A Novel Role of Sp1 and Sp3 in the Interferon-γ-mediated Suppression of Macrophage Lipoprotein Lipase Gene Transcription*

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The regulation of macrophage lipoprotein lipase by cytokines is of potentially crucial importance in the pathogenesis of atherosclerosis. We have shown previously that macrophage lipoprotein lipase expression is suppressed by interferon-γ (IFN-γ) at the transcriptional level. We investigated the regulatory sequence elements and the transcription factors that are involved in this response. We demonstrated that the −31/187 sequence contains the minimal IFN-γ-responsive elements. Electrophoretic mobility shift assays showed that the binding of proteins to two regions in the −31/187 sequence was reduced dramatically when the cells were exposed to IFN-γ. Both competition electrophoretic mobility shift assays and antibody supershift assays showed that the interacting proteins were composed of Sp1 and Sp3. Mutations of the Sp1/Sp3-binding sites in the minimal IFN-γ-responsive elements abolished the IFN-γ-mediated suppression of promoter activity, whereas multimers of the sequence were able to impart the response to a heterologous promoter. Western blot analysis showed that IFN-γ reduced the steady state levels of Sp3 protein. In contrast, the cytokine decreased the DNA binding activity of Sp1 without affecting the protein levels. These studies therefore reveal a novel mechanism for IFN-γ-mediated regulation of macrophage gene transcription.

Lipoprotein lipase (LPL1; EC 3.1.1.34) plays a central role in lipid metabolism and transport by catalyzing the hydrolysis of the triacylglycerol component of lipoprotein particles, thereby providing non-esterified fatty acids and 2-monacylglycerol for tissue utilization (1). LPL is expressed by the parenchymal cells of several extrahepatic tissues and is subject to regulation through the action of hormones, cytokines, and lipid metabolite products (2). The LPL expressed by macrophages is of major importance because of its crucial role in atherogenesis (see Ref. 3 for a recent review). LPL is expressed in the lesion where macrophage-derived foam cells represent the predominant site for the synthesis of the enzyme (4, 5). In addition, inbred murine strains with elevated levels of macrophage LPL show an increased susceptibility to atherosclerosis (6). More recently, the importance of LPL in the promotion of foam cell formation and atherosclerosis in vivo has been substantiated by three independent transplantation studies in irradiated mouse model systems using donor macrophages from different backgrounds (7–9). In those mice receiving macrophages from homozygous and heterozygous LPL-deficient donors, the mean lesion area of diet-induced atherosclerosis was reduced substantially compared with those receiving macrophages that express LPL normally (7–9). Further support for a pro-atherogenic role of macrophage LPL has been provided by Clee et al. (10) through an alternative approach. Such an atherogenic role of LPL predominantly involves a non-catalytic bridging action in which the enzyme serves as a ligand for mediating the interaction of lipoproteins to cell surface receptors and/or proteoglycans and their subsequent uptake by the cells (3).

The cellular changes in the vascular wall during the initiation and the development of atherosclerosis, including the transformation of macrophages into foam cells, are affected by many factors that are known to be present in the lesion, such as cytokines, growth factors, and modified lipoproteins (11, 12). The action of such factors on the expression of macrophage LPL has been implicated in the modulation of the atherosclerotic process (3) and has, therefore, been studied in detail in several laboratories including our own. Macrophage LPL expression is induced by platelet-derived growth factor, macrophage colony-stimulating factor, glucose, and activators of peroxisome proliferator-activated receptors and is suppressed by certain cytokines (13–17). Among these cytokines, interferon-γ (IFN-γ) possesses a unique ability both to prime macrophages and to synergize with other mediators in the regulation of LPL (18–19).

IFN-γ plays an important and complex role in atherogenesis with both pro- and anti-atherogenic actions being reported. IFN-γ-receptor/apoE double knockout mice show reduced lesion formation (20). In addition, IFN-γ stimulates the expression of vascular cell adhesion molecule on endothelial cells and class II major histocompatibility antigens in macrophages and smooth muscle cells (21, 22). On the other hand, IFN-γ decreases collagen synthesis on smooth muscle cells, blocks smooth muscle cell proliferation, and inhibits macrophage foam cell formation by both preventing the oxidation of low density lipoproteins (LDL) and suppressing the expression of several lipoprotein receptors, including type A scavenger receptor, very low density lipoprotein receptor, LDL receptor-related protein, and scavenger receptor CD36 (23–30).

In the light of the IFN-γ-mediated inhibition of the expression of several genes in macrophages that are involved in lipoprotein uptake, a detailed understanding is necessary of
the mechanisms that are in operation. This will not only better our understanding of the molecular basis of foam cell formation and atherogenesis but, in the longer term, may also lead to the identification of novel targets for therapeutic intervention.

With respect to macrophage LPL expression, we have demonstrated previously (31) that the IFN-γ action is mediated at the transcriptional level. We show here that IFN-γ decreases macrophage LPL gene transcription through a reduction in the binding of the transcription factor Sp1, and the related member Sp3, to the promoter region. In addition, we identify the potential mechanisms that are responsible for this action of IFN-γ on Sp1 and Sp3. The studies identify a novel mechanism for IFN-γ-regulated gene transcription.

**EXPERIMENTAL PROCEDURES**

**Reagents**—The human myeloid leukemia U937 and the mouse macrophage J774.2 cell lines were from the European Collection of Animal Cell Cultures, and the rat alveolar macrophage NR8383 cell line was obtained from the American Collection of Animal Cell Cultures. Recombinant human and mouse IFN-γ was from PeproTech. All the cell culture reagents were purchased from Greiner, Helena Biosciences, or Invitrogen. Anti sera against Sp1 and Sp3 were from Santa 

**Cell Culture**—The cell lines were maintained in either Dulbecco's modified Eagle's medium (J774.2), RPMI 1640 (U937), or Ham's F-12 medium (NR8383), which was supplemented with 10% (v/v) heat-inactivated fetal calf serum (HI-FCS; 56 °C, 30 min), 100 units/ml penicillin, and 100 µg/ml streptomycin. The cultures were maintained at 37 °C in a humidified atmosphere containing 5% (v/v) CO₂ in air. Alveolar macrophages were isolated from in-house-bred adult male Sprague-Dawley rats by bronchoalveolar lavage (32, 33). Alveoli were┌───────────────────────────────────────────────────────────────────────────────────────────────────────────────────────────────────────────────────────────────────────────────────────────────────┐

Before stimulation with IFN-γ, the cells were preincubated for 4 h in medium containing reduced (0.5%) HI-FCS (17–19). For experiments involving the use of apigenin or emodin, the inhibitors were added to the cells 1 h before the addition of IFN-γ (i.e., pretreatment).

**LPL Activity Assay**—**Assay and Northern Blot Analysis**—The heparin-releasable LPL activity in conditioned medium was determined as described previously (17). Total RNA was prepared from cells using Tri-Reagent LS (Molecular Research Center) according to the manufacturer's instructions. Samples of RNA (15 µg) were size-fractionated by electrophoresis on denaturing agarose gels, transferred to nylon membranes (38), and hybridized to radiolabeled LPL or β-actin cDNA inserts, as described previously (37–39).

**Preparation of Manipulated LPL Promoter-Reporter DNA Constructs**—The deletion series, containing 5'-truncations of the LPL promoter that are linked to the luciferase reporter gene in the vector p19, was a generous gift from Dr. J. M. Gimble (34). The other LPL promoter-luciferase DNA constructs were prepared using PCR as described previously (35–37). The PCR products were used to generate transfected promoter-luciferase DNA constructs and 0.5 µg of cytomegalovirus-β-galactosidase plasmid as an internal control for transfection efficiency (35–37), and 8 µl of Superfect™ were then prepared as described by the manufacturer and added to the wells. These were then differentiated into macrophages for 12 h using phorbol 12-myristate 13-acetate (PMA, 1 µM) or dexamethasone (10 µM) in the presence of IFN-γ (1,000 units/ml) for 48 h. The luciferase and the β-galactosidase activities in the cell extracts were then determined using commercially available kits (Promega). The luciferase activity was normalized to the β-galactosidase values (35–37), with each transfection being carried out in triplicate and repeated at least three times.

**Western Blot Analysis**—**Nuclear and whole cell extracts were prepared as described previously (38). Protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1 µg/ml pepstatin A, 10 µg/ml aprotinin, 10 µg/ml 1-S soybean trypsin inhibitor) and dithiothreitol (0.5 mM) were added to all the buffers before use. The concentration of proteins in the extracts was determined using the microBCA protein assay kit as described by the manufacturer (Pierce).

**Electrophoretic Mobility Shift Assays (EMSA)**—EMSAs were carried out essentially as reported previously (36, 40–43). The sequences of the oligonucleotides used for EMSA are as follows: 31-1/5, 5'-GTCACCT-
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RESULTS

IFN-γ Suppresses LPL mRNA Expression and Enzymatic Activity in a Range of Macrophage Sources—We have shown previously (17) that IFN-γ produces dose-dependent reductions in the enzymatic activity, mRNA expression, and protein levels of LPL in the murine macrophage J774.2 cell line. In addition, we have shown that IFN-γ produces a time-dependent reduction in LPL enzymatic activity in these cells (17). To investigate whether there were similar changes at the level of LPL mRNA expression, time course Northern blot analysis was carried out. As shown in Fig. 1A, a time-dependent decrease in steady state LPL mRNA levels was observed with the profile and overall extent of decrease being similar to that seen at the level of enzymatic activity (17).

The IFN-γ-mediated decrease in LPL activity and mRNA expression in J774.2 macrophages was of ~60% and seen only after a 24 h incubation period (Fig. 1A and Ref. 17). To evaluate whether a similar profile also occurred in other macrophage sources, the dose-response experiments on LPL enzymatic activity were repeated with primary cultures of rat alveolar macrophages using the rat alveolar macrophage NR8383 cell line for comparison. As shown in Fig. 1, the rat alveolar macrophage NR8383 cell line was more potent at decreasing LPL activity in these cells compared with J774.2 macrophages, and additionally, the maximal reduction in activity that was produced was greater than 80%. These results, along with previous studies (30–47) in other laboratory series, confirm the dose-dependent and time-dependent regulation of LPL transcription by IFN-γ.

Identification of the Minimal Region in the LPL Promoter That Is Required for the IFN-γ Response—We have shown previously (31) that the IFN-γ-mediated decrease in LPL expression was due to a reduction in gene transcription rather than any changes in mRNA stability. To evaluate whether the LPL promoter contained sufficient information for this response, transient transfection experiments were initiated using a LPL promoter-luciferase DNA construct that contains the -187 region in the p19 vector (34). Initial experiments using J774.2 macrophages showed that, similar to the experiments in other laboratory (48), these cells could not be transfected efficiently with exogenous DNA. We therefore tested a range of monocyte/macrophage cell lines and found that the human U937 myeloid leukemia cell line could be transfected most efficiently with DNA. Indeed, these cells have been used widely to investigate the regulation of macrophage gene transcription (49–51). The LPL promoter was induced dramatically when the transfected monocytes were differentiated into macrophages using PMA (data not shown), which is consistent with the results shown in the figure.
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with previous reports (52, 53) that LPL is expressed at virtually undetectable levels in monocytes and induced transiently at the transcriptional level during their differentiation into macrophages. When the transfected monocytes were differentiated with PMA for 12 h in the presence of IFN-γ, an ~65% reduction in LPL promoter activity was observed compared with cells that were only treated with PMA (Fig. 2, construct −1824/+187). A similar reduction in LPL promoter activity was also seen when the transfected cells were first differentiated for 12 h in the presence of PMA and then exposed to IFN-γ for 12 h (data not shown). These results therefore show that the −1824/+187 LPL promoter sequence contains sufficient information for the IFN-γ response. We decided to investigate the mechanisms that are involved in this action of IFN-γ in more detail.

To map the IFN-γ-responsive elements (IFN-γREs) in the LPL promoter, a 5′-deletion series, containing a common 3′ end at position +187, was transfected into U937 cells, and the relative LPL promoter activity was determined in the absence or the presence of IFN-γ. Deletion of the −1824 to the −101 region had little effect on the basal promoter activity obtained in the absence of IFN-γ. A further deletion to −54 produced an approximate halving of this activity, which was not affected further by a truncation to −31, thereby indicating the existence of important regulatory elements between the −101 to the −54 region that are essential for basal promoter activity. However, an IFN-γ-mediated reduction in LPL promoter activity of between 52 and 67%, which was comparable with the decrease in endogenous LPL mRNA levels produced in J774.2 macrophages by this cytokine (Ref. 17 and Fig. 1A), was seen with all the LPL promoter-luciferase DNA constructs used, including the −31/+187 region (Fig. 2). This suggests that the −31 to +187 LPL promoter region contains the minimal IFN-γREs and was therefore investigated in detail. However, instead of mapping further the precise sequences that are involved in the IFN-γ response by analyzing the effect of finer deletions or specific mutations in the −31/+187 region, we decided to first investigate the interaction of proteins with this promoter region by EMSA using extracts from cells that were either untreated or exposed to this cytokine. It was hoped that such a strategy would identify sub-regions in the −31 to +187 sequence to which the interaction of DNA-binding proteins is affected following exposure of the cells to IFN-γ and therefore form the foundation for further detailed investigation. In addition, EMSA would allow analysis of the action of IFN-γ in both J774.2 macrophages, where the original detailed studies on the action of IFN-γ were carried out Ref. 17 and Fig. 1A), and U937 cells that were used for the transfection studies (Fig. 2).

The Binding of Proteins to Two Regions Within the −31/+187 Sequence Is Reduced Following Exposure of the Cells to IFN-γ—EMSA were carried out using six double-stranded oligonucleotides that spanned the −31/+187 region (−31/+8, +9/+49, +46/+90, +88/+118, +119/+160, and +159/+195). Initial experiments using both nuclear and whole cell extracts from J774.2 macrophages showed a similar DNA-protein interaction pattern (data not shown), and the latter were therefore used for all subsequent experiments. Using the +88/+118, +119/+160, and +159/+195 oligonucleotides, no DNA-protein complexes could be detected even after long autoradiographic exposures (data not shown). In contrast specific DNA-protein complexes were seen with the other three oligonucleotides with binding to the +9/+49 and +46/+90 region being consistently reduced when extracts were used from cells that were treated with IFN-γ (Fig. 3A; other data not shown). Competition EMSA were carried out to evaluate whether the binding of proteins to the +9/+49 and +46/+90 region was specific and whether these two regions interacted with identical or distinct proteins. Fig. 3B shows the results with the +46/+90 oligonucleotide with a similar profile being seen with the +9/+49 region. Thus, the DNA protein complexes could be competed by an excess of oligonucleotide containing the self +46/+90 region but not by the −31/+8 or +88/+118 sequence. Interestingly, competition was also obtained using the +9/+49 sequence, thereby indicating that the +9/+49 and +46/+90 regions bound identical or related proteins (Fig. 3B).

To determine the time course of the IFN-γ-mediated decrease in binding to the +9/+49 and the +46/+90 region, EMSA were carried out using extracts from J774.2 macrophages that were exposed to IFN-γ for 15 min and 3, 6, 12, and 24 h with untreated cells at the start and the end of the experiment being used as controls (0 and 24h-). As shown in Fig. 3C, a decrease in binding of proteins to both regions was seen after exposure of the cells for 12 h with maximal reduction being attained at 24 h. Although a slight reduction in DNA binding was seen with the +46/+90 region in this experiment using extracts from untreated cells at 24 h, this was not reproducible, and in any case, the binding was substantially greater than that seen for cells exposed to IFN-γ for 24 h. The kinetics of the IFN-γ-mediated decreases in DNA binding to the +9/+49 and the +46/+90 regions were also comparable with the decrease in endogenous LPL mRNA expression seen in J774.2 macrophages (Fig. 1A). A similar IFN-γ-mediated reduction in DNA binding was also observed when the EMSA were repeated using extracts from the U937 cell line that were exposed to PMA for 12 and 24 h, either in the absence or the presence of this cytokine (data not shown). Thus, the IFN-γ-mediated reduction in binding of factors to the +9/+49 and +46/+90 sequence was seen with extracts from two different macrophage sources that are derived from different species.

Sp1 and Sp3 Interact with the IFN-γRE in the LPL Promoter—A computer analysis of the +9 to +90 region of the promoter using the GCG and the MatInspector version 2.2 data bases (54) showed the presence of putative consensus sites for four factors that have been shown previously to regulate macrophage gene expression, C/EBP, PU.1, STAT, and Sp1. In order to investigate whether any of these factors interacted with the IFN-γREs in the LPL promoter, competition EMSA experiments were carried out using an excess of oligonucleo-
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Fig. 3. Interactions of DNA-binding proteins with sequences in the −31/+187 region. A, EMSA was carried out using radiolabeled oligonucleotides against the +9/+49 and +46/+90 sequence and extracts from J774.2 macrophages that were either left untreated (−IFN-γ) or exposed to the cytokine for 24 h (+IFN-γ). B, competition experiments were performed using radiolabeled +46/+90 sequence and extracts from untreated cells (−IFN-γ) in the presence of a 500-fold molar excess of double-stranded oligonucleotides against the −31/+8, +9/+49, +46/+90 and +88/+118 sequence, as indicated. +IFN-γ represents the profile obtained with extracts from cells exposed to this cytokine for 24 h. C, EMSA were carried out using the +9/+49 and the +46/+90 oligonucleotides and extracts from J774.2 macrophages that were exposed to IFN-γ for 15 min and 3, 6, 12, and 24 h. Extracts from untreated cells at the start and the end of the experiment were included for comparison (0 and 24 h, respectively). P represents the profile obtained with the free probe alone, which has migrated off the gel, and the DNA-protein complexes are shown by the vertical line labeled C. The results are representative of three independent experimental series.

tides containing binding sites for these factors with the AP-1-binding site oligonucleotide being employed as a nonspecific competitor. As shown in Fig. 4A, the DNA-protein complexes formed using the +9/+49 and the +46/+90 regions could be competed using an excess of an Sp1-binding site oligonucleotide but not by the other sequences, thereby indicating that a Sp1-like factor bound to the IFN-γREs in the LPL promoter.

Sp1 was the first identified member of what is now a family of transcription factors in which Sp1 and Sp3 represent the major and most extensively characterized members that tend to be co-expressed in a number of tissues/cell types and able to interact with the same recognition sequence in many gene promoters (55). To further investigate whether Sp1 and Sp3 also interacted with the +9/+49 and +46/+90 regions, antibody supershift experiments were carried out using specific antisera against Sp1 and Sp3. Non-immune serum and that for C/EBPβ, which has been used extensively for previous studies in the laboratory (40, 42, 45), were included as controls. As shown in Fig. 4B, the production of a slower migrating, antibody-protein-DNA “super-shift” complex was seen with antisera against Sp1 and Sp3 but not C/EBPβ or non-immune serum, thereby indicating that both members interact with the LPL promoter. A closer examination of the data reveals the existence of three DNA-protein complexes, with complex C1 consisting predominantly of Sp1, whereas complexes C2 and C3 were composed mainly of Sp3.

IFN-γ Decreases the Binding of Sp1 and Sp3 to a Number of Recognition Sequences—Although GGGCGGGG (GC element) and CACCC (or GGCTG) boxes have been proposed as consensus Sp1/Sp3-binding sites, the factors have also been shown to interact with sequences divergent from the consensus (55). For example, there is an additional Sp1/Sp3 site in the LPL promoter (position −91 to −83) that contains a CT-rich sequence (5′-CCTCCCCC-3′) (56). We wondered whether the IFN-γ-mediated decrease in DNA binding seen in EMSA was specific to the +9/+90 LPL promoter sequence or could also be seen with a number of other Sp1/Sp3 recognition sequences. EMSA were therefore carried out using extracts from untreated or IFN-γ-stimulated J774.2 macrophages and several different Sp1/Sp3-binding sites. These included the upstream CT-rich sequence from the LPL promoter (56), the downstream sites in the +9/+49 and +46/+90 sequences, and the consensus Sp1 site (contains the GC element). In addition, the Sp1 site
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radioactivity. with the other probes despite an equal amount of input CT element in the LPL promoter was less intense compared the signals obtained from the binding of factors to the upstream /H9251 gene, consensus Sp1-binding site (57). and its promoter region also contains important binding sites for the Sp1 family members (36, 58–59). Fig. 5 shows that exposure of the cells to IFN-γ reduced the binding of factors to all the Sp1/Sp3 recognition sequences investigated. However, some subtle differences were identified. For example, a faster migrating complex, whose intensity was increased in extracts from IFN-γ-treated cells, was seen with the consensus Sp1-binding site (indicated by an asterisk in Fig. 5). Additionally, the signals obtained from the binding of factors to the upstream CT element in the LPL promoter was less intense compared with the other probes despite an equal amount of input radioactivity.

Mutations of All Three Sp1/Sp3 Sites in the +9 to +90 Region Decrease Basal Promoter Activity and Abolish the IFN-γ Response—The +9/+90 region contains three Sp1/Sp3 sites centered at positions +44 to +51 (antisense strand), +62 to +67 (antisense strand), and +65 to +71 (sense strand) that are highly conserved between the mouse, human, and rat LPL gene promoters (Fig. 6A). To evaluate the importance of these sites in the IFN-γ response, three DNA constructs were prepared that contained mutations in these Sp1/Sp3 sites, in the −31/+187 context, as follows: (i) mutation in the site at +44 to +51 (Sp1 M44); (ii) mutations in the overlapping sites at +62 to +67 and +65 to +71 (SpM62/65); and (iii) mutations in all three sites (Sp1M44/62/65) (see Fig. 6A). The DNA constructs were then transfected into U937 macrophages, and the relative luciferase activity in the absence or the presence of IFN-γ was determined. Mutations of these Sp1 sites not only produced a reduction of basal promoter activity but also abolished the IFN-γ response (Fig. 6B). Interestingly, although mutation of the Sp1 sites at +62 and +65 leads to a reduction of basal LPL activity that was similar to that obtained using the promoterless pGL2-Basic vector, the activity of the DNA construct containing mutations of all three Sp1/Sp3 sites (+44, +62 and +65) was greater, with the values obtained in the presence of IFN-γ also being slightly higher than that seen with the wild-type −31/+188 construct in the absence of the cytokine. Although the precise reason(s) for such changes are currently unclear, it is possible that some complex interactions may occur between Sp1/Sp3 that is bound to the three sites. The most important conclusion is clearly that mutation of these Sp1 sites leads to an abolition of the IFN-γ response.

The Sp1/Sp3 Sites in the LPL Promoter Can Impart the IFN-γ Response to a Heterologous Promoter—To evaluate whether the Sp1/Sp3 sites in the LPL promoter can impart the IFN-γ response to a heterologous promoter, four copies of the sites centered at positions +44 and +62/65 were linked to the minimal SV40 promoter in the pGL2-promoter vector. A similar construct was also prepared using the +35 to +75 region that harbors all three Sp1/Sp3 sequences. The DNA constructs were then transfected into U937 cells, and the relative luciferase activity was determined in the absence or the presence of
IFN-γ. The values obtained using the pGL2-promoter vector were subtracted from those obtained using the various LPL constructs. As shown in Fig. 6C, an IFN-γ-mediated reduction in reporter gene activity was obtained with all the LPL promoter sequences used, with maximal reduction being seen when all the three Sp1 sites were present in their normal context. The Steady State Levels of Sp3 but Not Sp1 Are Reduced in Macrophages Following Exposure to IFN-γ—The IFN-γ-mediated reduction in binding of Sp1/Sp3 to the LPL promoter region may either be because of a decrease in the steady state levels of the corresponding proteins and/or a cytokine-mediated suppression of DNA binding activity. To investigate the former possibility, Western blot analysis was carried out using J774.2 macrophages that were either left untreated or exposed to IFN-γ for various times. Fig. 7 shows a representative result from four independent experiments. The description of the trend in Sp1 and Sp3 expression described here is based on all four experiments. A single immunoreactive complex was seen with antisera against Sp1, the levels of which were not affected by exposure of the cells to IFN-γ. Antisera against Sp3 detected four complexes that formed two doublets with approximate molecular masses of 115 and 70 kDa (Fig. 7). Both alternative use of translation initiation codons and post-translational modifications may account for the four polypeptides. Indeed, Sp3 mRNA has been shown previously to specify for three polypeptides, which could be due to either their increased degradation or decreased synthesis. More recently, we have confirmed that IFN-γ induces CK2 kinase activity in J774.2 macrophages, and this is abolished in the presence of the inhibitors.²

² J. R. Mead and D. P. Ramji, unpublished data.
DISCUSSION

We report in this paper studies on the mechanisms responsible for the IFN-γ-mediated suppression of macrophage LPL gene transcription. Promoter-dissection experiments revealed that the −31/+187 region contained the IFN-γ REs (Fig. 2). EMSA identified the interaction of proteins to two sub-regions (+9/+49 and +46/+90), the binding of which was reduced dramatically following incubation of the cells with the cytokine (Fig. 3). These two regions contained three evolutionarily conserved Sp1 recognition sequences (Fig. 6A), and both competition EMSA and antibody supershift experiments revealed the interaction of Sp1 and Sp3 with these sites (Fig. 4). The binding of factors to a number of other Sp1/Sp3 recognition sequences was also found to decrease following incubation of the cells with IFN-γ (Fig. 5). This IFN-γ-mediated suppression was mediated, at least in part, by a reduction of Sp3 polypeptide levels and a decrease in the binding of Sp1 without any changes in the protein levels (Fig. 7). CK2 was found to be involved in the regulation of both Sp1 and Sp3 (Fig. 8).

Sp1 was originally identified as a ubiquitous transcription factor that was implicated in the constitutive expression of several genes (55). However, recent studies (55) have revealed the existence of an Sp1 family, with Sp1 and Sp3 being characterized extensively and known to be co-expressed in several tissues/cell types and to interact with an identical consensus sequence. In addition, the Sp1 family has now been shown to be involved in inducible gene transcription that includes responses to glucose (71, 72), serum (73), epidermal growth factor (74), platelet-derived growth factor (75), and transforming growth factor-β (76, 77). Furthermore, recent work has revealed that Sp1 plays a prominent role in the regulation of many genes in macrophages, including urokinase-type plasminogen activator receptor (51), interleukin-10 (78), hematopoietic cell kinase (79), acid sphingomyelinase (80), lysosomal acid lipase (81), CCAAT-enhancer binding protein (C/EBP) β (50), carboxylesterase (82), proto-oncogene c-fes (83), myeloid integrin CD11b (49), and the membrane glycoprotein CD14 (84). However, the precise role of the Sp1 family in the suppression of gene transcription has hitherto not been investigated in detail. The findings in this paper therefore identify a novel action of the Sp1 family in the IFN-γ-mediated suppression of macrophage LPL gene transcription. Such a mechanism may be widely applicable to other IFN-γ-regulated genes and indicate a need for further detailed studies. Indeed, Fig. 5 and other experiments in our laboratory indicate that such a mechanism may also be involved in the IFN-γ-mediated transcriptional suppression of the C/EBPα gene, the promoter region of which contains important binding site(s) for members of the Sp1 family of transcription factors (36, 58, 59).

The importance of Sp1 and Sp3 in the constitutive expression of LPL has been reported previously (56, 85) in relation to a common, naturally occurring −397/G transition that is associated with reduced promoter activity. Sp1 and Sp3 were shown to bind to an evolutionarily conserved CT element between positions −91 and −83 and activate transcription in THP-1 macrophages (56). In addition, a synergistic action of Sp1/Sp3 and sterol regulatory element-binding protein (SREBP)-1 was observed, which may provide a mechanism for cross-talk between cholesterol and triglyceride metabolic pathways (86). This Sp1/Sp3 site may have also contributed to the halving of the basal activity with deletion of the −101 to −54 LPL promoter region seen in this study (Fig. 2). However, this site was not involved in the IFN-γ response because its deletion does not abolish the cytokine-mediated suppression of LPL promoter activity (Fig. 2).

Although Sp1 acts exclusively as an activator of gene transcription, Sp3 contains a transcriptionally repressive domain and can act as a transcriptional activator or a repressor, depending on the promoter and cell type (55, 87). Thus, changes in the Sp1 to Sp3 ratio may represent one mechanism in the regulation of gene transcription. However, such a mechanism is clearly not involved in the IFN-γ-mediated suppression of LPL gene transcription because Western blot analysis (Fig. 7) shows that the cytokine decreases Sp3 levels. In addition, previous studies (56) have shown that both Sp1 and Sp3 are able to activate the LPL promoter in macrophages. The results presented in this paper instead suggest the existence of two distinct mechanisms for IFN-γ action, both of which require the protein kinase CK2 (Fig. 8). First, the cytokine produces a time-dependent reduction in the abundance of Sp3 polypeptides (Fig. 7). Whether such a reduction is produced via decreased synthesis or increased degradation remains to be determined. It is possible that similar to Sp1 (55, 88), Sp3 also undergoes a proteosome-mediated degradation in response to phosphorylation by CK2. Second, although the abundance of Sp1 does not change following incubation of the cells with IFN-γ, the DNA binding activity of complex C1 in EMSA, which is composed of this factor as judged by antibody supershift assays (see Fig. 4B), decreases. Thus, an IFN-γ-mediated phosphorylation of Sp1 via CK2 is likely to be responsible for its reduced binding.

CK2 is a serine/threonine kinase, which is ubiquitously expressed in both the cytoplasm and the nucleus of eukaryotic cells, and exists as a tetramer composed of two larger catalytic subunits (α and/or α’, 37–44 kDa) and two smaller regulatory β subunits (24–28 kDa) (89). The enzyme phosphorylates serine- and threonine residues in acidic domains, with (S/T)XX(D/E) being the canonical motif (89). The primary sequence of both Sp1 and Sp3 contains a number of consensus sites for CK2 (data not shown). Whether Sp3 is directly phosphorylated by CK2 remains to be determined. For Sp1, however, the CK2 consensus sequence at amino acid 579 in the second zinc finger motif has been shown to be phosphorylated by the enzyme, and this results in a decrease in its DNA binding activity (64). Such CK2-mediated phosphorylation has been implicated in the decrease in Sp1 binding activity during terminal differentiation of the liver and in the transcriptional attenuation of two genes encoding the D-site-binding protein and acetyl-coenzyme A carboxylase (64, 65, 90).

Binding sites for Sp1 members are present in the promoter regions of a large number of class II genes (55). Despite this, such genes are subject to differential regulation by IFN-γ, with the cytokine either inducing or inhibiting their expression or having no effect (see below). For example, we have shown previously (57, 91) that IFN-γ induces the expression of the OEBP/CEBPβ and c-jun genes in J774.2 macrophages, albeit with different kinetics and magnitude of activation. Although the promoter regions of these genes each contain important Sp1 recognition sequence(s) (50, 92–94), they are up-regulated by IFN-γ in the same cellular system where this cytokine suppresses the expression of the LPL gene. These findings therefore raise questions on the mechanisms that are responsible for such differential gene regulation by IFN-γ and the reason(s) why the cytokine does not produce a global reduction in the expression of all class II genes whose promoters bind Sp1 members. The Sp1 family has been shown to interact with sequences that are quite diverged from the proposed consensus (GC element and CACCC boxes) (55). For example, Sp1 interacts with a CT-rich sequence in the promoter regions of the LPL, LDL receptor, and c-myc genes (56, 95–96). It is therefore possible that variations in the Sp1-binding sites between dif-

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3 P. Foka, S. A. Irvine and D. P. Ramji, unpublished data.
different promoters may be responsible for the differential action of IFN-γ. Thus, the affinity of Sp1 members for the various sites may be different, and this could be regulated further by the cytokine. However, EMSA failed to reveal any gross differences in the binding profiles of several such sites when extracts were used from J774.2 macrophages that are either left untreated or exposed to IFN-γ for 24 h (Fig. 5).

The most likely explanation for the differential action of IFN-γ in the regulation of genes whose promoters contain Sp1 recognition sequence is therefore the presence of binding sites for other transcription factors that play a more prominent role in the response. Indeed, both promoter-dissection and DNA-protein interaction studies on the C/EBPβ, C/EBPδ, and c-Jun genes (which have been found to be up-regulated in J774.2 macrophages by IFN-γ(57, 91)) contain regulatory sites for Sp1 that act in concert with other factors: CREB/ATF (C/EBPα) (82), C/EBPγ (lactoferrin) (105), AP-1 and NF-kB (62). It should, however, be noted that the kinetics of IFN-γ action on these genes are different from those for the suppression of LPL, with their transient activation occurring immediately following exposure of the cells to the cytokine, and much earlier than the changes seen in the binding of Sp1/Sp3 to the LPL promoter (97).

In addition to IFN-γ-regulated promoters, the regulatory regions of a large number of genes that are expressed at high levels in monocytes/macrophages and modulated by specific signals also contain binding sites for Sp1 and other factors: AP-1 (acid sphingomyelinase and lysosomal acid lipase) (80, 81), SAF (serum amyloid A) (103), PU.1 (mannose receptor) (104), PU.1 and a novel factor (c-fes) (83), IRBP (carboxyesterase) (82), C/EBP (lactoferin) (105), AP-1 and NF-κB (tissue factor) (106), and c-Jun (CD11C) (107). Even the previously identified Sp1 recognition sequence in the promoter region of the LPL gene has been shown to act in synergy with binding sites for SREBP (86).

In conclusion, we have identified a novel mechanism through which IFN-γ suppresses macrophage LPL gene transcription. Although IFN-γ regulation of gene transcription is mainly mediated through Stat1 (97), several recent studies (108, 109) have indicated the existence of alternative pathways. The regulation through Sp1 and Sp3, as identified in this study, could represent one such mechanism, at least as far as the suppression of gene transcription is concerned. It is also possible that such a mechanism could extend to the action of other cytokines. For example, tumor necrosis factor-α has recently been shown to down-regulate murine hepatic growth hormone receptor expression by inhibiting Sp1 and Sp3 binding (110).

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REFERENCES

1. Goldberg, I. J., and Merkl, M. (2001) Front. Biosci. 6, 388–405
2. Eenensback, and Gimble, J. M. (1995) Biochim. Biophys. Acta 1168, 107–125
3. Mead, J. R., Cayer, A., and Ramji, D. P. (1999) FEBS Lett. 462, 1–6
4. Ts-Agktas, and J. Clin. Invest. 98, 1544–1556
5. Benzer, G., Skamer, K., Deutsch, J. B., and Radzioch, D. (1993) Arterioscler. Thromb. 13, 190–196
6. Babyseba, V. R., Fazio, S., Gleave, H. C., and Semenkovich, C. F., and Lipton, M. A. (1999) J. Clin. Invest. 103, 1697–1705
7. Babyseba, V. R., Patel, M. B., Semenkovich, C. F., Fazio, S., and Lipton, M. F. (2000) J. Biol. Chem. 275, 26293–26299
8. Van Eck, M., Zimmermann, R., Groot, P. H., Zeelen, R., and Van Berkel, T. J. (2000) Arterioscler. Thromb. Vasc. Biol. 20, 1553–1562
