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p38-dependent Activation of Interferon Regulatory Factor 3 by Lipopolysaccharide*

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Interferon regulatory factor 3 (IRF3) is known to participate in the transcriptional induction of interferon (IFN) α and IFNβ genes, as well as of a number of interferon-stimulated genes (ISGs), as a result of viral infection. In the present study we demonstrate the activation of IRF3 followed by ISG induction after exposure of cells to the bacterial cell wall component lipopolysaccharide. Engagement of Toll-like receptors by lipopolysaccharide triggered the nuclear translocation of IRF3, followed by its DNA binding and the subsequent induction of several interferon-regulated genes. Transcriptional activation of ISGs occurred in a protein synthesis independent manner, but was sensitive to inhibition of the stress-activated protein kinase, p38. The activation of IRF3 by viral particles or bacterial membrane components suggests that this signaling pathway might contribute to the evolutionary conserved innate immune response.

Interferons α/β (IFNα/β)[1] induce gene expression by activating members of the signal transducers and activators of transcription (STAT) family of protein via their tyrosine phosphorylation, a process that involves the tyrosine kinases Jak1 and Tyk2 (1). Cooperative binding of STATs 1 and 2 in conjunction with p48 ISGF3γ to the interferon-stimulated response element (ISRE), an IFNα/β inducible enhancer, is necessary and sufficient for the induction of IFNα/β-stimulated gene expression (2–4).

Numerous IFNα/β-induced genes (ISGs) were identified that contain an ISRE. They represent components of the antiviral defense such as the 2′-5′ poly(A) synthase (5, 6) and the double-stranded RNA activated protein kinase (7), cell surface proteins such as the immunoglobulin (Ig) superfamily cell adhesion molecule (8, 9) or the major histocompatibility complex class I and II molecules (10), genes encoding chemokines such as the *ISG15* and the *IP10* gene (2, 11), as well as many other genes of yet unknown functions such as *ISG54*, *ISG56* (4), *GBP* (12), or *6-16* (13).

The DNA binding adapter p48 ISGF3γ shares strong sequence homology in its DNA interaction domain with members of the interferon regulatory factor (IRF) family. Indeed, these proteins are able to bind to the ISRE and activate a specific subset of the genes typically activated by IFNα/β (14, 15). The ubiquitously expressed IRF3 has been found to be an important cellular response factor to viral infection. We have previously demonstrated that infection of fibroblasts with human cytomegalovirus causes nuclear translocation of IRF3 and cooperative DNA binding with the transcriptional co-activator CBP/p300 (16). This is followed by subsequent induction of a distinct subset of ISRE containing genes. Other labs reported similar observations after infection of cells with Newcastle disease virus or Sendai virus (17–19). Surprisingly, even transfection of cells with double-stranded RNA is able to trigger the formation of an IRF3 containing DNA binding complex (20).

While the connection between viral infection and the interferon system is well established, it is largely unclear if or to what extent activation of ISGs plays a role in the response to bacterial infection. Vertebrates and invertebrates respond to bacterial invasion by activation of a defense mechanism that is part of the innate immune response. This response is triggered by the recognition of bacterial cell wall lipopolysaccharides (LPS) by cells of the immune system. In mammals, it is primarily monocytes and macrophages that respond to LPS, releasing cytokines and chemokines that provoke an inflammatory response. Excessive amounts of LPS can result in a fatal syndrome known as septic shock (21).

In *Drosophila*, which relies entirely on innate immune defense, four Toll family members have been reported. They are involved in the regulation of dorsal-ventral patterning during development, and have been shown to control immune response to bacterial and fungal infection in the adult fly by inducing antimicrobial peptides (21). During the last year two cell surface receptors have been proven to essentially mediate the LPS response. These transmembrane proteins, referred to as the Toll-like receptors, can confer sensitivity toward LPS when ectopically expressed in otherwise LPS unresponsive cells (22–24). Responsiveness to LPS also requires the presence of the glycophosphatidylinositol-anchored CD14 myeloid cell membrane protein. Significant progress has been made over the last few years in the identification of the signaling molecules involved in LPS-induced gene expression, however, most of the work focused on the signaling pathway leading to the activation of the nuclear factor NFκB. However, in addition to the NFκB pathway, Toll-like receptors also stimulate signaling cascades that lead to the activation of the stress-activated protein kinases (SAPK) JNK and p38 via MKK4 (MEK4) (21). As in the case of the activation pathway of NFκB, the SAPK pathway is highly conserved between mammals and invertebrates.

We were interested in evaluating whether LPS stimulation would result in IRF3 activation and ISG induction similar to that observed after human cytomegalovirus infection. The re-

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[1] The abbreviations used are: IFNα/β, interferon α/β; STAT, signal transducers and activators of transcription; ISRE, interferon-stimulated response element; ISG, interferon-stimulated genes; IRF, interferon regulatory factor; LPS, lipopolysaccharide; SAPK, stress-activated protein kinase; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid.

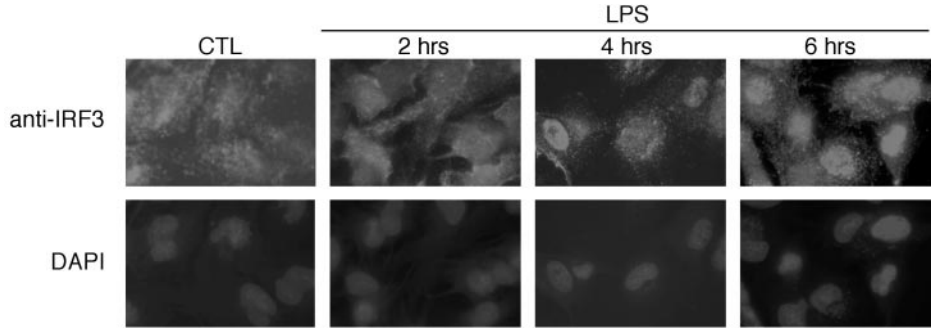

FIG. 1. **Nuclear translocation of IRF3 in response to LPS.** U373 cells were incubated for the indicated times with LPS, and subcellular localization of IRF3 was analyzed using a rabbit polyclonal antibody against IRF-3 (*upper panel*). Nuclei were counterstained with DAPI (*lower panel*). *DAPI*, 4,6-diamidino-2-phenylindole.

sults presented here demonstrate that LPS induces the nuclear translocation and the ISRE binding of IRF-3, followed by the induction of several ISGs. Furthermore, we find that LPS mediated activation of ISGs depends on the activity of p38 SAPK, but occurs independent of protein synthesis. Thus, our findings propose a central role for IRF3 in the innate immune response following viral or bacterial infection.

MATERIALS AND METHODS

Cells—Human U373 astrocytoma cells (U-373 MG) were obtained from the American Type Culture Collection and maintained in Dulbecco's modified media supplemented with 10% fetal bovine serum, penicillin, and streptomycin (Irvine Scientific).

Interferons and Reagents—IFNα was a generous gift from Hoffman LaRoche. LPS was purchased from Sigma. SB202190 and PD98059 were obtained from Calbiochem. Glutathione *S*-transferase fused to full-length human IRF-3 was expressed in DH5α cells and used to immunize white New Zealand rabbits. The resulting IRF-3 antisera was tested by Western blotting and supershift experiments for reactivity with recombinant IRF3, and for lack of cross-reactivity with recombinant IRF1.

Nuclear Extracts—Following treatment, cells were washed with PBS and lysed on ice for 15 min in hypotonic buffer (10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 1 mM Na_3VO_4, 20 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride). Lysates were vortexed vigorously and centifuged at 15,000 $\times$ *g* for 5 min at 4 °C. The pellet was washed once with cold phosphate-buffered saline, and then the nuclei extracted in a high salt buffer (25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM Na_3VO_4, 20 mM sodium fluoride) and shaken for 30 min at 4 °C. Nuclear extracts were obtained by centrifugation at 15,000 $\times$ *g* for 5 min. Protein concentration was measured by the Bio-Rad protein assay.

Electrophoretic Mobility Shift Assay—Electrophoretic mobility shift assays were performed using an ^{32}P end-labeled probe corresponding to the ISRE of the ISG15 promoter (5′-GATCCATGCCTCGGGAAAGGGAAACCGAAACTGAAGCC-3′). Equal amounts of protein were incubated with poly(dI-dC) and labeled oligonucleotides in ISRE binding buffer (40 mM KCl, 20 mM Hepes, pH 7.0, 1 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM dithiothreitol, 4% Ficoll, 0.02% Nonidet P-40). Electrophoresis was performed on 6% nondenaturing TBE polyacrylamide gel electrophoresis, and the gels were dried and subjected to autoradiography. For competition experiments, unlabeled oligonucleotides were added to the reaction mixture prior to the addition of the labeled oligonucleotides. The oligonucleotide used as a competitor corresponded to either the *ISG15*-ISRE or the GRR (IFNγ response region) sequence in the promoter of the high affinity FcγRI receptor (5′-AATTAGCATGTTTCAAGGATTTGAGATGTATTTCCCAGAAAAG-3′) For supershift experiments, nuclear extracts were incubated on ice with the specified antiserum for 1 h at 4 °C prior to the addition of the labeled oligonucleotide.

RNase Protection Assay Analysis—U373 cells were cultured and stimulated as described above. Total RNA was isolated using TRIzol Reagent (Life Technologies, Inc.). ^{32}P-Labeled antisense riboprobes were generated by transcription of the linearized plasmid *in vitro* using T7 or SP6 RNA polymerase (Promega). Labeled riboprobe and 10 μg of RNA were incubated in hybridization buffer (4:1 formamide and 5$\times$ stock; 5$\times$ stock was 200 mM PIPES, pH 6.4, 2 M NaCl, 5 mM EDTA) overnight at 56 °C before digestion with T1 RNase (Life Technologies, Inc.) for 1 h at 37 °C. After phenol extraction and ethanol precipitation, protected fragments were solubilized in RNA loading buffer (98% formamide, 10 mM EDTA, pH 8, 1 mg/ml bromphenol blue, 1 mg/ml xylene cyanol), boiled for 2 min, and subjected to electrophoresis on a 4.5% polyacrylamide/urea gel.

Immunofluorescence—U373 cells were seeded onto glass coverslips in 6-well plates and incubated overnight at 37 °C under 5% CO_2 in DMEM supplemented with 10% fetal bovine serum. After treatment, cells were rinsed once with PBS and once with 1$\times$ PIPES buffer (100 mM PIPES, pH 6.8, 2 mM $MgCl_2$, 2 mM EGTA). Cells were fixed in 4% paraformaldeyde/PBS for 6 min at room temperature. Nuclei were permeabilized by incubating with 0.5% Triton X-100/PBS for 10 min at room temperature. After rinsing three times with PBS, the fixed cells were blocked in 10% goat serum in PBS for 35 min at room temperature and incubated with rabbit anti-IRF3 serum overnight at 4 °C. Cells were rinsed 4 times for 5 min in PBS before incubation with a goat anti-rabbit Cy3-conjugated secondary antibody (Chemicon International, Inc.) for 40 min at room temperature and mounted after rinsing on glass slides with 50% glycerol, PBS.

RESULTS AND DISCUSSION

Several laboratories including our own recently reported similar results regarding the activation of IRF3 following infection with human cytomegalovirus, vesicular stomatitis virus, Newcastle disease virus, or after transfection of cells with double-stranded RNA (16–18). We reasoned that IRF3 activation may represent a general cellular host-defense mechanism that could also be triggered by contact with pathogens other than viruses. We therefore decided to investigate whether the Gram-negative bacterial cell wall component LPS is capable of initiating a cellular response similar to that observed after contact with viral particles.

The human astrocytoma cell line U373 was chosen as it is a well accepted model for investigating cellular responses to LPS. One of the early events in IRF3 activation is its nuclear translocation following appropriate phosphorylation on serine and threonine residues. We therefore decided to test whether LPS stimulation would trigger a change in the subcellular distribution of IRF3. U373 cells plated on coverslips were subjected to stimulation with 1 μg/ml LPS for 2, 4, or 6 h, and the subcellular localization of IRF3 was determined by immunofluorescence using rabbit anti-IRF3 antiserum. In order to exclude the possibility that IRF3 activation might occur as a consequence of LPS-mediated cytokine release, the experiments were performed in the presence of 100 μg/ml cycloheximide. As shown in Fig. 1, LPS caused the protein synthesis independent nuclear translocation of IRF3 within 4–6 h, a kinetic similar to that observed after viral infection.

Next, we decided to elucidate whether the nuclear translocated IRF3 was able to form an ISRE binding complex. U373 cells were stimulated for 6 h with 1 μg/ml LPS in the presence of 100 μg/ml cycloheximide, and nuclear extracts were subjected to electrophoretic mobility shift assays using the ISG15-ISRE as a probe. As anticipated, LPS induced the formation of a DNA binding complex whose migration is similar to that of the previously reported human cytomegalovirus-induced CIF complex (16), but is distinct from the IFNα/β-induced ISGF3 complex (Fig. 2*A, lane 2*). Competition experiments using 10–50-fold molar excess of unlabeled ISRE (*lanes 3* and *4*) or the unrelated GRR (*lanes 5–7*) oligo confirmed the binding specificity of the LPS-induced complex for the ISRE (the slower

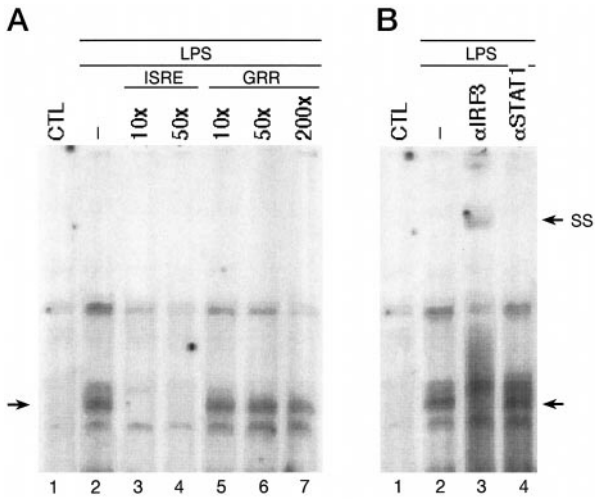

FIG. 2. **LPS induces ISRE binding of IRF3.** Nuclear extracts were prepared from untreated and LPS-stimulated cells, and electrophoretic mobility shift assays were perfomed using the end-labeled ISRE as a probe. *A,* competition experiments were performed with the indicated fold-molar excess of unlabeled ISRE (*lanes 3* and *4*) or GRR (*lanes 5–7*) oligo. The LPS-induced complex is indicated by the *arrow*. *B,* supershift assays on the LPS-induced ISRE binding complex were performed with either IRF3 (*lane 3*) or STAT1 (*lane 4*) antiserum (*SS,* supershift).

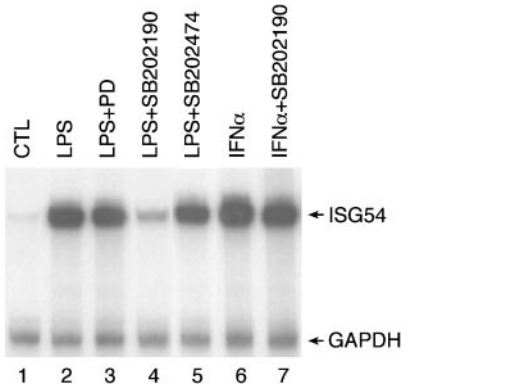

FIG. 3. **LPS stimulates p38-dependent induction of ISG54.** Total RNA was isolated from LPS (*lanes 2–5*) or IFNα (*lanes 6* and *7*) stimulated U373 cells with or without preincubation with 10 μM of the indicated inhibitors (*lanes 3, 4,* and *6*). RNase protection assays were performed using a probe corresponding to the human *ISG54* gene. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal standard.

migrating band competing with the unlabeled ISRE was identified as IRF1). In order to verify that the observed DNA binding complex indeed contains IRF3, we performed supershift experiments with anti-IRF3 serum. As shown in Fig. 2*B*, incubation of cell lysates with anti-IRF3 antibodies results in a supershift of the LPS-induced complex (*lane 3*), whereas the STAT1 antiserum which was used as a negative control was without effect (*lane 4*). These results clearly demonstrate that LPS stimulation of U373 cells is capable of inducing the nuclear translocation and the ISRE binding of IRF3 in the absence of protein synthesis.

In order to test whether IRF3 is capable of mediating LPS-induced ISG transcription in a manner similar to virus-induced gene transcription, we performed RNase protection assays using several ISRE driven genes as probes. Again, all experiments were done in the presence of 100 μg/ml cycloheximide to exclude synthesis of signaling intermediates. Indeed, LPS was able to induce the transcriptional activation of the ISRE containing genes *ISG54* and *GBP* within 6 h in a protein synthesis independent manner (Fig. 3, *lane 2*). The LPS-induced ISG was not accompanied by formation of the ISGF3 complex, nor did we detect any tyrosine phosphorylation of STAT proteins involved in ISG induction (data not shown). Furthermore, LPS induction of *ISG54* was also observed in GRE cells[2] which carry a deletion of the type I interferon locus (25). Thus, it is reasonable to assume that the observed ISG induction is indeed mediated through the activation of IRF3.

LPS or viral infection trigger the activation of several common signaling pathways, some of which may be involved in the activation of IRF3. One such pathway leads to the activation of the transcription factor NFκB. However, previous studies and our own results showed that the kinases leading to NFκB activation such as IRAK, NIK, or IKK are unable to phosphorylate IRF3 (27).[2] Another signaling cascade activated upon viral infection as well as after LPS stimulation leads to the activation of the stress kinase p38 (21). Indeed, we did observe activation of p38 after LPS stimulation of U373 cells as previously reported (26).[2] To explore the role of p38 in IRF3 mediated ISG induction, we treated U373 cells with the specific p38 inhibitor SB202190 prior to stimulation with LPS. As shown in Fig. 3 (*lane 4*), SB202190 (SB) significantly inhibits LPS-induced *ISG54* induction, whereas the structurally related, negative control compound SB202474 did not interfere with the LPS-induced ISG induction (*lane 5*). Furthermore, SB202190 did not alter *ISG54* induction in response to IFNα, demonstrating that the compound does not exert a general inhibitory effect on ISG transcription (Fig. 3, *lanes 6* and *7*). Last, we found that the mitogen-activated protein kinase specific inhibitor PD98059 (PD) was also without effect on LPS-induced ISG activation (*lane 3*).

During the preparation of this manuscript, Juang *et al.* (28) reported that LPS inhibits the virus-mediated activation of IRF3. These findings were obtained by exposure of cells to LPS for up to 1 h, followed by removal of the LPS and subsequent viral infection. Although mixed infections are a commonly found clinical manifestation, it is somewhat unlikely that cells are exposed to LPS for only 1 h followed by viral contact. Since our results demonstrate that the LPS mediated activation of IRF3 and the induction of ISGs, although protein synthesis independent, require at least 4 h of stimulation, it is possible that the differences in the experimental approach are responsible for the observed discrepancies.

In summary, we demonstrate that LPS stimulation is able to induce the nuclear translocation and the ISRE binding of IRF3. Subsequent induction of ISGs was found to occur in the absence of protein synthesis, but requires the kinase activity of the p38 stress kinase. The activation of IRF3 upon viral infection as well as after engagement of the Toll-like receptors suggests that IRF3 may be part of the evolutionary conserved innate immune response. It is possible that the protein synthesis independent activation of genes involved in the cellular self-defense mechanism may enable a cell to respond quickly to an encounter with viral or bacterial particles, whereas subsequent IFN production and release may primarily benefit neighboring, yet uninfected, cells.

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