The Role of Sp1 and NF-κB in Regulating CD40 Gene Expression*

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CD40 is a member of the tumor necrosis factor receptor superfamily and is a key signaling molecule expressed by antigen-presenting cells of the immune system. In a previous paper, we demonstrated that the expression of CD40 is regulated by both post-transcriptional and post-translational processes. In this paper, we show that basal (constitutive) CD40 gene expression is regulated by a TATA-less promoter, with Sp1 as a key transcription factor. Two Sp1 binding regions were identified in the mouse CD40 promoter at positions −59 to −50 and −74 to −66. Surprisingly, Sp1-mediated CD40 transcription was reduced following lipopolysaccharide stimulation and was associated with a time-dependent reduction in Sp1 DNA binding activity. This reduction seemed to be mediated by phosphorylation of the Sp1 molecule. We also showed here that CD40 expression in lipopolysaccharide-stimulated cells is up-regulated by NF-κB through two distinct sites. One of these sites (−128 to −119) was shown to bind p50 and p65 members of the NF-κB family, while the other site (−562 to −553) bound only p65. Transfectants of p65 were generated using RAW 264 cells, and it was shown that the up-regulation of CD40 mRNA expression was dependent on the presence of the p65 molecule.

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1 The abbreviations used are: TNF, tumor necrosis factor; NF-κB, nuclear factor-κB; JNK, Jun N-terminal kinase (JNK) and Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways (8–11).

Signaling through CD40 also up-regulates the expression of certain cytokines such as interleukin (IL)-12 (12) and as a result can promote CD4+ T helper 1 cell growth and differentiation (12, 13) in both health and disease. The pivotal position of CD40 as an orchestrator of T cell-mediated immune responses requires that its activity be carefully regulated. Recently, we have demonstrated that CD40 function is controlled by post-transcriptional and post-translational regulatory mechanisms (14). We identified five CD40 isoforms that were generated by alternative splicing. Type I CD40 is the functional form, which contains a signal-transducing cytoplasmic domain. Type II CD40 lacks the membrane-associated endodomain and seems to inhibit the expression of signal-transducing CD40 on the cell surface. Type III and IV CD40 are membrane-bound CD40 isoforms with cytoplasmic domains not capable of signal transduction. These two membrane-bound isoforms might function as dominant negative inhibitors of the Type I CD40 isoform.

Little is currently known of the transcriptional regulation of the CD40 gene. Nguyen and Benveniste (15) have shown that CD40 transcription is regulated by STAT-1 and Ets in IFN-γ stimulated cells. Otherwise, little is known of the mechanisms underlying transcriptional regulation of CD40 gene expression in nonstimulated resting cells or in cells stimulated by microbial products such as lipopolysaccharide (LPS).

In this paper, we show, in the macrophage cell line RAW 264 and in bone marrow-derived dendritic cells (bmDC), that Sp1 is a key transcription factor in the basal expression of CD40 and that the transcription factor NF-κB has an important role in up-regulating CD40 expression following LPS stimulation. We also demonstrate that in LPS-stimulated cells, Sp1-mediated CD40 promoter activity seems to be down-regulated by the phosphorylation of Sp1.

EXPERIMENTAL PROCEDURES

Reverse Transcriptase (RT)-PCR—RT-PCR was performed as described previously (16). Briefly, cDNA was prepared using 1 μg of total RNA and an oligo(dT) primer. This reaction mixture (20 μl) was diluted with 180 μl of water, and 0, 2.5, 5, or 10 μl of the cDNA solution was used for PCR. The PCR primers used were CD40 sense: GGAGATGGAAGATTATCCCGG; CD40 antisense: GCATGAGATGTGTCAC; p65 sense: GGTCCCTTCCTCAGCCATGG; and p65 antisense: TTAAAAGCTTTGCTAGACGGGTTCGCTGTCAGCAC and hypoxanthine phosphoribosyltransferase (HPRT) (16) primers. PCR cycle numbers were kept low to perform semi-quantitative PCR (HPRT, 18 cycles; Fig. 1A, CD40 19 cycles; Fig. 1B, CD40 16 cycles; Fig. 9, CD40 20 cycles and p65 15 cycles). The amplified products were then detected by Southern blot hybridization using cDNA probes.

Mapping of Transcription Start Sites—CD40 mRNA start sites were determined by the rapid amplification of cDNA ends procedure (RACE) as described previously (16). cDNA was prepared using an antisense primer (CTTGTCGAGGATACAGCTTTCG) CD40 cDNA containing the 5′-ends was amplified using a poly C primer as the sense primer and

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an antisense primer (GTTGACAGGAACTTCCCGTG). CD40 Promoter Constructs and Luciferase Assay—Fourteen CD40 promoter fragments (Fig. 2) and Sp1 (Fig. 4) and NF-κB (Fig. 7) knock-out promoter fragments were cloned into the pGL3-Basic Vector. Luciferase assays were performed using the mouse macrophage cell line RAW 264 (10^6 SL2 cells transfected with 0.5 μg of a luciferase reporter plasmid containing a CD40 promoter fragment (~195 to +22), and differing amounts of pPac (expression vector), pPacSp1 (Sp1 expression plasmid), and/or pPacUSp3 (Sp3 expression plasmid). These expression plasmids were kindly gifts from Dr. G. Suske (Philips-Universitat, Marburg, Germany).

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA) — Nuclear extracts for EMSA were prepared from RAW 264 and bmDC. Preparation of bmDC has been described previously (14). Both cell types were cultured with or without LPS (20 μg/ml) and nuclear extracts were prepared as described previously (16). EMSA was performed with 7 μg of nuclear extract in 20 μl of EMSA reaction buffer containing 2 μg of poly(dI·dC)·poly(dI·dC), 20 mM Heps, pH 7.9, 1 mM MgCl₂, 40 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 12% glycerol. For the competition assay, a 100-fold excess of unlabeled competitor was added to EMSA reaction mixtures. Samples were analyzed on a polyacrylamide gel containing 89 μl Tris borate, 20 mM EDTA, and 10% glycerol. To perform the super shift assay, nuclear extracts in EMSA reaction buffer were incubated with anti-Sp1 (Santa Cruz, C6), anti-Sp3 (Santa Cruz, D-20), anti-NF-κB p65 (Santa Cruz, C-20), and/or anti-NF-κB p50 (Santa Cruz, D-17) antibodies for 15 min, at which time probes were then added.

To examine the probe binding capacity of Sp1 in its dephosphorylated state, 7 μg of nuclear extract was treated with 0.2 units calf intestinal alkaline phosphatase (CIP) in EMSA buffer at room temperature for 30 min. The reaction was then terminated by adding 1 μl of phosphatase inhibitor cocktail 2 (Sigma). To prepare the control nuclear extract, phosphatase inhibitor cocktail 2 was added before the addition of CIP and incubated. A 32P-labeled oligonucleotide probe was then added into each reaction and incubated for another 20 min. To prepare a labeled probe for this assay, a sense oligonucleotide (~83 to ~61) and antisense oligonucleotide (~81 to ~59) were annealed and labeled using a Klenow fragment with [α-³²P]dCTP. Activities of CIP and the inhibitor were checked using a 32P-labeled probe.

Immunoblotting—10 μg of nuclear extract was electrophoresed on a 6% SDS-polyacrylamide gel and transferred to a nitrocellulose filter. This protein filter was treated with anti-Sp1 rabbit polyclonal IgG (Santa Cruz, PEP2), and Sp1 was detected using peroxidase-conjugated swine anti-rabbit antibody (Dako) and ECL plus Western Blotting Detection System (Amersham Biosciences, Inc.).

In Vitro Transcription Assay—To analyze Sp1 activity, an in vitro transcription assay was performed using the AdML G-less cassette (17). This vector was a kind gift from Dr. J. Portugal (Instituto de Biologia Molecular de Barcelona, Spain). For cloning, a sense oligonucleotide (AATTTGCGGAAGGCCTCCGTCCCTCCT; and an antisense oligonucleotide (AATTAGAAGAAAGCGGGGTCGCGCCCGG) containing the Sp1 site (Sp1/A) in the CD40 promoter were annealed and phosphorylated. This fragment was then cloned into the EcoRI site in the AdML G-less cassette upstream of the basal AdML promoter, and a plasmid containing three copies of the Sp1 binding sequence was selected by DNA sequencing. The plasmid structure is shown in Fig. 10A. 2 μg of the resulting plasmid and the AdML G-less cassette (as a negative control) were incubated with 25 μg of nuclear extracts in 32 μl of reaction buffer containing 20 mM Heps, pH 7.9, 40 mM KCl, 0.5 mM MgCl₂, 0.2 mM EDTA, 10% glycerol, 2 μl dithiothreitol, 0.31 μM of ATP and CTP, 0.78 μM 3⁰-3'-O-methyl-GTP (Amersham Biosciences, Inc.), 20 μCi [α-³²P]UTP (800 Ci/μmol, Amersham Biosciences, Inc.) and RNase T1 (Roche). The reaction mixtures were incubated at 30 °C for 60 min. The reaction was then terminated by adding phenol/chloroform. Unrelated [³²P]-labeled RNA (270 nucleotides in length) was added as an internal-control to assess for the recovery yield of RNA. After

phenol/chloroform and chloroform extractions, transcribed products were ethanol-precipitated. These purified transcripts were then electrophoresed on an 8% sequencing gel and analyzed by autoradiography and phosphorimaging.

Generation of p65 Transfectants—To construct the NF-κB p65 expression plasmid, NF-κB p65 cDNA was amplified and cloned into an expression vector (pMTF) containing the elongation factor-1α promoter and a neomycin resistance gene. RAW 264 cells were then transfected with either the p65 expression plasmid or with vector alone (controls). Stable transfectants were selected by using G418 (1 mg/ml). p65 expression levels were then analyzed by RT-PCR and EMSA.

RESULTS

Expression of CD40 mRNA—CD40 expression is observed in a wide range of cells, including macrophages and dendritic cells. CD40 mRNA levels were analyzed by RT-PCR. Amplified cDNA was detected by Southern blot hybridization using CD40 and HPRT cDNA probes. A, for PCR amplification (CD40, 19 cycles), 0 μl (lane 0), 2.5 μl (lane 1), 5 μl (lane 2), and 10 μl (lane 3) of cDNA samples were used. B, 10 μl of cDNA samples were used for PCR (CD40, 16 cycles). LFS stimulation times are indicated above the blot. C, RT-PCR results shown in B were also analyzed using phosphorimaging. mRNA levels of CD40 were then compared with those of HPRT.

![Fig. 1. Expression of CD40 mRNA in RAW 264 cells. CD40 mRNA levels in nonstimulated (0 h) and LPS-stimulated (2 and 24 h) were analyzed by RT-PCR. Amplified cDNA was detected by Southern blot hybridization using CD40 and HPRT cDNA probes. A, for PCR amplification (CD40, 19 cycles), 0 μl (lane 0), 2.5 μl (lane 1), 5 μl (lane 2), and 10 μl (lane 3) of cDNA samples were used. B, 10 μl of cDNA samples were used for PCR (CD40, 16 cycles). LPS stimulation times are indicated above the blot. C, RT-PCR results shown in B were also analyzed using phosphorimaging. mRNA levels of CD40 were then compared with those of HPRT.](http://www.iarc.fr/.../26.2018)
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Fig. 2. CD40 promoter activity of the CD40 gene. CD40 promoter activity was analyzed by luciferase assays. Luciferase activities generated using the reporter plasmids D1 to D14 were compared with that generated using the negative control plasmid (no insert) pGL3-Basic vector (Basic) in nonstimulated (Non) and LPS-stimulated (LPS) RAW 264 cells. These assays were repeated at least three times, and the activities were normalized using Renilla luciferase activities (internal control). A, structure of luciferase reporter plasmids. B, luciferase activities generated using the reporter plasmids D1-D4. C, luciferase activities generated using the reporter plasmids D3-D9. D, luciferase activities generated using the reporter plasmids D7-D14.

mapped between −52 and +26. No TATA box sequence was found in the region 30 bp upstream of any of the transcription start sites, suggesting that CD40 gene expression is regulated by a TATA-less promoter.

Basal CD40 Gene Expression Is Regulated through Two Sp1 Sites—To investigate cis-acting elements in the CD40 promoter, we performed luciferase reporter assays using deletion mutants of the CD40 promoter (Fig. 2). Significant reduction of promoter activity was observed by deletion of a 31-bp sequence from −91 (D3) to −60 (D2) in both nonstimulated and LPS-stimulated cells (Fig. 2B), suggesting that constitutively expressed transcription factors might bind to this region. EMSA was performed using three probes (P1-P3), which contained sequences in the identified region and its flanking sequence (Fig. 3A). Three slowly migrating complexes were detected with probe P2 (Fig. 3B), and DNA-dependent binding was confirmed by a competition assay (Fig. 3C). We found a typical Sp1 consensus sequence (ACCCCGGCC) in the P2 sequence (Fig. 3A) and confirmed binding of Sp1 and Sp3 to this probe by a super shift assay using the anti-Sp1 family antibodies (anti-Sp1, Sp2, Sp3, and Sp4) (Fig. 3D). We have named this region the Sp1/A site.

In addition, by using the transcription factor data base, a typical Sp1 consensus sequence was also found at position −59 to −50 (TGGGCGGCC) (named the Sp1/B site). However, no complex formation was detected with probe P3 containing the Sp1/B site by EMSA (Fig. 3B). To further characterize this site, the binding of Sp1 and Sp3 to the Sp1/B site was analyzed by a competition assay using probe P2 (containing the Sp1/A site) and the competitor P3 (containing the Sp1/B site). A reduction of complex formation with 32P-labeled P2 probe was observed when a 100-fold excess of unlabeled competitor P3 was added (Fig. 3C), indicating that Sp1 and Sp3 can also bind to the Sp1/B site (in the competitor P3), but the binding affinity to this site is much lower than to the Sp1/A site.

Although Sp1 and Sp3 bind to the Sp1/B site, promoter activity was not detected with the small fragment D2 containing this site (Fig. 2B). Since the two Sp1 sites are closely positioned, we hypothesized that they might cooperate with each other. To further explore this possibility, luciferase reporter plasmids were constructed using two longer promoter fragments in which Sp1/A and Sp1/B sites were then mutated. Mutation in the Sp1/A site reduced the promoter activities in both fragments by either 88% (217-bp fragment, −195 to +22) or 73% (723-bp fragment, −701 to +22), respectively (Fig. 4, A and B). Surprisingly, a mutation in the weak Sp1 binding site (Sp1/B) also reduced promoter activity by 60% (Fig. 4, A and B). Only 5% (−195 to +22) and 8% (−701 to +22) of the wild type promoter activities were detected on the Sp1/A and B double knockout promoter (Fig. 4, A and B). These results suggest that Sp1/A and B sites act as key cis-acting elements for regulating basal CD40 promoter activity, and that, in addition, they act in a cooperative manner.

Sp3 Enhances Sp1-mediated CD40 Transcription—The involvement of Sp1 and Sp3 in regulating CD40 transcription was also investigated using Drosophila SL2 cells, which lack endogenous Sp factors. The CD40 promoter (−195 to +22) luciferase reporter plasmid was cotransfected with either the Sp1 (pPacSp1) or Sp3 (pPacUSp3) expression plasmid (Fig. 4C). CD40 promoter activity in the reporter plasmid was up-regulated by cotransfection with Sp1 (Fig. 4C, Sp1) but not with Sp3 (Fig. 4C, Sp3), suggesting that Sp1 alone could regulate CD40 transcription while Sp3 alone could not. However, when the Sp3 expression plasmid (pPacUSp3, 0–2 μg) was cotransfected with a fixed amount of the Sp1 expression plas-
NF-κB transcription. Sp3, then, is capable of enhancing Sp1-mediated CD40 increased in a dose-dependent manner (Fig. 4 C (Sp1 Sp3)). Total plasmid amount was adjusted to 2/μH11001/μH9262/luciferase plasmid with the indicated amount of pPacSp1 (Sp1), pPacUSp3 (Sp3), or pPacSp1 (0.4 μg)pPacUSp3 (Sp1+Sp3). Total plasmid amount was adjusted to 2 μg (Sp1 and Sp3) or 2.4 μg (Sp1+Sp3) with pPac (no insert) plasmid. Luciferase activities generated from the CD40 luciferase construct in SL2 cells cotransfected with pPacSp1, pPacUSp3, or pPacSp1+pPacUSp3 were compared with that in SL2 cells cotransfected with pPac control plasmid. These assays were repeated at least three times.

mid (pPacSp1, 0.4 μg), the CD40 promoter activity was increased in a dose-dependent manner (Fig. 4C, Sp1+Sp3). Sp3, then, is capable of enhancing Sp1-mediated CD40 transcription.

**CD40 Gene Expression Is Regulated through Two Distinct NF-κB Sites**—In LPS-stimulated cells, a significant reduction (3.5-fold) of CD40 promoter activity was also detected by a 66-bp deletion from −619 (D11) to −553 (D10) (Fig. 2D). Slowly migrating complexes were detected with probe P12 by EMSA (Fig. 6, A and B), and DNA-dependent complex formation was confirmed by a competition assay (Fig. 6C). This probe also contained a potential NF-κB recognition sequence, AGGAATTC. A super shift assay was then performed using anti-p50, p52, p65, rel-B, and c-rel antibodies. A super shifted band was easily detected using the anti-p65 antibody, but only weakly detected with the anti-p50 (Fig. 6D). No super shifted bands were detected with the other antibodies. We can therefore conclude that NF-κB binds to this region, and we have named this region the NF-κB/B site.

We have thus far identified two NF-κB sites in the CD40 promoter by EMSA and luciferase assay using promoter deletion mutants. To determine whether CD40 promoter activity is regulated through these NF-κB sites, additional promoter constructs were generated in which site NF-κB/A and/or site NF-κB/B were mutated (Fig. 7). Mutations in the NF-κB/A site and/or the NF-κB/B caused a reduction in CD40 promoter activity (Fig. 7B), suggesting that CD40 transcription is regulated, at least in part, through both of the two NF-κB sites we have identified here.

To investigate whether the nuclear translocation of NF-κB varies with the amount of LPS stimulation, NF-κB binding was measured by EMSA in nuclear extracts from cells that were stimulated with LPS for different amounts of time (Fig. 8A, RAW). The intensity of complex formation with NF-κB/A site (in probe P5) increased in cells stimulated with LPS for 2 and 6 h but then decreased in samples with longer stimulation times (Fig. 8A, RAW). These changes were also observed using nuclear extracts from LPS-stimulated bmDC (Fig. 8A, bmDC). To analyze p50 and p65 protein levels, super shift assays were performed using probe P5 (Fig. 8A, RAW p50 + Ab, p65 + Ab). Complex formation between p50 and probe P5 was detected in...
were already present in the nucleus of nonstimulated cells (Fig. 8A), although increased levels of CD40 promoter activity with NF-κB/A sites in these cells were not detected (Fig. 2C, D4–D5, Non), indicating that p50 alone (p50 homodimer) cannot transactivate the CD40 promoter. This suggests that p65 (p50/p65 heterodimer and/or p65/p65 homodimer) might be a key transcription factor used to regulate CD40 expression. To examine this possibility, p65 transfectants were generated using RAW 264 cells and a p65 expression plasmid. Two transfectants, RAW/p65–1 (low) and RAW/p65–2 (high), were selected for their different expression levels of p65 mRNA (Fig. 8A). A control transfectant (RAW/Con) was generated by transfection with vector alone. In these nonstimulated transfectants, CD40 mRNA expression increased as the level of p65 increased (Fig. 9A). Without LPS stimulation, p65 seems to be translocated to the nucleus simply by overexpression of the p65 molecule in these cells. To confirm this, an EMSA was performed using nuclear extracts from the nonstimulated RAW/p65–2 to ana-
lyze the nuclear translocation patterns of p50 and p65 (Fig. 9B). Increased complex formation with p65 and probe P5 was observed in RAW/p65–2 (Fig. 9B, with anti-p65 Ab). On the other hand, complex formation with p50 was detected in both control and RAW/p65–2 cells (Fig. 9B with anti-p50 Ab). Taken together, these results suggest that p65 regulates CD40 gene expression.

Sp1-mediated CD40 Transcription Is Down-regulated in LPS-stimulated Cells—We have demonstrated that basal CD40 promoter activity is regulated by Sp1 and Sp3, which are known to be expressed constitutively and ubiquitously. We also investigated the role of Sp1 and Sp3 involvement in CD40 transcription in LPS-stimulated cells. Complex formation was easily detected in nuclear extracts from nonstimulated cells by EMSA using probe P2 (containing Sp1/A site) (Fig. 8C RAW 0 h). However, the intensity of these complexes was reduced considerably in nuclear extracts from LPS-stimulated cells and was dependent on the duration of LPS stimulation (Fig. 8C RAW 2–24 h). These changes were also observed in LPS-stimulated bmDC (Fig. 8C, bmDC). Similar amounts of Sp1 were detected in all nuclear extracts by immunoblotting (Fig. 8D). Taken together, these results suggest that the reduction of complex formation after LPS stimulation was due to reduced DNA binding activity of Sp1 rather than to a decrease in the amount of Sp1 protein.

Reduction of the Sp1-mediated CD40 promoter activity was also detected by an in vitro transcription assay (Fig. 10). To construct a template plasmid for this assay, we used the AdML G-less cassette (17), which contains a 50-bp AdML basal promoter upstream of a 190-bp G-less sequence. Structure of the template DNA is shown in Fig. 10C. After normalization with an internal control (to assess for the RNA recovery yield), the amount of transcripts produced by the promoter containing the three CD40 Sp1 sites was compared with that from the basal promoter (without the Sp1 site) by phosphorimaging. Sp1-mediated transcription was clearly detected with the nuclear extract from nonstimulated cells, but was down-regulated by LPS stimulation in a manner dependent on the length of LPS stimulation, such that by 24 h after LPS stimulation, Sp1-mediated promoter activity was reduced to 35% of that in nonstimulated cells (Fig. 10C).

Sp1 DNA Binding Activity Is Reduced by Phosphorylation—It has recently been shown that the DNA binding and transcriptional activity of Sp1 is regulated by modifications such as phosphorylation of the Sp1 protein (see “Discussion”). We considered whether or not phosphorylation might be the mechanism by which Sp1 DNA binding activity is decreased after LPS stimulation. This was indeed shown to be the case when we analyzed Sp1 binding activity to the Sp1/A site using nuclear extracts that were treated with an alkaline phosphatase, CIP. Increased levels of Sp1 binding activity were detected in CIP-treated nuclear extracts from cells stimulated with LPS for either 6 or 12 h (Fig. 8E), suggesting that Sp1 binding to the CD40 promoter is regulated by phosphorylation.

DISCUSSION

In a previous paper (14), we have shown that the signal-transducing CD40 isoform is expressed on nonstimulated cells and is required for the up-regulation of IL-12 p40 expression in these cells. This constitutive CD40 expression seems to be maintained by Sp1, which is known to be constitutively ex-
pressed and to bind to the G+C-rich consensus sequence (KRG-GCGKRRY). Recently, we found that Sp1 can also bind to the CCTCCT motif in the IL-10 promoter and plays a key role in IL-10 transcriptional regulation in a wide range of cell types (16). Sp1-mediated transcription, therefore, is an important player in the regulation of the immune response.

We have also shown previously that the CD40 gene is expressed as a series of CD40 isoforms (Type I-V) generated by alternative splicing. Type I CD40 is the normal signal-transducing receptor, and signaling through this receptor is blocked by coexpression of the Type II, III, and IV isoforms. Interestingly, up-regulation of CD40 expression by LPS stimulation is blocked by coexpression of the Type II isoform (14), suggesting that CD40 signaling requires its own up-regulation. Since macrophages and dendritic cells express the ligand for CD40 (CD40L), it is possible that CD40 expression is up-regulated by LPS-mediated CD40L interaction with CD40 on the same cells. Signaling through CD40 is known to activate the NF-κB pathway, and in this paper we have demonstrated that CD40 expression is also regulated by NF-κB. Taken together, the up-regulation of CD40 expression might be regulated through a positive feedback mechanism using this CD40/NF-κB activation pathway.

In LPS-stimulated cells, increased levels of promoter activity (about 2-fold) were observed by addition of a 48-bp sequence (-553 to -505) (Fig. 2D, D9-D10). Binding of the transcription factor STAT-1 to this region and involvement of this region in CD40 transcriptional regulation in IFN-γ-stimulated cells has been reported (15). It has also been reported that STAT-1 is activated by LPS stimulation through a signal transduction pathway distinct from that used by IFN-γ (18). It would seem, therefore, that CD40 expression in LPS-stimulated cells might also be up-regulated by STAT-1.

We show that NF-κB up-regulates CD40 expression in LPS-stimulated cells. However, this strong NF-κB-mediated promoter activity was reduced to less than 10% of the baseline by mutations in the Sp1 sites in the CD40 promoter, suggesting that the CD40 promoter does not function properly without Sp1. We also show that Sp1 DNA binding activity is decreased by phosphorylation of this transcription factor in LPS-stimulated macrophages and dendritic cells. We speculate, therefore, that the phosphorylation of Sp1 after LPS activation of macrophages and dendritic cells might result in a reduced capacity for CD40 transcription. There are two known mechanisms of post-translational modification of Sp1. One involves glycosylation and the other phosphorylation. Sp1 contains O-linked N-acetyl-glucosamine residues in the N-terminal half of this protein (19), but this glycosylation has no effect on the DNA binding function of Sp1. The N terminus of Sp1 can be phosphorylated also by DNA-dependent protein kinase (20). This, however, also does not appear to affect either its transcriptional or DNA binding activities. Phosphorylation with cAMP-dependent protein kinase, on the other hand, results in increased transcriptional activity and DNA binding activity (21). DNA binding activity and transcriptional activity of the phosphorylated form of Sp1 seem to be dependent on the actual site of phosphorylation, which in turn may depend on the particular kinase. In the case of CD40 expression, the phosphorylation of Sp1 seems to down-regulate Sp1-mediated CD40 transcription. In support of these findings, decreased Sp1 DNA binding activity by phosphorylation in rat liver cells has also been reported (22). It has been reported also that the C terminus of Sp1 is phosphorylated by casein kinase II, and DNA binding activity is decreased due to this phosphorylation (23). The reduction of Sp1 binding to the CD40 promoter in LPS-stimulated RAW 264 cells is perhaps also mediated by casein kinase II. Indeed, activation of casein kinase II in LPS-stimulated RAW 264 cells has been reported (24). It is therefore possible that Sp1-mediated CD40 transcription may be regulated by the casein kinase activation pathway. Further research is needed to explore this possibility.

We have demonstrated that CD40 promoter activity is reduced by LPS-mediated phosphorylation of Sp1. Since NF-κB regulates CD40 gene expression, IκB can block this transactivation (25). We know that CD40 transcription is also stimulated by STAT-1 in IFN-γ-stimulated cells (15), and presumably in LPS-stimulated cells as well. We have already shown that in IFN-γ-stimulated cells, up-regulation of CD40 gene expression is blocked by the suppressor of cytokine signaling-1 (SOCS-1) (14), an inhibitor of the JAK/STAT pathway. CD40 transcription, therefore, might be controlled by three different negative feedback mechanisms: IκB, phosphorylation of Sp1, and SOCS-1.

Based on our results and results shown by Nguyen and Benveniste (15), we suggest the following sequence of events during CD40 expression. (i) Nonstimulated macrophages and dendritic cells express low levels of CD40 mRNA generated via Sp1-mediated basal transcription. (ii) CD40 mRNA expression is substantially increased through the activation of NF-κB, the latter itself being sustained through CD40L/CD40 interaction. Signaling through the IFN-γ receptor activates STAT-1, which then also contributes to the enhancement of CD40 expression. Maximum transactivation, however, is maintained only for a short period. (iii) Eventually, IκB inactivates NF-κB, STAT-1 activation is down-regulated by SOCS-1, and Sp1 DNA binding activity is decreased by LPS-mediated phosphorylation. CD40 transcription is then decreased by the combination of these negative feed back pathways. (iv) In addition, by this time, post-transcriptional regulation has become involved in the form of alternative splicing to produce the CD40 isoforms Type II, III, and IV (half of pre-CD40 RNA is spliced out to these CD40 mRNA isoforms at 24 h after LPS stimulation) (14). (v) By a mechanism of post-translational regulation, the protein products from these CD40 isoforms block signaling through the CD40 Type I receptor.

The CD40 molecule is a pivotal receptor influencing the activity of many cell types, and its expression must therefore be tightly controlled. We have show in this and a previous (14) paper that CD40 gene expression occurs through a range of processes that operate at three different levels of gene expression: transcriptional, post-transcriptional, and post-translational regulation.

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