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A Novel STAT-Like Factor Mediates Lipopolysaccharide, Interleukin 1 (IL-1), and IL-6 Signaling and Recognizes a Gamma Interferon Activation Site-Like Element in the *IL1B* Gene

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Binding of many cytokines to their cognate receptors immediately activates Jak tyrosine kinases and their substrates, STAT (signal transducers and activators of transcription) DNA-binding proteins. The DNA binding targets of STATs are sequence elements related to the archetypal gamma interferon activation site, GAS. However, association of interleukin 1 (IL-1) with Jak-STAT signaling has remained unresolved. We now report an element termed LILRE (lipopolysaccharide [LPS] and IL-1-responsive element) in the human prointerleukin 1β gene (*IL1B*) which can be immediately induced by either lipopolysaccharide (LPS) or IL-1 protein to bind a tyrosine-phosphorylated protein. This LPS- and IL-1-induced factor (LIL factor) is recognized by an antibody raised against the N terminus of Stat1, but not by those specific for either the C terminus of Stat1 or any other GAS-binding STAT. Phosphotyrosine (P-Tyr) specifically inhibits formation of the LIL factor-DNA complex, suggesting the importance of P-Tyr for the DNA-binding activity, as has been found for all STAT dimers. Analysis of DNA-binding specificity demonstrates that the LIL factor possesses a novel GAS-like binding activity that contrasts with those of other STATs in a requirement for a G residue at position 8 (TTCCTGAGA). Further investigation has revealed that IL-6, but neither IL-4 nor gamma interferon, activates the LIL factor. Thus, the existence of such a STAT-like factor (LIL-Stat) relates the LPS and IL-1 signaling pathway to other cytokine receptor signaling pathways via the activation of STATs. Moreover, the unique DNA-binding specificity and antigenicity of this factor suggest that LPS, IL-1, and IL-6 may use a common signaling pathway.

The cytokines which regulate mammalian cellular activation, growth, and differentiation have been demonstrated to exert their effects via a collection of specific membrane receptors. Many of these receptors belong to the cytokine receptor superfamily, which has structurally related extracellular domains, a single transmembrane hydrophobic anchor, and a cytoplasmic portion lacking intrinsic protein kinases (3, 43). Although numerous cellular signals have been reported to be elicited by cell treatment with cytokines, a recent unifying phenomenon has been the immediate activation of members of the STAT (signal transducers and activators of transcription) family of DNA-binding proteins. The preexisting STATs are activated by association with specific phosphotyrosine peptides located in the receptor cytoplasmic domains following ligand binding. The ligand-induced receptor phosphorylation (as well as the subsequent phosphorylation of associated STATs) has been shown to be mediated by Jak tyrosine kinases (17). The DNA binding target for STAT dimers is the consensus gamma interferon (IFN-γ) activation site (GAS) [TT(C/A)CNNNAA], which was initially reported to bind the IFN-γ-induced Stat1 (5, 17).

Interleukin 1 receptor (IL-1R), like the other cytokine receptors, possesses a single transmembrane domain and a cytoplasmic domain not related to protein kinases, although its extracellular ligand binding domain belongs to the immunoglobulin receptor superfamily. The interaction of IL-1 with IL-1R has been previously shown to activate NF-κB and NF-IL6 (6, 7). However, the association of IL-1 with the Jak-STAT signaling pathway has remained obscure. In addition, IL-1 and lipopolysaccharide (LPS), in contrast with other cytokines, have been reported to be incapable of directly inducing any factors capable of binding a GAS sequence (22). Also, the observation that IL-6 can directly induce a STAT protein, Stat3/APRF (acute-phase response factor), that requires a specific variation of the GAS consensus sequence (48) argues that not all STATs bind the canonical GAS sequence.

In the process of studying the responsiveness of the upstream induction sequence (positions −3134 through −2729, upstream of the transcription start site) (34, 44) of the human prointerleukin 1β gene (*IL1B*) to either LPS or IL-1β, we have discovered that the upstream induction sequence contains sequences (see Fig. 1) (LPS- and IL-1-responsive element [LILRE]) similar to both the consensus GAS and the distinct ISRE (IFN-stimulated response element). ISRE sites are directly recognized by the ISGF/IRF family such as the IFN-α/β-activated ISGF3γ-containing complex (5). We report here that a protein-LILRE complex can be immediately (15 min

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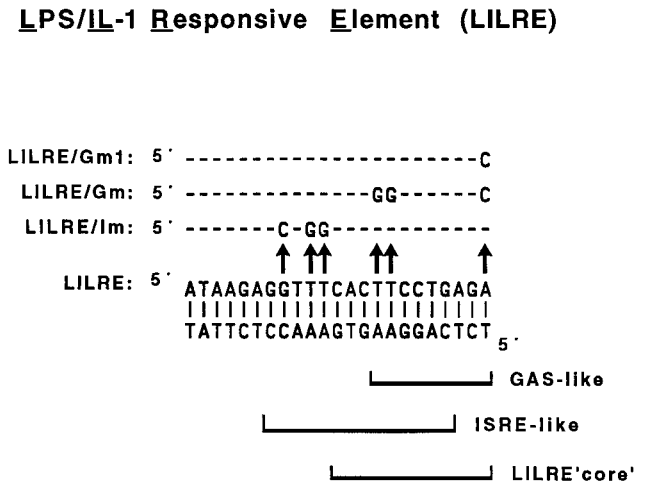


FIG. 1. The LILRE contained within the human *IL1B* gene. The sequence corresponds to positions −2863 to −2841 of the gene (34, 44) (GenBank accession no. L06808). The GAS- and ISRE-like motifs are shown below the sequence. Specific nucleotide substitutions contained in the three distinct oligonucleotides are indicated by arrows. These mutations are located at specific sites known to be critical for GAS and ISRE function (26, 27, 42). LILRE 'core' shows an *IL1B* sequence used as a probe in some experiments (described in Materials and Methods) to examine specific binding of LIL factor to the LILRE GAS-like motif.

after stimulation in the presence of a protein synthesis inhibitor) induced by both LPS and IL-1β. This LPS- and IL-1-induced protein (LIL factor) is a tyrosine-phosphorylated DNA-binding factor. The LIL factor-DNA complex is inhibited by phosphotyrosine (P-Tyr), but not by either phosphoserine (P-Ser) or phosphothreonine (P-Thr), suggesting the presence of a protein–P-Tyr interaction essential for DNA-binding activity and dimerization. Further characterization of the LIL factor revealed that it resembles the STAT family of transcriptional factors. This factor (referred to as LIL-Stat) is distinct from other STATs. For example, LIL-Stat is recognized by an antibody (Ab) raised against the amino terminus of Stat1 (Stat1N Ab) but is not recognized by Abs raised against various other STATs and against a peptide near the carboxyl terminus of Stat1 (Stat1C Ab). Furthermore, unlike other known STATs, LIL-Stat requires a G residue at position 8 (LILRE GAS-like site; TTCCTGAGA) of the GAS-like sequence in order to bind its target DNA efficiently. In addition, as a result of examining the abilities of IL-4, IL-6, and IFN-γ to activate LIL-Stat, IL-6 can also induce a protein-DNA complex identical to that induced by LPS and IL-1. These binding data agree well with chloramphenicol acetyltransferase (CAT) data which show that the LILRE mediates transcriptional induction in response to IL-1, IL-6, and LPS. Thus, the fact that a STAT-like protein can be activated in common by LPS, IL-1, and IL-6 is evidence for the involvement of a common and rapid signal transduction mechanism possibly extending to the ligand binding components.

MATERIALS AND METHODS

Endotoxin tests. All materials and solutions, including plasmid preparations, phosphate-buffered saline, media, and fetal bovine serum for tissue culture and transfection, were tested for endotoxin by a *Limulus* amebocyte lysate assay (QCL-1000; Whittaker Bioproducts, Inc., Walkersville, Md.) as described previously (44).

Oligonucleotides and phosphopeptides. The double-stranded oligonucleotides used in the present study were as follows (the core recognition sequence of each oligonucleotide is underlined): LILRE, 5′-AGCTTATAAGAGGTTTCACTTC CTGAGAGTCGA-3′; GAS derived from the FcγRI gene, 5′-CGATCGAGAT GTATTTCCCAGAAAAGTCGA-3′ (35); mutated GAS, 5′-CGATCGAGAT GTATggCCCAGAcAAGTCGA-3′; ISRE, 5′-TCGACGGCTTAGTTTCACTT TCCCTACTAT-3′ (5); and hSIE (high-affinity *sis*-inducible element), 5′-AGCT TGTGCATTTCCCGTAAATCTTGTCGTCGA-3′ (45). The LILRE mutant sequences LILRE/Im, LILRE/Gm, and LILRE/Gm1 were identical to the wild-type LILRE oligonucleotide but contained the substitutions shown in Fig. 1. LILRE core oligonucleotide (5′-AGCTTCACTTCCTGAGAGTCGA-3′) contained the GAS-like sequence of the LILRE plus three 5′ nucleotides derived from the *IL1B* gene (Fig. 1) as well as *Hin*dIII (5′) and *Sal*I (3′) sites for labeling. Three kinds of mutated LILRE core oligonucleotides contained a single nucleotide substitution of G to T, A, or C at position 8 of the underlined GAS-like sequence, respectively. For example, a G-to-A mutation resulted in the sequence 5′-AGCTTCACTTCCTGAaAGTCGA-3′. LILRE core oligonucleotide which contained a G-to-T mutation at position 6, LILRE core (G_6→T), resulted in the sequence 5′-AGCTTCACTTCCTtAGAGTCGA-3′.

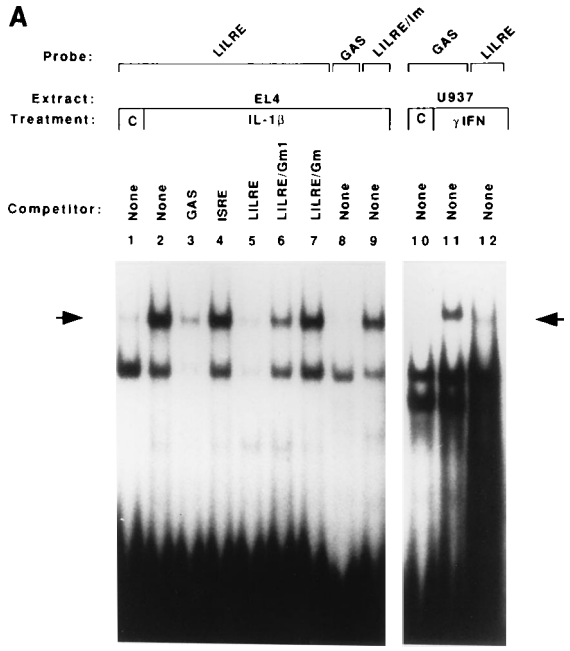
Synthetic phosphotyrosine 12-mer peptides were synthesized by using fluorenylmethoxycarbonyl chemistry on an Applied Biosystems synthesizer. The human IFN-γ receptor (IFNγR) α-chain sequence (Y_{440}) corresponded to residues 436 to 447 (TSFGYDKPHVLV, with P-Tyr underlined), and the human IL-1 type I receptor sequence (Y_{496}) corresponded to residues 493 to 504 (IQDYEK MPESIK).

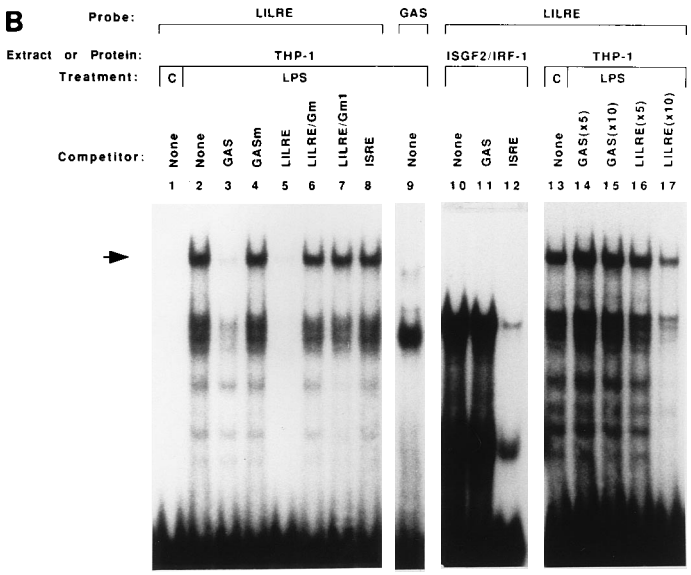
Recombinant proteins and Abs. Human recombinant IL-1β (10^7 U/mg by a thymocyte proliferation assay), IL-4 (10^7 U/mg by a human peripheral blood T-lymphocyte assay), IL-6 (4×10^6 U/mg by a T-1165 cell bioassay), and IFN-γ (2.5×10^7 NIH human IFN-γ reference standard units per mg) proteins were used in the present study. IL-1β was provided by Cistron Biotechnology (Pine Brook, N.J.) and contained an endotoxin level of less than 0.8 ng/mg of IL-1β protein. Such a low level of endotoxin does not affect our assay system. IL-4, IL-6, and IFN-γ were purchased from Genzyme (Cambridge, Mass). IL-1β, IL-4, IL-6, IFN-γ, and LPS were used at final concentrations of 20 ng/ml, 10 ng/ml, 5 ng/ml, 16 ng/ml, and 10 μg/ml, respectively, to make nuclear extracts. Purified ISGF2/IRF-1 protein was produced by expression in *Escherichia coli* and was a gift from T. Maniatis (Harvard University). The amino-terminus-specific Stat1N Ab (anti-Stat1N Ab) was purchased from Transduction Laboratories, Lexington, Ky. (catalog no. G16930). This antibody was raised against the amino-terminal 194 amino acids of Stat1. The ISGF3γ (anti-ISGF3γ Ab) and carboxyl terminus-specific Stat1C (anti-Stat1C Ab) Abs were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, Calif.). The anti-Stat1C Ab (catalog no. sc-346) was raised against a peptide containing amino acids 688 to 700 of Stat1 and is reported to react with both Stat1α (p91) and Stat1β (p84). Anti-Stat3 Ab was kindly provided by S. Akira (Osaka University, Osaka, Japan). This Ab was raised in rabbits against a synthetic peptide corresponding to the sequence of Stat3 between amino acids 626 and 640 (1). Anti-Stat4 and anti-Stat5 antibodies were kindly provided by J. N. Ihle (St. Jude Children's Research Hospital, Memphis, Tenn.). The anti-phosphotyrosine Ab (anti-P-Tyr Ab) was purchased from Upstate Biotechnology Inc. (Lake Placid, N.Y.).

Cell culture and nuclear extracts. Murine EL4 6.1.C10 thymoma cells (a gift from L. Gehrke) (24) were grown in culture dishes (150 by 25 mm) each containing 50 ml of endotoxin-free complete RPMI 1640 medium supplemented with 10% fetal calf serum and 0.5% penicillin and streptomycin. We have detected 16,600 IL-1Rs per cell in a receptor binding assay (data not shown). Cells were split at 1:3 dilution every 2 or 3 days to avoid overcrowding and were further split at 1:3 on the day before preparation of the nuclear extract. Cells were stimulated by the method reported by Akira et al. (1) with some modifications. Before stimulation, cells were washed at least three times with serum-free medium and were then incubated at a density of 5×10^6/ml at 37°C for 1 h in the presence of 3.55 mM cycloheximide. We have previously reported that the addition of 3.55 mM cycloheximide to cell cultures results in inhibition of protein synthesis by greater than 90% (10). IL-1β (at a final concentration of 20 ng/ml) was added to the cells and was further incubated for 15 min. Human THP-1 (ATCC TIB 202) and U937 (ATCC CRL 1593) monocytic cell lines were grown as previously described (20, 34). Human Hep3B hepatoma cell line (ATCC HB 8064) was grown as recommended by the American Type Culture Collection. Human UT7 hematopoietic cells were kindly provided by D. Wen (Brigham and Women's Hospital, Boston, Mass.). Nuclear extracts were prepared as previously described (34) except that 1 mM $ZnCl_2$, 1 mM sodium orthovanadate, and 10 mM NaF were used to inhibit phosphatase activities.

Electrophoretic mobility shift assay (EMSA). Oligonucleotides were labeled with DNA polymerase Klenow fragment and [α-^{32}P]dTTP at 3,000 Ci/mmol (DuPont-NEN). Unincorporated deoxynucleoside triphosphates were removed by use of G-25 spin columns (5 Prime-3 Prime, Inc., Boulder, Colo.). Radiolabeled probes were further purified on a 10% polyacrylamide gel. Binding reactions were performed as previously described (34) and then analyzed on a 4% polyacrylamide gel running in 0.5× TBE buffer (45 mM Tris-borate and 1 mM EDTA).

Plasmids. Human IL-1β genomic DNA fragments were derived from clone BDC454 (4). We have used the identical sequence numbering as that described in our recent report (44). The LILRE sequence located between positions −2863 and −2841 bp upstream of the transcription start site was inserted into a CAT gene plasmid vector containing a minimal (−59 to +105) murine c-*fos* promoter (*fos*CAT) (23). The LILRE/*fos*CAT construct was verified by sequencing as described previously (34).





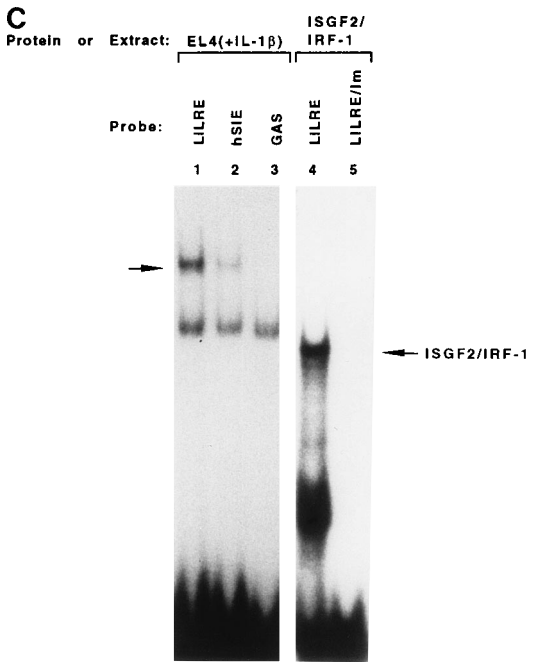


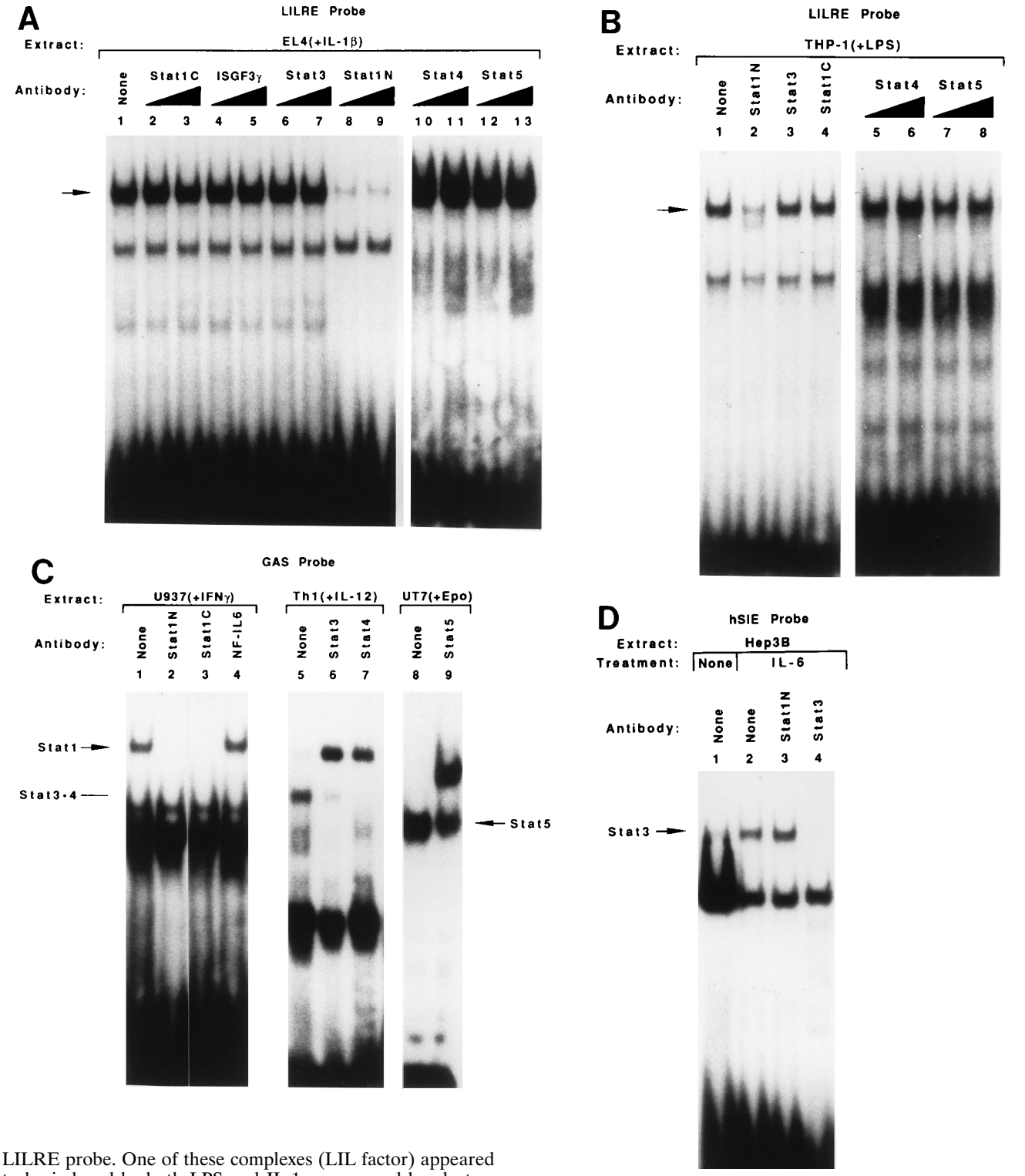
FIG. 2. EMSA of DNA-binding proteins induced by IL-1β (A and C) and LPS (B). Nuclear extracts were prepared from control cells (C) and treated (as indicated over the lanes) murine EL4 thymoma and human monocyte (U937 and THP-1) cells. The oligonucleotide sequences and ISGF2/IRF-1 protein were shown in Fig. 1 and described in Materials and Methods. Unless otherwise indicated [by (×5) or (×10) for 5- or 10-fold molar excess], unlabeled competitor oligonucleotides were used at a 50-fold molar excess over the concentration of radiolabeled probe. (A) IL-1β-induced binding of LIL factor to LILRE; (B) LPS-induced binding of LIL factor to LILRE; (C) relative affinities of the LIL factor for three different GAS-like sites (LILRE, hSIE, and GAS). The unlabeled arrow indicates the position of the inducible factor common to both IL-1β and LPS induction.

Transfection and CAT assay. The human THP-1 monocytic cell line was transfected by the DEAE-dextran method as described previously (34). Transfections of the human Hep3B hepatoma and murine EL4 thymoma cell lines were carried out by a calcium phosphate precipitation procedure (20). THP-1 cells (10^7 cells per plate) were transfected with 10 μg of plasmids. Adherent Hep3B (60% confluent in a dish [10 by 2 cm]) and nonadherent EL4 (10^7 cells per plate) cells were transfected with 20 μg of plasmids. After transfection, THP-1 cells were treated with 100 ng of LPS per ml, 10 ng of IL-4 per ml, or 20 ng of IFN-γ per ml for 16 h. Hep3B cells were treated with 10 ng of IL-1β per ml or 5 ng of IL-6 per ml for 16 h. EL4 cells were treated with 10 ng of IL-1β per ml for 16 h. The CAT assays were carried out by a liquid scintillation method (38) with 100 μg of THP-1, 50 μg of EL4, or 40 μg of Hep3B cell lysate. The protein concentrations for extracts were determined with a Bradford protein assay kit (Bio-Rad, Melville, N.Y.). CAT activities were determined by calculating slopes from plots of time versus counts per minute within a linear range of the response as described previously (34).

Affinity purification and characterization of LILRE-binding proteins. Whole-cell extract of IL-1β (10 ng/ml)-stimulated EL4 cells was prepared by treatment of 2×10^{10} cells with lysis buffer (1 mM $MgCl_2$, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.9], 10 mM KCl, 300 mM NaCl, 0.1% Triton X-100, 20% glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 1 mM $ZnCl_2$, 1 mM Na orthovanadate, 1 mg of aprotinin per ml, 1 mg of antipain per ml, 1 mg of chymostatin per ml, 1 mg of leupeptin per ml, and 1 mg of pepstatin A per ml). LILRE-binding proteins were purified by ammonium sulfate fractionation (25 to 50% cut) followed by chromatography on a DNA affinity column (0.8-ml bed volume) consisting of double-stranded 5′-biotinylated LILRE oligonucleotide coupled to avidin agarose (Sigma, St. Louis, Mo.). After extensive washes with 10 column volumes of buffer (20 mM HEPES [pH 7.9], 1 mM EDTA, 5 mM $MgCl_2$, 0.05% Nonidet P-40), bound proteins were eluted via salt wash (20 mM HEPES [pH 7.9], 1 mM EDTA, 5 mM $MgCl_2$, 0.05% Nonidet P-40, 1 M KCl). The purified proteins were precipitated with trichloroacetic acid, resolved on a sodium dodecyl sulfate–10% polyacrylamide gel, and detected by Western blot (immunoblot) analysis (25) using anti-Stat1C, anti-Stat1N, and anti-P-Tyr Abs described above. A human fibroblast cell lysate obtained from Transduction Laboratories was used as a positive control for Stat1. Immunoreactive bands were visualized with the Amersham (Arlington Heights, Ill.) epichemiluminescence Western blotting system.

RESULTS

A protein-GAS complex is induced by either IL-1 or LPS. Sequence analysis of the LILRE suggested two possible protein binding sites (Fig. 1). One is a GAS-like site, TTCCT GAGA. Another resembles an ISRE, GGTTTCACTTCCTG. In order to examine proteins which bind to the LILRE, induction of nuclear factors from LPS- and IL-1-treated cells was evaluated by EMSA using radiolabeled DNA probes containing a consensus GAS site derived from the FcγRI gene as well as the LILRE. Nuclear extracts from IL-1-treated EL4 and LPS-treated THP-1 cells which were incubated for 15 min with inducer in the presence of a protein synthesis inhibitor (cycloheximide) revealed induction-specific complexes with the



LILRE probe. One of these complexes (LIL factor) appeared to be induced by both LPS and IL-1, as assessed by electrophoretic migration and competition with various unlabeled oligonucleotides (Fig. 2, unlabeled arrows). Competition with a 50-fold molar excess of unlabeled GAS DNA (Fig. 2A, lane 3, and Fig. 2B, lane 3), but not with an ISRE (Fig. 2A, lane 4, and Fig. 2B, lane 8), suggests the involvement of a STAT-like dimer. However, this complex was not competed for by either a 5- or 10-fold molar excess of GAS DNA, in contrast to similar amounts of LILRE competitor (Fig. 2B, lanes 13 to 17), demonstrating a large difference in binding affinity between the two sequences. Pearse et al. have reported that an A-to-C mutation at position 9 of the GAS sequence (TTCCNNNAA) resulted in a significant reduction in Stat1-binding activity (26). The fact that both a single point mutation at position 9 (LILRE/Gm1; Fig. 2A, lane 6, and Fig. 2B, lane 7) and a

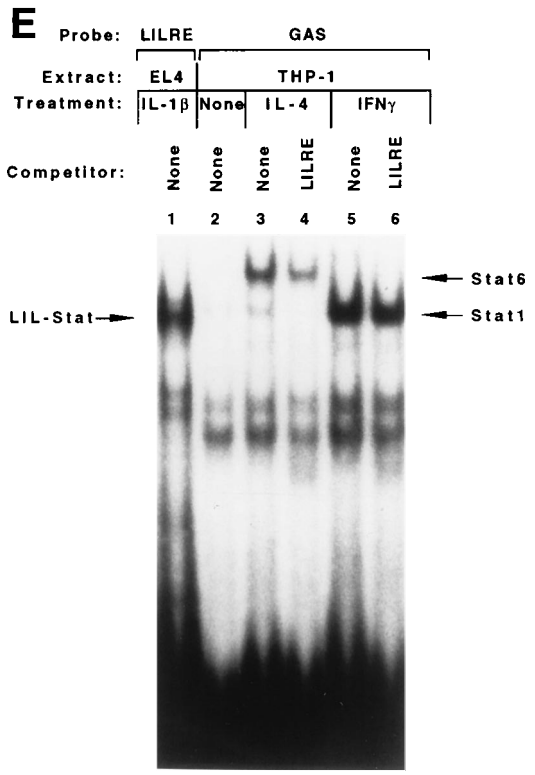


FIG. 3. The IL-1β- and LPS-induced DNA-binding protein is distinct from other STATs. EMSAs were conducted as described in the legend to Fig. 2. All binding reactions with Ab were performed at 4°C for 1 h. The arrows indicate the positions of STATs induced by the corresponding stimulants. (A) IL-1β-induced EL4 extract was incubated with either 1 and 2 μl (lanes 2 to 9) or 0.5 and 1 μl (lanes 10 to 13) of the indicated Ab and then assayed for binding activity to the LILRE probe. Triangles indicate increasing Ab concentration. (B) LPS-induced THP-1 extract was incubated with either 2 μl (lanes 2 to 4) or 0.5 and 1 μl of the indicated Ab and then assayed for binding activity to the LILRE probe. (C) IFN-γ-induced U937 extract containing Stat1 (lanes 1 to 4) was incubated with 2 μl of the indicated Ab. IL-12-treated mouse Th1 cell extract (kindly provided by N. G. Jacobson and K. M. Murphy) (lanes 5 to 7) was incubated with 1 μl of either Stat3 or Stat4 Ab. Human UT7 cell extract (lanes 8 and 9) was incubated with 1 μl of Stat5 Ab. All samples were assayed for binding activity to GAS probe. Epo, erythropoietin. (D) Nuclear extracts were prepared from untreated (None) and IL-6-treated Hep3B cells. Stat3 from the IL-6-induced extract was incubated with 2 μl of the indicated antibody and then assayed for binding activity to the hSIE probe. (E) Nuclear extracts were prepared from untreated THP-1 cells (None) and treated (as indicated over the lanes) EL4 and THP-1 cells. Unlabeled competitor oligonucleotides were used at a 50-fold molar excess over the concentration of radiolabeled probe.

multiple mutation (LILRE/Gm; Fig. 2A, lane 7, and Fig. 2B, lane 6) within the LILRE GAS-like site significantly reduced the affinity of the LILRE for the LIL factor argues that the LIL factor targets the GAS-like site within the LILRE. This argument is further supported by the observation that a LILRE oligonucleotide containing a mutated ISRE motif retained the affinity for the LIL factor (LILRE/Im; Fig. 2A, lane 9). On the other hand, the LILRE was capable of specifically binding the ISRE-binding protein ISGF2/IRF-1 (Fig. 2B, lanes 10 to 12). The mutation of the ISRE site within the LILRE completely blocked ISGF2/IRF-1 binding (Fig. 2C, lane 5), further evidence for a functional ISRE site.

We further examined IFN-γ-induced Stat1 binding to the LILRE as well as the GAS probe in our EMSA system. As shown in lanes 10 and 11 of Fig. 2A, the GAS probe bound its cognate Stat1 derived from IFN-γ-treated cells. However, the GAS probe could not efficiently bind the LIL factor (Fig. 2A, lane 8). Also, the LILRE probe did not efficiently bind Stat1 (Fig. 2A, lane 12), suggesting that the LIL factor possesses a DNA-binding specificity distinct from that observed for Stat1.

The LIL factor (LIL-Stat) is a member of the STAT family distinct from other STATs. Figure 2C shows that when a radiolabeled hSIE probe known to have a high avidity for Stat3 was used with IL-1-induced extract, a weak complex which comigrated with the LIL factor was detected (lane 2). These data suggest a minimal relationship between the LIL factor and Stat3. The identity of the LIL factor was further determined by specific Abs raised against Stat1, Stat3, Stat4, Stat5, and the ISRE-binding factor ISGF3γ. As shown in Figure 3A and B, binding studies conducted in the presence of these Abs revealed that only the Stat1N Ab specific for the amino-terminal 194 amino acids cross-reacted with the LIL factor. In contrast, the Abs raised against the other STATs and ISGF3γ did not cross-react. Among the nonreactive Abs was Stat1C Ab which was raised against amino acids 688 to 700 of Stat1. The Stat1N and Stat1C Abs completely blocked IFN-γ-induced Stat1 binding to the GAS probe (Fig. 3C, lanes 2 and 3), and the Stat3, Stat4, and Stat5 Abs specifically supershifted the Stat3 · 4 heterodimer from IL-12-treated T helper type 1 cells and Stat5 from erythropoietin-treated UT7 hematopoietic cells as previously described (11, 18) (Fig. 3C, lanes 5 to 9). Finally, IL-6-induced Stat3-hSIE complex was abrogated by Stat3 Ab, but not by Stat1N Ab (Fig. 3D, lanes 3 and 4). Both Ab-induced abrogation and supershifting of STAT-DNA complex have been reported by others (1, 21, 29, 35).

Recently, a new member of the STAT family, Stat6/IL-4Stat, has been reported to be activated by IL-4 in THP-1 cells (16). This factor binds efficiently to the FcγRI GAS probe used in the present study and generates a complex with a slower mobility by EMSA than that of Stat1 as previously reported (21). We also observed that Stat6 from IL-4-induced THP-1 cells binds to the GAS probe and migrates slower than complexes of Stat1 with the same probe and the LIL factor with a LILRE probe of similar length (Fig. 3E, lane 3). The IL-4-inducible Stat6 complex was resistant to competition by a 50-fold molar excess of LILRE competitor (lane 4). In addition, the LILRE did not significantly compete for the Stat1-GAS complex (lane 6), consistent with the data derived from the LILRE probe incubated with IFN-γ-treated cell extract (Fig. 2A). These results clearly demonstrate that the LIL factor (now referred to as LIL-Stat) is related to, but not identical with Stat1, and distinct from all other known GAS-binding STATs.

LIL-Stat contains at least one phosphotyrosine residue essential for DNA binding. The abrogation of DNA binding by anti-P-Tyr Ab (Fig. 4A, lane 2) demonstrates that LIL-Stat, like other STAT family members, is a substrate for tyrosine kinases. However, it is not clear whether this phosphorylation mediates Src homology 2 (SH2) domain-dependent dimerization as required for DNA binding by other STATs. Figure 4B and C show that P-Tyr, but not P-Ser or P-Thr, could abrogate DNA binding of LIL-Stat at concentrations similar to that observed for other STATs (29). Phenyl phosphate could also abrogate binding at similar concentrations (data not shown). This suggests that P-Tyr is important for DNA binding by LIL-Stat, consistent with the presence of an essential SH2-like P-Tyr binding domain found in other STATs (35). It has been reported that P-Tyr can disrupt STAT dimerization and abrogate DNA binding, probably as a result of competition for an essential SH2–P-Tyr interaction (29). These results further support the argument that the LIL factor is likely a member of the STAT family. A faster-migrating protein-LILRE complex which intensified with the addition of P-Tyr was seen in both induced and untreated extracts (Fig. 4B and C). Consequently,

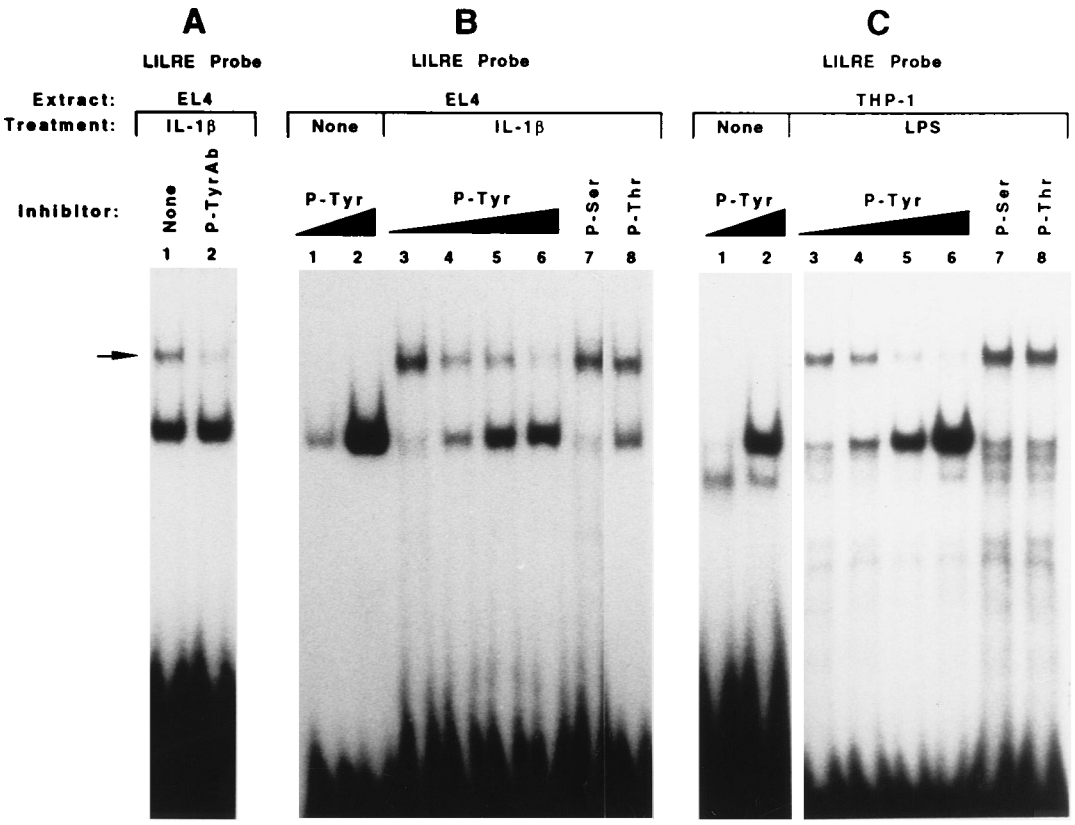


FIG. 4. LIL-Stat contains both a phosphotyrosine binding domain and at least one phosphotyrosine residue. EMSAs were conducted as described in the legend to Fig. 2. Nuclear extracts were prepared from control cells (None) and treated (as indicated over the lanes) EL4 and THP-1 cells. (A) P-Tyr Ab (1.5 μl) was incubated with IL-1-induced EL4 extract under the same conditions as those described in the legend to Fig. 3. (B and C) Phosphotyrosine (P-Tyr) ranging from 0 to 25 mM (indicated by triangle) was incubated with nuclear extracts from treated (as indicated over the lanes) EL4 (lanes 3 to 6 in panel B) or THP-1 (lanes 3 to 6 in panel C) cells at 4°C for 1 h. Phosphoserine (P-Ser) and -threonine (P-Thr) were used at 25 mM as a control. DNA-binding activities of untreated EL4 (B) and THP-1 (C) extracts were also examined in the absence (lane 1) and presence (lane 2) of P-Tyr (25 mM). The arrow indicates the position of LIL-Stat.

this LILRE-protein complex appears to be unrelated to LIL-Stat activation. Furthermore, the fact that this faster-migrating protein was not recognized by P-Tyr Ab (Fig. 4A) is evidence that this protein is not STAT-like.

The P-Tyr binding domain of LIL-Stat is distinct from that of Stat1. The observation that LIL-Stat cross-reacts with an Ab raised against the amino terminus of Stat1, but not with an Ab raised against residues 688 to 700 of Stat1 which is located adjacent to and between the Stat1 SH2 and P-Tyr domains, suggests that LIL-Stat may not possess Stat1-like SH2 binding specificity. In order to test this hypothesis, Stat1 derived from IFN-γ-treated U937 cells was assayed by EMSA for DNA binding inhibition resulting from incubation with a phosphotyrosine peptide derived from the IFNγR α chain. As previously reported (12), complete inhibition of GAS probe binding occurred at a low concentration (10 μM) of phosphopeptide (Fig. 5, lanes 2 to 5). An irrelevant phosphopeptide did not inhibit binding (lane 1). In contrast, the IFNγR phosphopeptide had no effect on LIL-Stat binding to the LILRE when used at a concentration at least 40-fold higher than that required for complete inhibition of Stat1 binding to GAS (Fig. 5, lanes 6 to 9). Consequently, LIL-Stat does not appear to possess a Stat-1-like SH2 domain binding specificity, evidence for a distinct sequence in the protein in the vicinity of the Stat1C Ab epitope.

Characterization of a novel STAT-like protein by affinity chromatography. An immobilized LILRE oligonucleotide was used as an affinity matrix in order to enrich for a protein that could be demonstrated to possess the characteristics of LIL-Stat. Figure 6 shows Western blots for enriched fractions derived from LILRE affinity chromatography (LILRE+; lanes 2, 3, and 6) as well as a control cell lysate containing both forms of Stat1 (i.e., p91 or Stat1α and p84 or Stat1β, lanes 1 and 5). The use of three different Abs demonstrates that only one protein (apparent molecular weight of 52,000 and labeled p52) reacts with both the anti-Stat1N and anti-P-Tyr Abs, but not with anti-Stat1C Ab. This profile of Ab reactivity matches that observed for the novel LIL-Stat DNA-binding activity observed by EMSA (Fig. 3 and 4).

A single G-to-A point mutation at position 8 of the LILRE GAS-like element eliminates LIL-Stat binding and increases affinity for Stat1. As shown above (Fig. 2), LILRE bound the LIL-Stat factor with a higher avidity than Stat1. In contrast, the FcγRI GAS preferentially bound Stat1. The LILRE GAS-like sequence (TTCCTGAGA) differs from the GAS consensus sequence [TT(C/A)CNNNAA] only at position 8. The G residue at position 8 (G_8) of the LILRE has not been previously reported in any GAS sequence. In order to determine the significance of G_8, LILRE core oligonucleotides (i.e., LILRE oligonucleotides not containing a functional ISRE element [Fig. 1 and Materials and Methods]) were mutated at position 8 and assayed for the ability to compete for LIL-Stat, Stat1, Stat3, and Stat6. As shown in Fig. 7, a G_8-to-A mutation increased affinity of the LILRE core oligonucleotide for both Stat1 (Fig. 7B) and Stat3 (Fig. 7C), whereas only the wild-type

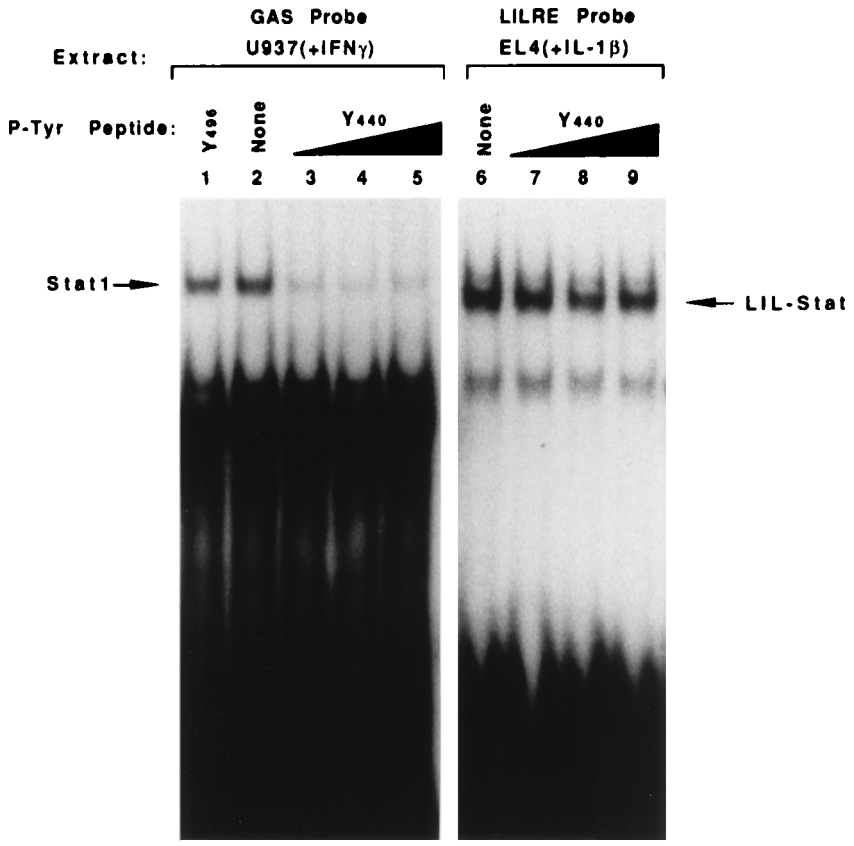


FIG. 5. LIL-Stat and Stat1 possess distinct SH2 phosphopeptide binding specificities. Stat1 binding to GAS probe (lanes 1 to 5) was generated with nuclear extracts from IFN-γ-treated U937 cells and LIL-Stat binding to the LILRE probe with nuclear extracts from IL-1-treated EL4 cells (lanes 6 to 9). Lane 1 contained a phosphotyrosine peptide (100 μM) derived from the IL-1 type I receptor (Y_{496}). The solid triangles indicate increasing concentrations for a phosphotyrosine peptide (Y_{440}) derived from the IFNγR α chain (10, 30, and 100 μM for lanes 3 to 5, respectively, and 100, 200, and 400 μM for lanes 7 to 9, respectively).

LILRE core containing G_8 efficiently competed for the LIL-Stat (Fig. 7A). These data clearly show that LIL-Stat, in contrast to Stat1 and Stat3, requires G_8 to bind to its target site. On the other hand, Stat6 requires a distinct variation of the GAS sequence, because only weak competition for Stat6 was observed for the wild-type LILRE core even at a 20-fold molar excess, while the other oligonucleotides did not compete (Fig. 7D). In order to confirm the specific binding affinity of the wild-type LILRE core for LIL-Stat, we carried out EMSAs using the wild-type LILRE core as a radiolabeled probe with IL-1-induced EL4 nuclear extract. As shown in Fig. 8, consistent with LIL-Stat involvement, the protein-DNA complex was recognized by Stat1N Ab, but not by the Stat1C and Stat3 Abs. In addition, the complex was specifically inhibited by P-Tyr, but not by either P-Ser or P-Thr. These results further confirmed that LIL-Stat binding is specific for the LILRE GAS-like sequence and not the ISRE element.

In the case of the Stat3 SIE-binding sequence, a single point mutation of G to T at position 6 ($G_6 \rightarrow T$) has been shown to completely block Stat3 binding (45). Interestingly, LILRE also possesses a G_6. In order to investigate the importance of G_6 in the LILRE, a $G_6 \rightarrow T$ transversion similar to that reported for Stat3 (45) was assayed by EMSA. As shown in Fig. 9, $G_6 \rightarrow T$ did not significantly change the affinity of the LILRE GAS-like site for LIL-Stat, further evidence for LIL-Stat and Stat3 being two distinct factors.

IL-6 is also able to activate LIL-Stat. In the liver, several factors are known to induce the synthesis of the acute-phase

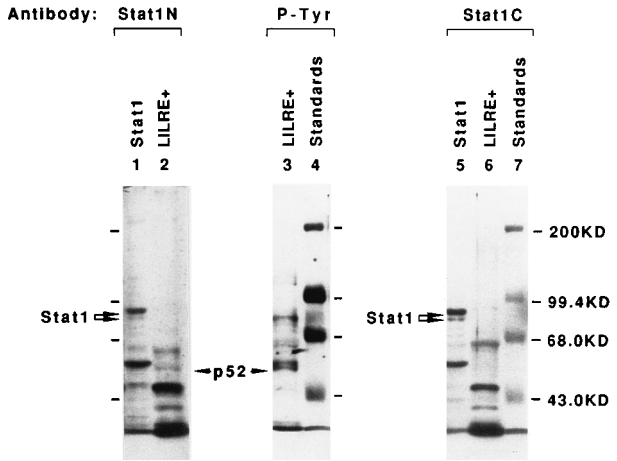


FIG. 6. Western blot analysis of LILRE-binding proteins. Whole-cell extracts from EL4 cells treated with 10 ng of IL-1β per ml were purified by ammonium sulfate fractionation and DNA affinity chromatography (lanes 2, 3, and 6). Duplicate and or stripped blots were probed with Abs as indicated over the lanes. An unstimulated dermal fibroblast extract (lanes 1 and 5) was used as a source of the p91 and p84 forms of Stat1 (double arrows). The p52 label indicates the migration position of the only protein that is reactive with anti-Stat1N and anti-P-Tyr Ab but unreactive with anti-Stat1C Ab. Prestained protein standards were used for determining molecular mass (in kilodaltons) (lanes 4 and 7).

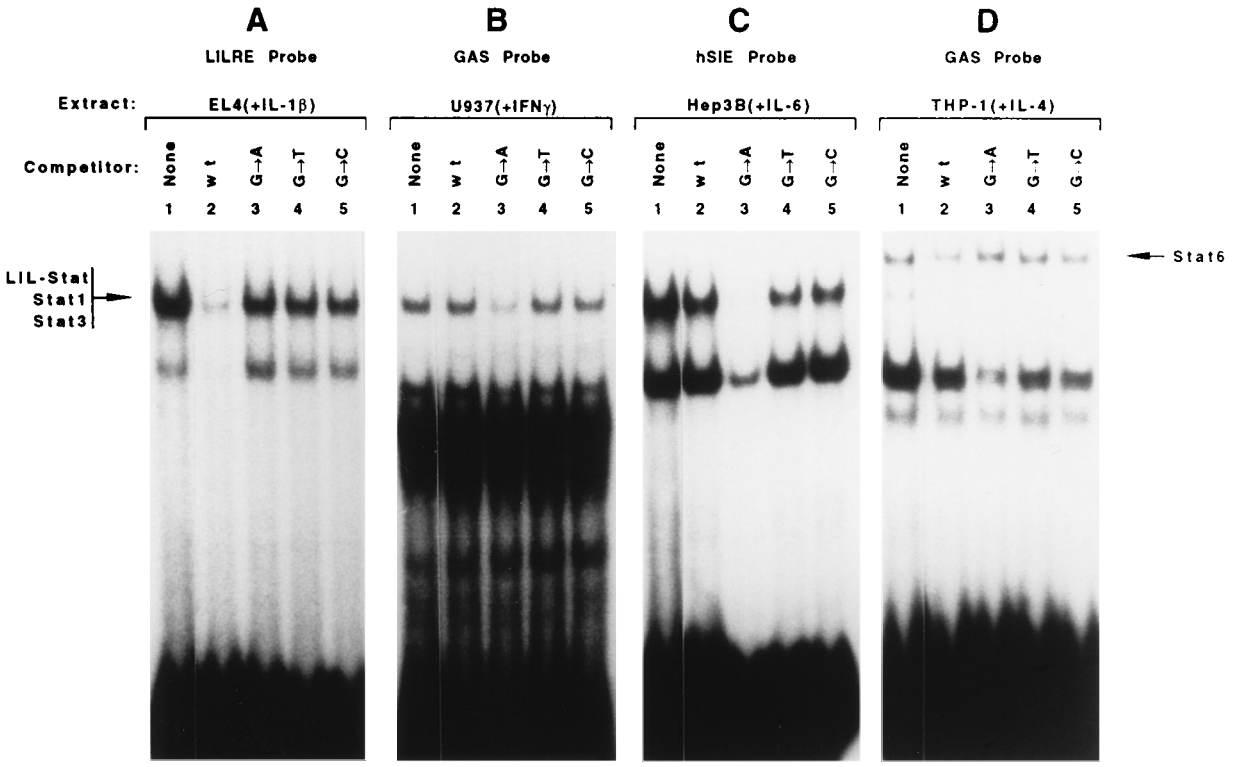


FIG. 7. LIL-Stat, unlike Stat1 and Stat3, requires G_8 within the LILRE GAS-like sequence to recognize its target site. EMSAs were conducted as described in the legend to Fig. 2. Nuclear extracts prepared from treated (as indicated over the lanes) murine EL4 thymoma, human Hep3B hepatoma, and human monocyte (U937 and THP-1) cells were incubated with the corresponding radiolabeled DNA probes. Unlabeled LILRE core (described in Materials and Methods) mutated at position 8 was assayed for the ability to compete for LIL-Stat (A), Stat1 (B), Stat3 (C), and Stat6 (D). The arrows indicate the positions of STATs induced by their corresponding stimulants. Unlabeled competitor oligonucleotides were used at a 20-fold molar excess over the concentration of radiolabeled probe. wt, wild type.

plasma proteins. These factors are LPS, IL-1, IL-6, IL-11, leukemia inhibitory factor, oncostatin M, and ciliary neurotrophic factor. IL-6 is an important inducer of the acute-phase response and possesses several overlapping biological effects with IL-1 (19). Therefore, we tested the ability of IL-6 to activate LIL-Stat in a human Hep3B hepatoma cell line. As shown in Fig. 10A (lanes 1 and 2), B (lanes 1 and 3), and C (lanes 1 and 6), a protein-LILRE complex was induced after treatment with IL-6. Similar intensities of the IL-6-inducible complex (arrows) were observed in Fig. 10. However, Fig. 10C reveals a reduced amount of the faster-migrating nonspecific complex observed in Fig. 10A and B. The IL-6-inducible factor was recognized by Stat1N Ab, but not by Stat3 Ab (arrow; Fig. 10C, lanes 3 and 5). Unlike Stat3, this IL-6-induced factor showed the highest affinity for the wild-type LILRE core sequence containing G_8 (Fig. 10A). Consistent with STAT involvement, P-Tyr specifically blocked its DNA-binding activity (Fig. 10B). The IL-6 used in the study was not contaminated by significant levels of endotoxin (<10 ng of endotoxin per mg of IL-6 protein was detected via the *Limulus* amebocyte assay). In contrast, neither IL-4 (data not shown) nor IFN-γ (Fig. 2) could induce significant LIL-Stat binding to the LILRE. Also, the IL-6-treated Hep3B extract was demonstrated by EMSA (not shown) to bind to an hSIE probe and generate all previously identified (29) STAT complexes (i.e., Stat1 and Stat3 homodimers as well as the Stat1 · 3 heterodimer). These results demonstrate that the IL-6-induced LILRE-binding protein is not Stat3 but is likely LIL-Stat. Furthermore, these results reveal that LILRE and hSIE are specific binding sites for LIL-Stat and Stat3, respectively.

The LILRE can support transcriptional activation. To examine the function of the LILRE in response to specific activators, a single copy of the sequence was inserted into an enhancer-dependent CAT construct by employing the minimal murine c-*fos* gene promoter (23). The vector was transfected into various cell types, and CAT activity was measured after treatment with either IL-1, IL-4, IL-6, IFN-γ, or LPS (Fig. 11, black bars). The Hep3B hepatoma cell line, which induces LIL-Stat in response to IL-6 treatment (Fig. 10) and has been reported to be responsive to IL-1 (39), was used in addition to EL-4 cells for IL-1 induction studies. As shown in Fig. 11A, treatment of Hep3B cells with IL-1 and IL-6 resulted in 4- and 2.5-fold increases in CAT activity, respectively. Figure 11B also shows that IL-1-treated EL4 cells were strongly induced. Treatment of THP-1 monocytic cells with LPS resulted in an 11-fold increase in CAT activity (Fig. 11C). However, IL-4 and IFN-γ, which did not activate LIL-Stat, showed no significant induction of LILRE CAT activity (Fig. 11C). Furthermore, transfection of a similar vector containing the LILRE/Gm1 single point mutation shown in Fig. 1 resulted in a complete loss of activity in IL-1- and IL-6-treated Hep3B cells and LPS-treated THP-1 cells (Fig. 11, white bars). These data are consistent with the LILRE–LIL-Stat binding data and argue that LIL-Stat is a transcriptional activator that confers IL-1, IL-6, and LPS responsiveness through binding to the LILRE.

DISCUSSION

The cytokine superfamily of receptors has been recently demonstrated to mediate the rapid activation of the Jak-STAT

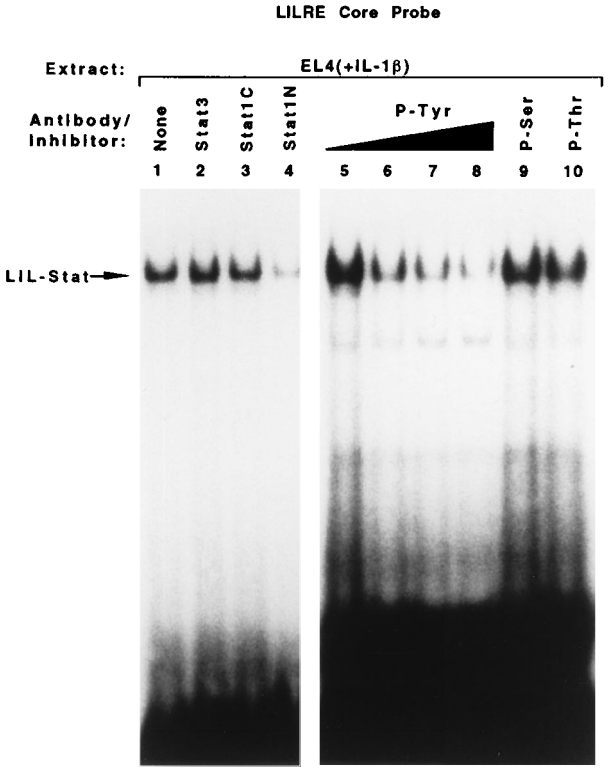


FIG. 8. The protein binding specificity of the GAS-like sequence in the LILRE is identical to that of the entire LILRE element. EMSAs were conducted as described in the legend to Fig. 2. Nuclear extract prepared from IL-1β-induced murine EL4 thymoma cells was incubated with radiolabeled LILRE core probe (described in Materials and Methods). Incubations of the EL4 extract with the indicated Ab (2 μl), P-Tyr (0 to 25 mM), P-Ser (25 mM), and P-Thr (25 mM) were carried out under the same conditions as those described in the legends to Fig. 3 and 5.

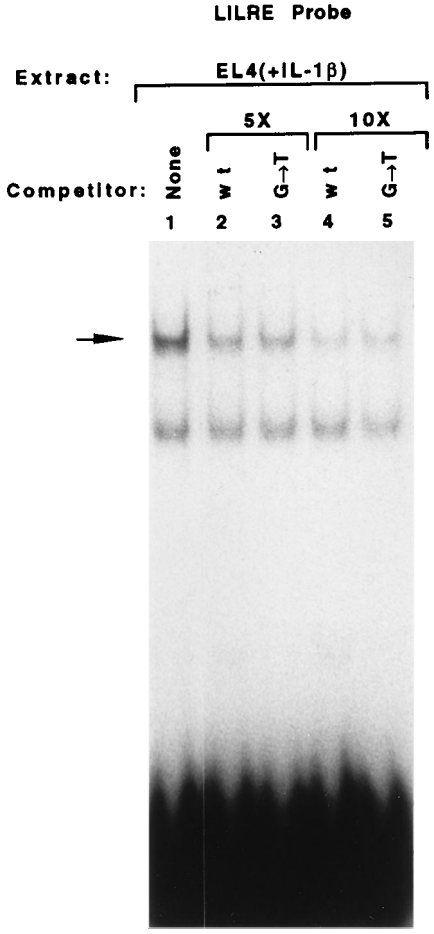


FIG. 9. A G_6 within the LILRE GAS-like sequence is not essential for LIL-Stat binding. EMSAs were conducted as described in the legend to Fig. 2. IL-1β-induced EL4 nuclear extract was incubated with radiolabeled LILRE probe. Unlabeled LILRE core containing a $G_6 \rightarrow T$ mutation of the GAS-like sequence (LILRE core [$G_6 \rightarrow T$] described in Materials and Methods) was assayed for the ability to compete for LIL-Stat. Unlabeled wild-type (wt) LILRE core was used as a control. 5× and 10×, 5- and 10-fold molar excess over the concentration of radiolabeled probe.

signal transduction pathway. Following specific ligand binding, latent cytoplasmic proteins termed STATs are activated through phosphorylation by Jak tyrosine kinases, which are physically associated with the membrane-proximal cytoplasmic domain of the receptors. The activated STATs dimerize and are translocated to the nucleus in order to transactivate genes by binding to target GAS sequences. This pathway transfers signals immediately to the nucleus without de novo protein synthesis and connects the cell surface events directly to gene regulation (5, 17). IL-1R, in contrast to most other cytokine receptors, belongs to the immunoglobulin superfamily (7). However, IL-1R, like the other cytokine receptors, has no intracellular sequence homology with known protein kinases (36, 37). LPS has also been reported to cause immediate activation of many genes, including induction of the *IL1B* gene (9). However, although efficient LPS binding to cells is mediated by CD14, a glycosylphosphatidylinositol-anchored extracellular membrane protein (47), the identity of the LPS signaling receptor has not yet been identified. A recent report (28) that LPS, in contrast to the rapid (15-min) induction by epidermal growth factor, could only induce Stat3 binding in mouse liver after 75 min of treatment does not argue for a direct relationship between LPS and STAT induction. Thus, the biological effects of IL-1 and LPS are well documented, but the molecular mechanisms of signal transduction for these two factors are not well understood.

In the present study, we have demonstrated that both IL-1 and LPS activate a common immediate-early (15 min after cell treatment) tyrosine-phosphorylated protein in the presence of a protein synthesis inhibitor. This protein possesses a minimum apparent molecular weight of 52,000, binds a GAS-like sequence within the *IL1B* gene, and is recognized by an antibody raised against the N terminus of Stat1. Moreover, the DNA-binding activity of this protein is specifically inhibited by P-Tyr, suggesting that P-Tyr mediates the obligate dimerization required for STAT DNA binding (29). From these results, this LPS- and IL-1-inducible protein is most likely a member of the STAT family (LPS/IL-1 Stat or LIL-Stat). The existence of such a factor relates the signaling pathway for IL-1 and LPS receptors to other cytokine receptors that mediate signaling via the immediate activation of STAT transcription factors. The activation of LIL-Stat by IL-1 is not restricted to EL4 T cells, because LIL-Stat activation can also be detected in human dermal primary fibroblasts following IL-1 treatment (data not shown).

The GAS sequences, like those that have been reported to respond to IFN-γ activation, are the DNA binding targets for STAT dimers (5). GAS sites share a common motif, TT(C/A)CNNNAA, as a minimal element. The FcγRI GAS site used

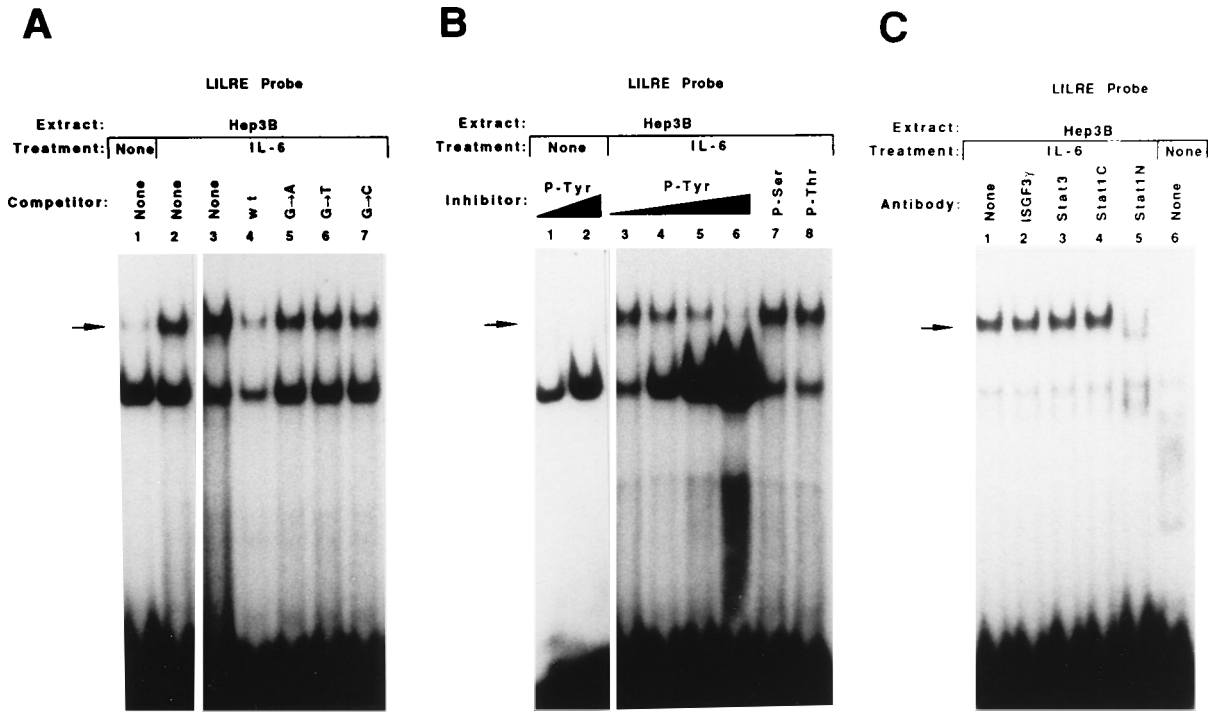


FIG. 10. LILRE-binding protein is induced by IL-6 in hepatocytes. EMSAs were conducted as described in the legend to Fig. 2. Untreated control (None) and IL-6-treated extracts were prepared from human Hep3B hepatoma cells. (A) Unlabeled LILRE core sequence (described in Materials and Methods) containing position 8 mutations was assayed for the ability to compete for IL-6-induced protein-LILRE complex. The unlabeled wild-type (wt) LILRE and position 8 mutant competitor oligonucleotides were used at a 20-fold molar excess over the concentration of radiolabeled probe. (B) Incubations of the IL-6-induced extract with P-Tyr (0 to 25 mM), P-Ser (25 mM), or P-Thr (25 mM) were carried out under the same conditions as those described in the legends to Fig. 5 and 8. DNA-binding activities of control (uninduced) extract were also examined in the absence (lane 1) or presence (lane 2) of P-Tyr (15 mM; corresponding to lane 5 for treated extract). (C) IL-6-induced extract was incubated with 2 μl of the indicated Ab. Arrows mark IL-6-inducible complex migration.

in the present study is a well-known Stat1 binding element (22, 35). We also have observed a significant induction of Stat1 binding to the FcγRI GAS following IFN-γ treatment. However, Stat3 can be activated in response to either granulocyte colony-stimulating factor, IL-6, or epidermal growth factor (1, 40, 48). Stat3 regulates c-*fos* gene expression through binding to a GAS-like SIE site (45). Although the sequence of SIE, TTCCCGTCA (and the sequence of the higher-avidity hSIE,

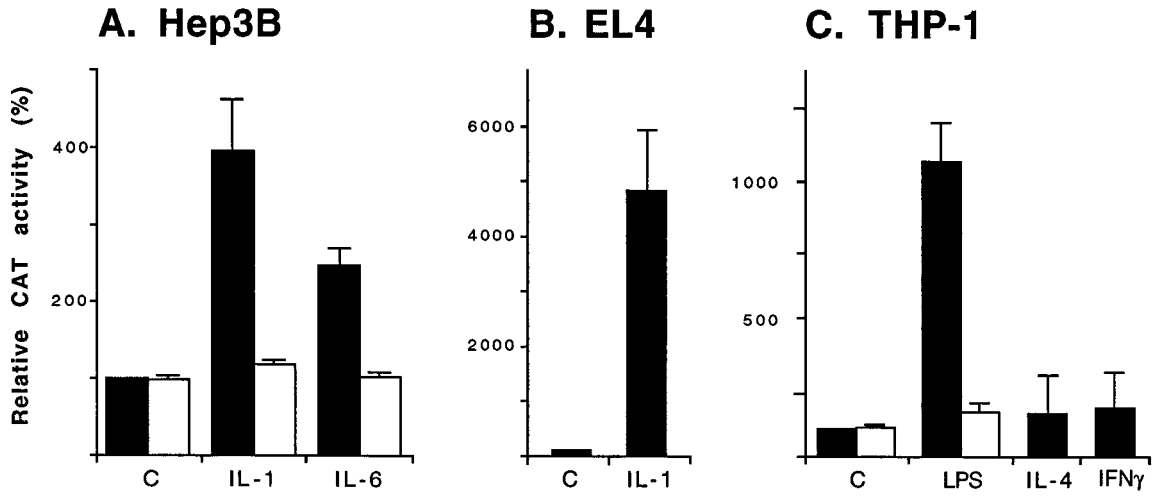


FIG. 11. CAT activity for LILRE/*fos*CAT in the presence of either IL-1β, IL-4, IL-6, IFN-γ, or LPS. LILRE/*fos*CAT was transfected into Hep3B (A), EL4 (B), or THP-1 (C) cells. After transfection, cells were either treated as indicated under the bars or left untreated. The CAT data were calculated as described in Materials and Methods. The background value in our CAT assay was determined by using the *fos*CAT reporter gene which did not contain any inducible transcriptional factor binding sites. CAT activity results from these assays were calculated by subtracting the background *fos*CAT value from LILRE/*fos*CAT data. Values represent the relative activity in treated cells compared with that in untreated cells (control [C]). Error bars represent the standard deviation from at least three independent experiments.

TTCCCGTAA), is similar to the consensus GAS element, Stat3 cannot efficiently recognize FcγRI GAS (TTCCCAG AA). This demonstrates that Stat3 requires a specific variation of the GAS consensus sequence to bind DNA (45, 48). In the present study, LIL-Stat binds to LILRE with high avidity but does not efficiently bind to either FcγRI GAS or hSIE, suggesting that LIL-Stat possesses a DNA-binding specificity distinct from that of Stat1 or Stat3. This argument is further supported by our observation that neither Stat1 nor Stat3 efficiently binds to the LILRE.

Our results argue that LIL-Stat is antigenically related to the Stat1 amino terminus, but not to either the Stat1 carboxyl end or to any other characterized STAT. In addition, the resistance of LIL-Stat DNA binding to IFNγR phosphopeptide inhibition further distinguishes the specificity of the Stat1 SH2 domain from the putative SH2 domain of LIL-Stat. Together these results suggest that LIL-Stat possesses unique DNA-binding specificity and SH2 domains. In Stat1, these domains are both located on the carboxyl side of the Stat1N Ab epitope. Therefore, LIL-Stat either is the product of a novel gene possessing homology with the Stat1 amino terminus or is derived from the Stat1 gene by alternative splicing. Because LIL-Stat possesses distinct DNA-binding and SH2 interaction specificities, alternative splicing would likely involve either a single splice replacing more than half of the coding region or two or more splices resulting in a more subtle alteration of the individual domains responsible for the unique LIL-Stat properties (i.e., DNA-binding specificity, SH2, and possibly the SH2-binding phosphotyrosine peptide located near the carboxyl terminus).

The presence of G_8 in the LILRE is novel for a GAS site. Many GAS elements possess a conserved A residue at this position (26). However, our EMSA data show that substitution of this G for any other residue greatly reduces the affinity of the LILRE for LIL-Stat. This result clearly shows that G_8 plays an important role in determining the binding affinity of the LILRE GAS-like site for LIL-Stat. Furthermore, the fact that Stat1 and Stat3 both show a significantly lower binding affinity for the wild-type LILRE than for a mutated LILRE with a consensus A substitution at position 8 further demonstrates the selectivity of the LILRE site. GAS sequence variations have been reported to be essential for STAT selectivity (15). It may also be noteworthy that this sequence variation is always dependent upon a G residue. For example, Stat3 requires G_6 (45, 48), whereas a G residue at position 7 has been reported to be essential for Stat5 (46). This G requirement is sufficient to distinguish Stat5 from LIL-Stat, because an A residue is found at position 7 of the LILRE.

Our binding studies have demonstrated that the LIL-Stat binding activity resembles but is not identical to that of Stat1, Stat3, Stat4, Stat5, or Stat6. Thus, LIL-Stat appears to be distinct from these known STATs. In addition, an anti-Stat2 Ab does not affect LIL-Stat complex migration by EMSA (not shown). Stat2 does not bind to GAS sequences, has been reported to associate only indirectly with DNA via protein-protein interaction with ISGF3γ at ISRE sites (31), and is not a likely component of LIL-Stat.

LPS, IL-1, IL-6, leukemia inhibitory factor, IL-11, oncostatin M, and ciliary neurotrophic factor are known mediators of the acute-phase response to bacterial infection (2, 19). In addition, LPS, IL-1, and IL-6 have been shown to induce several common genes, including plasminogen activator inhibitor 1, serum amyloid P component, and C-reactive protein (2, 8, 13, 30, 41), suggesting the existence of a common signal transduction pathway. Consistent with this, we observed the common induction of LIL-Stat by LPS, IL-1, and IL-6. In addition, weak protein binding was detected when a radiolabeled hSIE probe was incubated with IL-1-induced EL4 extract. Our studies using Stat1N and Stat3 Abs showed that the protein which weakly binds to hSIE following IL-1 treatment is likely Stat3 (data not shown), further evidence for a common signal pathway with IL-6. The IL-6 receptor system consists of a receptor component that confers cytokine specificity and the signal-transducing molecule, gp130 (14). IL-6-mediated activation of Stat3 is known to result from homodimerization of the gp130 and subsequent activation of gp130-associated Jak kinases (1, 19). On the basis of this evidence, it is possible that LIL-Stat activation by IL-6 may also utilize gp130.

Recent reports have demonstrated that differences exist among the various known STAT factors with regard to DNA target specificity (32, 33, 45, 46, 48). The data presented here demonstrate that the LIL-Stat binding activity is distinct from that of other STATs, further evidence for the regulation of STAT target gene expression by binding site selectivity. This selectivity and the distinct antigenic properties of LIL-Stat have allowed us to distinguish this novel binding activity from that of other STATs. The fact that all three ligands induce LIL-Stat and the precedent set for STAT activation by specific receptors argue for the possibility of a common signal transduction component. Therefore, the IL-1, IL-6, and LPS receptors likely recruit both LIL-Stat and a Jak kinase(s) either directly or via an associated subunit (perhaps gp130 or a related molecule). Consequently, characterization of LIL-Stat and upstream components of the LPS and IL-1 signaling pathways will be critical for delineating a refractory signal transduction pathway important in the regulation of inflammation.

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