may have different effects on transcription, e.g. c-Jun is an effective transcriptional activator, while JunB is not, and thus JunB may have an inhibitory function (26). These data emphasize the importance of the CRE and AP-1 sites and the CREB/ATF and AP-1 families of transcription factors in LPS-induced transcriptional activation of pro-inflammatory genes. These transcription factors and their binding sites may also play a significant role in PGN-induced inflammatory response.

Although both PGN (10) and LPS (36) bind to CD14 and activate cells through CD14 (8), there are several differences in the function of CD14 as the PGN and LPS receptor. In particular, both the binding sites for PGN and LPS on CD14 and the sites needed for cell activation are partially similar but partially different (8, 10), and only LPS-induced, but not PGN-induced cell activation and binding affinity for CD14 are enhanced by the LPS-binding protein (7, 10). Moreover, PGN and LPS induce differential activation of MAP kinases, with LPS strongly inducing all three families of kinases (ERK, JNK, and p38), but with PGN only inducing ERK and JNK, but not p38 (37). Furthermore, soluble CD14-LPS complexes activate CD14-negative cells, whereas soluble CD14-PGN complexes do not (16).

Despite these differences, in this study we did not detect any differences between PGN and LPS in the activation of CREB/ATF and AP-1 transcription factors. Therefore, our current and previous (8) results indicate that transcription factors NF-κB, CREB/ATF-1, and AP-1 are either induced by CD14-dependent signal transduction pathways that are common for PGN and LPS, or that different pathways activated by PGN and LPS converge to activate these three families of transcription factors. Such a convergence of initially different signal transduction pathways to activate the same transcription factors has been demonstrated in the activation of cells through cytokine receptors, e.g. IL-1 and TNF-α (38).

The signal transduction pathway(s) through which PGN and LPS activate CREB/ATF and AP-1 are still not clear. Activation of AP-1 is consistent with the strong activation of JNK and ERK1 and ERK2 by both PGN and LPS (37), since JNK can activate c-Jun and both JNK and ERK can induce c-Fos through activation of ternary complex factor/Etk-1 (39). The possible mechanism of activation of ATF-1 and CREB are less clear, since in other systems the main mechanism of activation of ATF-1 and CREB is through protein kinase A, but we could not show any activation of protein kinase A by PGN or LPS (37). Other possible mechanisms of activation could involve calmodulin kinase or the MAP kinases ERK1 and ERK2 (17, 18).

The functional significance of the activation of NF-κB, CREB/ATF, and AP-1 for the induction of cytokine genes by PGN is still not clear. As seen for LPS, these transcription factors are required for the induction of specific genes coding for pro-inflammatory molecules, and our preliminary data indicate that NF-κB, CREB, and ATF-1, but not AP-1, are required for PGN-induced transcriptional activation of TNF-α.² These transcription factors are also likely to play a role in the

² D. Gupta and Q. Wang, unpublished observation.
induction of several other pro-inflammatory molecules, such as cytokines, chemokines, and adhesion molecules in PGN-activated cells.

In summary, we demonstrate that activation of macrophages by PGN leads to the functional activation of the transcription factors CREB/ATF and AP-1 and that this activation is CD14-dependent.

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REFERENCES

1. Schleifer, K. H., and Kandler, O. (1972) Bacteriol. Rev. 36, 407–477
2. Heymer, B., Seidl, P. H., and Schleifer, K. H. (1985) in Immunology of the Bacterial Cell Envelope (Stewart-Tull, D. E., S., and Davies, M., eds) pp. 11–46, John Wiley & Sons, New York
3. Dziarski, R., Ulmer, A. J., and Gupta, D. (1999) in Glycobiology (Dyke, R. J., ed) Plenum Press, New York, in press
4. Raetz, C. R. H. (1990) Annu. Rev. Biochem. 59, 129–170
5. De Kimpe, S. J., Kengatharan, M., Thiemermann, C., and Vane, J. R. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10359–10363
6. Gupta, D., Jin, Y., and Dziarski, R. (1995) J. Immunol. 155, 2620–2630
7. Weidemann, B., Brade, H., Rietsema, E. T., Dziarski, R., Bazil, V., Kusumoto, S., Flad, H. D., and Ulmer, A. J. (1994) Infect. Immun. 62, 4709–4715
8. Gupta, D., Kirkland, T. N., Viriyakosol, S., and Dziarski, R. (1996) J. Biol. Chem. 271, 23310–23316
9. Weidemann, B., Slatter, J., Dziarski, R., Kusumoto, S., Stelter, F., Rietsema, E. T., Flad, H. D., and Ulmer, A. J. (1997) Infect. Immun. 65, 858–864
10. Dziarski, R., Tapping, R. I., and Tobias, P. S. (1998) J. Biol. Chem. 273, 8680–8690
11. Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J., and Mathison, J. C. (1996) Science 274, 1431–1433
12. Ulevitch, R. J., and Tobias, P. S. (1994) Curr. Opin. Immunol. 6, 125–130
13. Hailman, E., Lichenstein, H. S., Wurefel, M. M., Miller, D. S., Johnson, D. A., Kelly, M., Busse, L. A., Zukowski, M. M., and Wright, S. D. (1994) J. Exp. Med. 179, 269–277
14. Viriyakosol, S., and Kirkland, T. N. (1996) J. Biol. Chem. 270, 361–368
15. Juan, T. S. C., Kelly, M. J., Johnson, D. A., Busse, L. A., Hailman, E., Wright, S. D., and Lichenstein, H. S. (1995) J. Biol. Chem. 270, 1382–1387
16. Jin, Y., Gupta, D., and Dziarski, R. (1998) J. Infect. Dis. 177, 1629–1638
17. Brindle, P. K., and Montminy, M. R. (1992) Curr. Opin. Genet. Dev. 2, 199–204
18. Xing, J., Ginty, D. D., and Greenberg, M. E. (1996) Science 273, 959–963
19. Karin, M., Liu, Z.-G., and Zandi, E. (1997) Curr. Opin. Cell. Biol. 9, 240–246
20. Rosenthal, R. S., and Dziarski, R. (1994) Methods Enzymol. 235, 253–285
21. Dziarski, R. (1991) J. Biol. Chem. 266, 4713–4718
22. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1995) Current Protocols in Molecular Biology, Vol. 3, John Wiley and Sons, Inc., New York
23. Montminy, M. R., Sevarino, K. A., Wagner, J. A., Mandel, G., and Goodman, R. H. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6682–6686
24. Ahn, S., Olive, M., Aggarwal, S., Krylov, D., Ginty, D. D., and Vinson, C. (1998) Mol. Cell. Biol. 18, 967–977
25. Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R. J., Rahmsdorf, H. J., Jonat, C., Herrlich, P., and Karin, M. (1997) Cell 89, 729–739
26. Deng, T., and Karin, M. (1993) Genes Dev. 7, 479–490
27. Olive, M., Krylov, D., Echlin, D. B., Gardner, K., Taparowsky, E., and Vinson, C. (1997) J. Biol. Chem. 272, 18586–18594
28. Chandra, G., Cogswell, J. P., Miller, L. R., Godlevski, M. M., Stinnett, S. W., Noel, S. L., Kadwell, S. H., Kost, T. A., and Gray, J. G. (1995) J. Immunol. 155, 4535–4543
29. Fujiwara, M., Muroi, M., Muroi, Y., Ito, N., and Suzuki, T., (1993) J. Biol. Chem. 268, 14088–14095
30. Yao, J., Mackman, N., Edgington, E., and Fan, S.-T. (1997) J. Biol. Chem. 272, 17795–17801
31. Profitt, J., Crabtree, G., Grove, M., Daubersies, P., Baillieul, B., Wright, E., and Plumb, M. (1994) Gene (Amst.) 152, 173–179
32. Pan, J., Xia, L., Yao, L., and McEver, R. P. (1998) J. Biol. Chem. 273, 10608–10617
33. Gray, J. G., Chandra, G., Clay, W. C., Stinnett, S. W., Haneline, S. A., Lorenz, J. J., Patel, I. R., Wisely, G. B., Furdon, P. J., Taylor, D., and Kost, T. A. (1993) Mol. Cell. Biol. 13, 6678–6689
34. Mackman, N., Brand, K., and Edgington, E. (1991) J. Exp. Med. 174, 1517–1526
35. Camhi, S. L., Alam, J., Otterbein, L., Sylvester, S. L., and Chui, A. M. K. (1995) Am. J. Resp. Cell Mol. Biol. 13, 387–398
36. Tobias, P. S., Soldau, K., Kline, L., Lee, J. D., Kato, K., Martin, T. P., and Ulevitch, R. J. (1993) J. Immunol. 150, 3011–3021
37. Dziarski, R., Jin, Y., and Gupta, D. (1996) J. Infect. Dis. 174, 777–785
38. Kirschnung, C., Weshe, H., Ayres, T. M., and Roth, K. (1998) J. Exp. Med. 188, 2091–2097
39. Karin, M. (1995) J. Biol. Chem. 270, 16483–16486