Identification and Characterization of a Novel Ets-2-related Nuclear Complex Implicated in the Activation of the Human Interleukin-12 p40 Gene Promoter*

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Interleukin-12 (IL-12) is a proinflammatory cytokine produced by antigen-presenting cells in response to many microbial infections. IL-12 plays an important role in the generation of Th helper type-1 cells, which favor cell-mediated immune response. IL-12 is composed of two different subunits, p40 and p35, whose expression can be regulated concomitantly or differentially. Mono- cytic cells, the major producers of IL-12, can be primed by interferon-γ (IFN-γ) to produce optimal amounts of IL-12 in response to LPS stimulation as a consequence of bacterial infection. The priming effect is exerted primarily at the transcriptional level on the p40 promoter in conjunction with the effects of LPS, possibly by inducing specific transcription factors, which individually have no direct effect but which cooperatively can activate the promoter. We examined in detail one of these DNA-protein interactions observed around an Ets-2 element situated at −211/−207 of the p40 promoter, which is known to be a functionally critical site. This region interacts with a nuclear complex termed F1 that appears to be highly inducible by either IFN-γ treatment for 16 h or lipopolysaccharide stimulation for 8 h. F1 binding to the Ets-2 site requires a considerable amount of spacing around the Ets-2 site, as revealed by gel mobility shift and in vitro methylation assays. Supershift experiments and DNA affinity purification indicated that both Ets-2 and a novel, antigenically related protein with an approximate molecular mass of 109 kDa are part of the F1 complex, together with additional components including IRF-1 and c-Rel. This novel protein is designated GLp109 for its inducibility by IFN-γ or lipopolysaccharide. Its possible role in the activation of the IL-12 p40 promoter is discussed.

Interleukin-12 (IL-12) consists of a heavy chain (p40) and a light chain (p35) linked covalently to give rise to a heterodimeric (p70) molecule. IL-12 is produced by phagocytic cells and other antigen-presenting cells in response to stimulation by a variety of microorganisms as well as their products. IL-12 mediates its biologic activities mainly through T and NK cells by inducing their production of interferon-γ (IFN-γ), which augments their cytotoxicity, and by enhancing their proliferative potential. Through these functions, IL-12 plays a critical role in the early inflammatory response to infection and in the generation of T helper type 1 Th-1 cells, which favor cell-mediated immunity.

We previously (1) reported that both the p35 and p40 genes are regulated primarily at the transcriptional level by LPS and IFN-γ in human monocytes. The human IL-12 p40 gene promoter cloned in our laboratory as a 3.3-kb genomic fragment carries sufficient sequence information to confer transient cell type-specific expression and can respond to priming by IFN-γ followed by LPS stimulation in monocytic cells, in analogy to the endogenous promoter. One of the critical regions in the human IL-12 p40 promoter resides at −222/−204, as determined by 5′ deletion coupled with transient transfection in a murine macrophage-like cell line RAW264.7 (3). The promoter construct deleted to −222 still retains about 50% of the 3.3-kb promoter activity induced by the combination of LPS and IFN-γ, suggesting that additional upstream regulatory elements exist. Further deletion of the promoter down to −204 abolishes most but not all of the activity in response to LPS and IFN-γ, indicating that a downstream element may be responsible for this persistent inducibility. One of these potential cis elements may be the NFκB half-site located at −117 to −107, which has recently been characterized as being responsible for response to LPS stimulation in the murine macrophage J774 cell line (2). Within the −222 and −204 region, there is an Ets-like element, TTTCCCT or AGGAAA for its complement, between −212 and −207. The GGAA motif has been established as the consensus motif for the Ets family of transcription factors (3, 4). We showed that this Ets-like element in the p40 promoter is essential for the response of the promoter to LPS and IFN-γ stimulation, since a deletion of 5 out of 6 nucleotides from this element in the context of the entire 3.3-kb p40 promoter resulted in the loss of inducibility by LPS and IFN-γ; the element is also essential for activation of the promoter by Ets-2 (not Ets-1 or PU.1), since Ets-2 expressed from a cotransfected vector failed to activate the p40 promoter with the Ets-2 element deleted. However, activation of the p40 promoter by exogenous Ets-2 in the transient transfection system still depends on LPS or IFN-γ stimulation, suggesting the presence of additional inducible factors that may account for the activation of the IL-12 p40 promoter by IFN-γ and LPS.

In the present study, we investigated DNA-protein interactions at this element and analyzed some of the nuclear factors that interact with this region in monocytic cells primed with IFN-γ followed by stimulation with LPS. We describe here a nuclear factor, termed GLp109, which is inducible by either IFN-γ or LPS and which is part of a complex including IRF-1 and possibly NFκB c-Rel that interacts with the Ets-2 element in the p40 promoter.
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MATERIALS AND METHODS

Reagents

Murine IFN-γ was a generous gift from Dr. Gianni Garotta (Human Genome Sciences, Inc., Rockville, MD). LPS was purchased from Sigma. All antibodies used in supershift and Western blot experiments were purchased from Santa Cruz Biotechnology, Inc.

Nuclear Extract Preparation

Nuclear proteins were isolated from RAW and other cell lines according to Dignam et al. (5).

Gel Electrophoretic Mobility Shift Assay (EMSA)

End-labeled DNA probes (10,000 cpm/sample) were mixed with 2 μg of crude nuclear extracts and incubated at room temperature for 20–30 min in the presence of 1 μg of poly(dI-dC) (Boehringer Mannheim) in a volume of 10 μl of 0.5 × dialysis (D) buffer (10 mM Hepes, pH 7.9, 10% glycerol, 50 mM KCl, 0.1 mM EDTA, 0.25 mM dithiothreitol, 0.25 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin A). The mix was then fractionated through a 4% polyacrylamide gel in buffer containing 6.7 mM Tris-HCl, pH 7.5, 3.3 mM sodium acetate, 0.1 mM EDTA for 1.5 h at 200 V. The gel was dried and exposed in a PhosphorImager storage screen (Molecular Dynamics) and scanned. Supershift experiments were carried out by preincubating the nuclear extract with 1–2 μg of affinity-purified polyclonal antibodies for 30 min at 4 °C before the probe was added.

Methylation Interference

In vitro methylation interference assay was performed as described (30).

Western Blot

Reducing SDS-PAGE (10%) was performed according to Laemmli (6) with 20–30 μg of nuclear proteins in each sample. The gel was then electroblotted in Tris-glycine buffer containing 40% methanol onto a nitrocellulose membrane (Trans-blot, Bio-Rad). After blocking the membrane with TBS-T buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 5% milk powder for 1 h at room temperature, primary antibodies (rabbit anti-mouse IgG) were added at 1:1000 dilution (1 ng/ml) in TBS-T containing 1% milk powder for 1 h at room temperature. The membrane was washed three times for 7 min each with TBS-T, and incubated with secondary antibody (goat anti-rabbit IgG conjugated to horseradish peroxidase, Amersham) at 1:5000 dilution for 1 h at room temperature. After washing three times in TBS-T for 5 min each, and once in TBS for 5 min, the membrane was drained briefly and subjected to the enhanced chemiluminescence (ECL) detection procedure (Amersham).

DNA Affinity Purification

Annealing of Complementary Oligonucleotides—Equimolar amounts of two complementary oligonucleotides were mixed at a final concentration of 1 mg/ml in 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, heated at 90 °C for 2 min, and brought to room temperature over a period of 30 min to 1 h. The upper strand oligonucleotide represents the human IL-12 p40 promoter sequence −289 to −196, and the lower strand, −292 to −196. The control DNA ligand has the same upper strand, but the lower strand spans −245 to −196.

Biotin Tailing of the DNA Ligand—Double-stranded synthetic DNAs above were biotinylated at the 3′ end using terminal deoxynucleotidyl transferase and biotin-16-dUTP following the manufacturer’s protocol (Boehringer Mannheim). To monitor efficiency of biotinylation (generally 65–70%), 5 ng, 560,000 cpm of [32P]ATP 5′-end-labeled upper strand was included in the mix.

Coupling of Biotinylated DNA to Streptavidin-Agarose—Streptavidin-agarose slurry (Life Technologies, Inc.) was preabsorbed with 1 mg/ml BSA in 2 h at room temperature with constant agitation. Excess BSA was removed by a brief spin followed by the removal of supernatant, and the slurry was reconstituted in 0.5 × D buffer (see above). Biotinylated DNA (30 μg) was then mixed with 20 μl of the reconstituted streptavidin-agarose slurry in 0.5 × D buffer overnight with constant mixing at 4 °C. Efficiency of the coupling reaction (generally ~95%) was monitored based on the radioactivity derived from the 32P-end-labeled DNA ligand.

Purification of Nuclear Extracts—DNA ligand (30 μg) coupled to streptavidin-agarose in 20 μl of 0.5 × D buffer was mixed with 20 μg of poly(dI-dC) (Boehringer Mannheim) and 60 μg of RAW cell nuclear extract in 60 μl of D buffer, in a final volume of 140 μl for 30 min at room temperature with frequent gentle vortexing. The mixture was spun 10 s in an Eppendorf centrifuge, and the supernatant was collected (S1 fraction). After washing the resin with 50 μl of D buffer followed by centrifugation, supernatant was again collected (S2 fraction). The next four washes were: 50 μl of D buffer, 0.2 M KCl (S3); 50 μl of D buffer, 0.4 M KCl (S4); 50 μl of D buffer, 0.4 M KCl and 0.5% SDS (S5); and 50 μl of D buffer, 0.4 M KCl, and 0.5% SDS, 1% β-mercaptoethanol (S6). Each fraction (25 μl) was analyzed by SDS-PAGE and Western blot assay. The resin was regenerated by 10 washes with 0.5 × D buffer, reabsorbed with 1 mg/ml BSA, reconstituted in 0.5 × D buffer, and stored at 4 °C.

RESULTS

Sequence Comparison of the Human and Murine IL-12 p40 Promoters—The human IL-12 p40 gene promoter was recently cloned as a 3.3-kb genomic fragment. Sequence comparison of the proximal promoters of the human and the murine IL-12 p40 genes (Fig. 1) shows that the two promoters are quite homologous up to about −400 with respect to the transcription initiation site, where the homology breaks down with large gaps between them. Within the −400 proximal promoter region, several putative transcription factor binding sites are very well conserved: Ets-2, PU.1, and an NFXB-like element, which has been postulated to be a critical response element in the murine p40 promoter for LPS stimulation in macrophagic J774 cells (2).

Several Nuclear Factors Including Ets-2 Interact with the −292/−196 Region—To test whether the −222/−204 region interacts with any nuclear factors that are induced by IFN-γ and LPS, an oligonucleotide probe was made that spanned −222 to −196. The probe was constructed to include sequences downstream from −204 because the Ets-2-like element is quite close to −204 and thus might require additional space for the stabilization of potential DNA-protein interactions at this putative site. However, this probe did not yield any significant binding with either unfractionated nuclear extracts derived from stimulated RAW cells or with recombinant Ets-2. The only detectable binding with this probe was observed with unstimulated RAW nuclear extract. The complex was designated F3 (Fig. 2). When the probe was extended upstream to −292, it bound a low mobility nuclear complex (named F1) in RAW cells stimulated with either LPS or IFN-γ (Fig. 2). The F1 binding activity was first detected around 2 h after LPS stimulation and peaked at 8 h post-stimulation, coinciding with the transient p40 promoter activity in RAW cells (data not shown). Deletion from the 5′ end to −265 or −243 abrogated F1 binding, but also resulted in the appearance of a second factor, designated F2, which was inducible by IFN-γ but not by LPS, and which was induced synergistically by the combination of IFN-γ and LPS (Fig. 2). F3 binding was also detected with the −243/−196 probe in the unstimulated RAW cell nuclear extract (Fig. 3). Deletion from the 3′ end to −227 also resulted in the loss of all binding (data not shown), suggesting that both the 5′ and 3′ ends of the −292/−196 region are essential for F1 binding.

We first focused on the F1 complex, reasoning that F1 might contain F2 and that F3 does not appear to be part of F1 and might be a negative regulator of the p40 promoter. To establish the specificity of F1, competitive EMSA was performed using a number of homologous and heterologous oligonucleotides (Fig. 3A). The F1 complex present in IFN-γ- and LPS-stimulated RAW cells was efficiently competed off by a 20-fold molar excess of the −292/−196 DNA, but not by the consensus Ets-2 (Ets2-CS) oligonucleotide, or by the −222/−196 oligonucleotide containing the Ets site of the p40 promoter, or by the −735/−710 oligonucleotide of the p40 promoter containing an IRF-1-like element. The consensus IRF-1 oligonucleotide (IRF1-CS) did compete somewhat, although the efficiency was consider-
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Fig. 1. Comparison of the human and mouse IL-12 p40 promoters. The proximal sequences of the human (upper line) and mouse (lower line) IL-12 p40 promoters are shown with coordinates relative to their respective transcription initiation sites (horizontal arrows) determined by primer extension. Location of the exon-intron boundaries (vertical arrows) are inferred by comparing genomic and cDNA sequences. Putative transcription factor motifs are boxed.

Fig. 2. Nuclear factor binding to the −292/−196 region of the IL-12 p40 promoter. Nuclear extracts were isolated from RAW cells either unstimulated (medium) or stimulated with IFN-γ (1000 units/ml) for 16 h, LPS (1 μg/ml) for 8 h or IFN-γ for 8 h followed by LPS 8 h in the presence of IFN-γ. Nuclear extracts were mixed with the −292/−196, −243/−196, or −222/−196 probes and analyzed by electrophoretic mobility shift assay (EMSA). The sequences of the three 5′ deletion fragments (−292/−196, −243/−196, and −222/−196) used in the experiment are shown at the bottom with the Ets element boxed.
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Figure 3. Competitive and supershift EMSA. EMSA was performed with the −292/−196 probe and the RAW cell nuclear extract stimulated with both IFN-γ and LPS. A, various competitor DNAs (oligonucleotides) were mixed with the probe in a 20-fold molar excess of the competitor. B, prior to the addition of the probe, nuclear extract was mixed with affinity-purified polyclonal antibodies directed against the indicated transcription factors. After a 30-min incubation, the probe was added and incubation continued for an additional 30 min before electrophoresis. C, specificity of the anti-Ets-2 antibody. A 10-fold molar excess of the blocking peptide for anti-Ets-2 was added simultaneously.

DNA affinity column was prepared using the anti-Ets-2 antibody and nuclear extract. Excess of the blocking peptide for anti-Ets-2 was added simultaneously. The double-stranded DNA fragment was biotinylated and bound to streptavidin-agarose. Nuclear extracts from IFN-γ-stimulated RAW cells were mixed with the methylated probe, and the resulting F1 complex was excised from a native gel, electroeluted, and cleaved with piperidine, and resolved on a denaturing sequencing gel with sequence ladders of the same DNA fragment and a primer corresponding to the most 5′ end (~471) of this fragment. In the BSA lane, no F1 complex is present and the unbound probe was excised. The region of interest (~196 to −292) is indicated by the location of the two ends.

FIG. 4. Methylation interference assay. DNA methylation interference assay was performed with a probe spanning −196 to −471. Nuclear extracts from RAW cells treated as indicated were mixed with the methylated probe, and the resulting F1 complex was excised from a native gel, electroeluted, and cleaved with piperidine, and resolved on a denaturing sequencing gel with sequence ladders of the same DNA fragment and a primer corresponding to the most 5′ end (~471) of this fragment. In the BSA lane, no F1 complex is present and the unbound probe was excised. The region of interest (~196 to −292) is indicated by the location of the two ends.

stimulated RAW cells were mixed with the column resin, and flow-through and subsequent washes in increasing stringency were collected and subjected to reducing SDS-PAGE followed by Western blotting using α-Ets-2. The same nuclear extracts were subjected to a identical purification scheme with a control DNA column, which is identical to the −292/−196 probe in the sense strand, but different in the antisense strand in that it spanned −243 to −196. This probe was first tested for its inability to bind F1 before use. Nuclear extracts from unstimulated, IFN-γ-, LPS-, and IFN-γ + LPS-stimulated RAW cells were also directly analyzed by Western blot without purification (Fig. 5A). In unstimulated cells, the antibody detected only one strong band of about 54 kDa, the known size of Ets-2 (4). Upon induction with IFN-γ or LPS, two additional larger bands were detected, one of −58 kDa, the other −109 kDa. Stimulation with LPS resulted in yet more bands, notably at 52, 119, and 165 kDa. Results of bacterial alkaline phosphatase treatment suggested that the 52-kDa protein was likely to be the phosphorylated form of the 54-kDa Ets-2 (data not shown). The reason for the increased mobility of the 54-kDa Ets-2 upon phosphorylation is not clear. The 58- and 109-kDa proteins were induced with either LPS or IFN-γ, and are designated GLp58 and GLp109, respectively. GLp58 is likely an alternative form of Ets-2, since it is also present in recombinant murine Ets-2 translated from rabbit reticulocyte lysate. However, it is unlikely to be a phosphorylated form of the 52-kDa Ets-2, since bacterial alkaline phosphatase did not change its mobility (data not shown). The 119- and 165-kDa proteins were induced only by LPS, and are thus designated Lp119 and Lp165, respectively. Bacterial alkaline phosphatase treatment of these proteins also did not result in any mobility changes (data not shown), suggesting that they are probably not derived from phosphorylation at threonine, serine, or tyrosine residues. In addition, the specificity of the anti-Ets-2 antibody was confirmed by including the epitope peptide with the primary antibody in the procedure. The Ets-2 peptide to which the anti-Ets-2 antibody was directed specifically blocked the binding of
been reported that IFN-g, which is induced upon stimulation with IFN-related molecule in unstimulated RAW cells (F3), diminished upon stimulation of RAW cells with IFN-g, consistent with our observations that F3 (PU.1) binding is down-regulated by IFN-g which is certainly repressed by PU.1 binding to the PU box domain in its promoter. This site is essential for induction of the promoter by Ets-2 site located at 212 to 207 in the human IL-12 p40 promoter. We examined the physical protein-DNA interactions at the proximal promoters, suggesting their functional significance. We show here that several putative sequence motifs are highly conserved between the mouse and the human IL-12 p40 proximal promoters, suggesting their functional significance. We examined the physical protein-DNA interactions at the Ets-2 site located at -212 to -207 in the human IL-12 promoter. This site is essential for induction of the promoter by IFN-g and LPS in RAW cells and for the response of the promoter to activation directly by Ets-2. Although this sequence in the form of a 27-mer oligonucleotide with 10 base pairs flanking either side of the core motif TTTAAT (AGGAAA for the complement) failed to bind either recombinant Ets-2 or nuclear extracts derived from IFN-g- or LPS-stimulated RAW 264.7 cells. However, this probe did interact with PU.1 or a related molecule in unstimulated RAW cells (F3). F3 is diminished upon stimulation with IFN-g and LPS.

The functional role of F3 remains to be established. It has been reported that IFN-g, a cytokine that induces the expression of major histocompatibility complex class II molecules, down-regulates the expression of PU.1 (7), which is certainly consistent with our observations that F3 (PU.1) binding is diminished upon stimulation of RAW cells with IFN-g. Furthermore, Borras et al. (8) showed that I-Ag gene expression is repressed by PU.1 binding to the PU box domain in its promoter in both bone marrow-derived macrophages and the mouse B cell line A20-2J.

**DISCUSSION**

We show here that several putative sequence motifs are highly conserved between the mouse and the human IL-12 p40 proximal promoters, suggesting their functional significance. We examined the physical protein-DNA interactions at the Ets-2 site located at -212 to -207 in the human IL-12 promoter. This site is essential for induction of the promoter by IFN-g and LPS in RAW cells and for the response of the promoter to activation directly by Ets-2. Although this sequence in the form of a 27-mer oligonucleotide with 10 base pairs flanking either side of the core motif TTTAAT (AGGAAA for the complement) failed to bind either recombinant Ets-2 or nuclear extracts derived from IFN-g- or LPS-stimulated RAW 264.7 cells. However, this probe did interact with PU.1 or a related molecule in unstimulated RAW cells (F3). F3 is diminished upon stimulation with IFN-g and LPS.

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**FIG. 5.** Western blot analysis of RAW cell nuclear extracts with anti-Ets-2. A, nuclear extracts from RAW cells stimulated as indicated were analyzed by SDS-PAGE under reducing conditions. The gel was blotted and probed with the anti-Ets-2 antibody. Molecular weight markers are indicated on the left of the panel, and the various proteins described in the text are indicated by the arrows. B, specificity of anti-Ets-2 antibody. Western blot was performed as described in A using the anti-Ets-2 antibody and nuclear extracts from RAW cells stimulated as indicated. In the left panel, only the antibody was used; in the middle panel, the anti-Ets-2 antibody was mixed with a 20-fold molar excess of Ets-2 blocking peptide, i.e. the peptide to which the antibody was directed; and in the right panel, the antibody was mixed with a 20-fold molar excess of a second peptide directed against another antibody: Ets-1/2, which recognizes a separate epitope shared between Ets-1 and Ets-2. Arrows indicate the major Ets-2-related proteins.

**FIG. 6.** DNA affinity purification of F1. DNA affinity resins were made with either -292/-196 DNA or control DNA by biotinylation and subsequent coupling to streptavidin-agarose. Nuclear extracts from IFN-g- and LPS-stimulated RAW cells were mixed with the resin and flow-through, and subsequent washes in increasing stringency were collected and subjected to reducing SDS-PAGE followed by Western blotting using anti-Ets-2. A, the antibody was mixed with a 20-fold molar excess of a second peptide, Ets-1/2, which represents a separate epitope common to both Ets-1 and Ets-2. B, only the antibody was used; in the left panel, the antibody was mixed with a 20-fold molar excess of one antibody: Ets-2, had no effect on the action of the anti-Ets-2 antibody (Fig. 5B). When the fractions eluted from the DNA columns were examined by SDS-PAGE and Western blotting using α-Ets-2, most of the proteins that reacted with the antibody, namely, the 52-, 54-, 58-, 109-, 119-, and 165-kDa proteins, were present in the flow-through of the control DNA column, whereas the same proteins were bound tightly on the column and were eluted only by a combination of 0.4 M KCl, 1% SDS, and β-mercaptoethanol (Fig. 6), suggesting that these proteins specifically bound to the -292/-196 DNA.

Extension of the probe further upstream to -292 (the -292/-196 probe) resulted in a readily formed high molecular weight complex with extracts from either IFN-g- or LPS-induced RAW cells, but not from unstimulated cells. The apparent discrepancy between the ability of the -292/-196 region to bind F1 and its inability to enhance significantly the promoter activity over that of the -222/-196 region led us to test the possibility that F1 binding may require larger physical space than does the -222/-196 site to stabilize the DNA-protein complex due to its large size. We anchored the -222/-196 region in the context of the polylinker region of the PCRII plasmid (Invitrogen). This sequence was then excised along with varying lengths of the polylinker sequence. These fragments were used...
in EMSA with nuclear extracts from IFN-γ/LPS-induced RAW cells. Two requirements for F1 binding were established. First, the length of the probe has to be more than 66 base pairs. Second, the TTTCCT sequence is critical for F1 binding (data not shown). This indicates that F1 binding is specific for the Ets-2 site but requires large physical spacing around the core motif, suggesting that F1 may be a large complex composed of multiple factors. Indeed, when the −292/−196 probe was deleted to −243/−196, F1 binding was diminished and F2 appeared, which responded to IFN-γ stimulation more than to LPS.

In vitro methylation and footprint experiments to examine the −292/−196 region indicated that the complex induced by either IFN-γ or LPS covered quite extensive sequences. In vivo footprint assay also revealed strong and extensive protection around this region in either RAW cells or primary macrophages primed with IFN-γ followed by LPS stimulation (data not shown). Together, the data suggest that the region including and around the Ets-2 site actively interacts with a series of nuclear factors that may be involved in the activation of the IL-12 p40 promoter in response to specific stimulation.

The first indication of the presence of Ets-2 or its derivatives in the F1 complex came with supershift experiments in which an anti-Ets-2 polyclonal antibody was able to retard the mobility of the F1 complex. The F1 complex can also be supershifted by two IRF-1 antibodies directed against a very homologous epitope of human and mouse origins, respectively, as well as by the anti-NFκB C-Rel antibody, suggesting that F1 may be a highly sophisticated complex containing members of the Ets, IRF, and NFκB families of transcription factors. By contrast, the F2 complex was not affected at all by any of the antibodies whose antigens are well known IFN-γ-inducible factors (IRF-1, IRF-2, STAT-1, and STAT-4), indicating that F2 might be yet another new factor which responds to IFN-γ-stimulation. When nuclear extracts containing the F1 complex were partially puriﬁed through a DNA afﬁnity scheme using the −292/−196 sequence as the ligand together with Western blot analysis using the anti-Ets-2 antibody, several proteins antigenically related to Ets-2 were revealed. First, the 54-kDa Ets-2 protein seemed to be constitutively present in RAW cells. Upon induction with IFN-γ or LPS, a 52- and a 58-kDa protein appeared, which probably represent derivatives of Ets-2, since both of these species were also found in recombinant Ets-2 translated in vitro in rabbit reticulocyte extracts. In fact, the 54-kDa protein was shown to be derived from the 52-kDa protein after alkaline phosphatase treatment. In addition to the 52-, 54-, and 58-kDa proteins, which may be derived directly from Ets-2 via posttranslational modification, three other proteins were also identiﬁed in RAW cells: GLp109, Lp119, and Lp165, which responded to IFN-γ or LPS.

The form in which Ets-2 in RAW cells interacts with the Ets-2 site at −212 and −207 is presently not clear. However, several observations suggest that Ets-2 binds to that site only as part of a large complex. First, recombinant Ets-2 does not bind the −222/−196 probe, but does bind a consensus Ets-2 probe. Second, RAW cell nuclear extracts contain some binding activities which interact with the consensus Ets-2 probe constitutively (data not shown); this cannot account for the inducible binding of Ets-2 in the above complex. Third, DNA afﬁnity puriﬁcation of the F1 complex showed that Ets-2 is indeed tightly associated with several other Ets-2-related proteins. Finally, we have shown also data indicating the presence of IRF-1 and NFκB C-Rel in the F1 complex. Together, these data strongly suggest a multi-component nature of the F1 complex.

The Ets proteins contain a structural motif (winged helix-loop-helix) that is capable of binding DNA as a monomer. However, the DNA binding activity of several members of this family appears to be suboptimal in the absence of a partner protein. For example, Elk-1 activates transcription from the c-fos promoter in conjunction with the DNA-binding protein serum response factor. A speciﬁc protein–protein interaction stabilizes the binding of Elk-1 and serum response factor on the c-fos promoter (9–12). In the case of GA-binding protein (GABP), which is composed of an a subunit with an Ets domain and a non-DNA-binding b subunit, two a and two b subunits form a stable tetramer with augmented DNA binding activity on the herpes simplex virus type I immediate early gene promoter (13, 14). A domain has been mapped in Elk-1 and GABPα that is important in the protein–protein interaction, and only Ets proteins bearing that domain can interact with the partner (15, 16). Ets-1 and Ets-2 have also been reported to cooperate with several different putative partners in functional studies. Ets-1 works with Sp1 in activating transcription from the human T-cell lymphotropic virus type 1 enhancer (17), while on the immunoglobulin μ heavy chain enhancer, Ets-1 cooperates with PU.1 (18). Ets-1 also synergizes with AP-1 in activating the poliovirus enhancer (19). Myb and Ets-2 cooperate in activating the mim-1 promoter (20). In the Moloney murine leukemia virus and T cell receptor β-chain enhancers, adjacent binding sites for Ets and the poliovirus enhancer-binding protein-2 (PEBP2/core-binding factor (cbf) family of proteins are both required to constitute a phorbol ester response element (21). The B cell and macrophage-speciﬁc transcription factor PU.1 recruits the binding of a second B cell-restricted nuclear factor, NF-EM5, to a DNA site in the immunoglobulin κ 3 enhancer. DNA binding by NF-EM5 requires a protein–protein interaction with PU.1 and speciﬁc DNA contacts. Phosphorylation of PU.1 at Ser148 is necessary for interaction with NF-EM5 and for regulating transcriptional activity (22, 23). A recent study using radiolabeled PU.1 protein as a probe to screen a B cell cDNA expression library in search of PU.1-interacting proteins identiﬁed three DNA-binding proteins (NF-IL6δ, HMG-I(Y), and SSRP), a chaperon protein, and a multifunctional phosphatase (24). The physical interaction between PU.1 and NF-IL6δ requires the carboxyl-terminal 28 amino acids of PU.1. PU.1 and NF-IL6δ can bind adjacent sites simultaneously. In transient transfection assays, PU.1 and NF-IL6δ can functionally cooperate to activate transcription synergistically. A direct physical association between Ets-1 and AP-1 has been demonstrated in normal human T cells (25). This interaction is mediated by the binding of the basic domain of Jun to the Ets domain of Ets proteins. The associated Jun is capable of interacting with Fos family members to form a trimolecular protein complex. The physical association of Ets-1 and AP-1 is required for the transcriptional activity of enhancer elements containing adjacent Ets and AP-1 binding sites.

Our observation of the presence of IRF-1 and NFκB C-Rel in the F1 complex is not surprising. NFκB regulates a wide range of genes involved in immune function and inﬂammation responses. The interferon regulatory factor (IRF) family, on the other hand, plays a vital role in gene regulation mediated by interferons (type I and type II). Interactions between NFκB, HMG-I(Y), and an Ets-like protein (Elf-1) reportedly regulate expression of the IL-2 receptor α gene (26). The promoter of the VCAM-1 gene contains two essential NFκB sites, which are not sufﬁcient for activation, and may require an IRF-1 site that is located 3′ to the TATA box. Furthermore, IRF-1 physically interacts with NFκB subunits, and its binding is stimulated by the binding of NFκB and HMG-I(Y) (27). Based on the studies of the IFN-β gene regulation, Maniatis and colleagues have...
proposed a model in which interactions between bZip proteins (ATF-2 and c-Jun), IRF-1, HMG-I(Y), and NFκB form a higher order complex, which is required for full activation of the IFN-β promoter (28, 29).

In summary, our data strongly suggest that multiple nuclear factors induced by IFN-γ or LPS interact with the Ets-2 element of the IL-12 p40 promoter in the form of a large complex (F1). This complex consists of Ets-2 and some related proteins (GLp109, Lp119, and Lp165), IRF-1 and c-Rel, and probably of additional proteins yet to be identified. The size and multi-component nature of F1 may explain its extensive physical occupancy requirement. This interaction is necessary but may not be sufficient to activate the promoter since neither IFN-γ nor LPS is capable of inducing the promoter significantly, despite the fact that F1 can be stimulated by either of them. The −292/−196 region failed to enhance the activity of a minimal promoter (28, 29).

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