Flanking Sequences for the Human Intercellular Adhesion Molecule-1 NF-κB Response Element Are Necessary for Tumor Necrosis Factor α-Induced Gene Expression*

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The regulated expression of intercellular adhesion molecule-1 (ICAM-1) by cytokines such as tumor necrosis factor α (TNF-α) plays an important role in inflammation and immune responses. Induction of ICAM-1 gene transcription by TNF-α has previously been shown to be dependent upon a region of the ICAM-1 5′-flanking sequences that contains a modified κB site. We demonstrate here that this modified κB site alone is insufficient for induction of transcription by TNF-α. Site-directed mutagenesis of both the κB site and specific flanking nucleotides demonstrates that both the specific 5′- and 3′-flanking sequences and the modified κB site are necessary for TNF-α induction. Further, site-directed mutagenesis of this modified κB site to a consensus κB site allows it to mediate transcriptional activation in response to TNF-α, even in the absence of specific flanking sequences. Transcription through this minimal ICAM-1 TNF-α-responsive region can be driven by co-expression of p65, and the minimal response element interacts with p65 and p50 in supershift mobility shift assays. However, when in vitro transcription/translation products for the Rel proteins are used in an electrophoretic mobility shift assay, only p65 is capable of binding the minimal response element while both p50 and p65 bind a consensus κB oligonucleotide. Additionally, in the absence of the specific flanking nucleotides, the ICAM-1 κB site is incapable of DNA-protein complex formation in both electrophoretic mobility shift assay and UV cross-linking/SDS-polyacrylamide gel electrophoresis analysis. These results demonstrate the requirement for specific flanking sequences surrounding a κB binding site for functional transcription factor binding and transactivation and TNF-α-mediated induction of ICAM-1.

Interdependence adhesion molecule-1 (ICAM-1)§§ is a cell surface glycoprotein and member of the immunoglobulin superfamily (1, 2) As the counter-receptor for the leukocyte β2 integrins, ICAM-1 plays a central role in a number of inflammatory and immune responses. Although ICAM-1 is constitutively expressed on a variety of cell types, including hematopoietic cells, fibroblasts, and vascular endothelium (3), its regulated expression is fundamental to leukocyte trafficking. Up-regulated ICAM-1 expression on cytokine-activated vascular endothelial cells controls the targeted transmigration of leukocytes into specific areas of inflammation. In addition, ICAM-1 can be induced on some cell types in which it is not constitutively expressed (e.g. human keratinocytes (4)), further fine tuning the localized inflammatory reaction once leukocyte transmigration has occurred.

ICAM-1 expression can be induced by a variety of cytokines, including interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and interleukin-1, as well as bacterial lipopolysaccharide (5–8). Both IFN-γ and TNF-α have been shown to mediate this induction at the level of transcription (5, 9). The molecular events underlying the transcriptional activation of the ICAM-1 gene in response to TNF-α stimulation are not fully understood. In the up-regulation of other cellular adhesion molecules by TNF-α, it has been shown that TNF-α induces expression by activation of members of the NF-κB (Rel) family of transcription factors (10). There are five known members of the Rel family, NF-κB1 (p50), NF-κB2 (p52), RelA (p65), c-Rel, and RelB (11). The first NF-κB complex described consisted of p65, p50, and the inhibitor IκB-α pre-existent in the cytoplasm. Upon stimulation with TNF-α, IκB-α is degraded, unmasking nuclear translocation signals on p65 and p50. These proteins translocate to the nucleus, bind to DNA as a heterodimer (p65/p50) through a decameric consensus sequence (GGGRNNYYCC), and mediate transactivation (10, 12, 13). Since this original description, individual subunits of the NF-κB complex have been shown to regulate transcriptional activity as homodimers or as heterodimers with other members of the Rel family. Specifically, both p50 and p65 homodimers, as well as heterodimers with other members of the Rel family, are capable of moderating transcription. Further, this activation requires a DNA binding sequence that is specific for the particular dimer (9), and binding of both subunits is required for activation (14).

While a κB site has been implicated in induction of ICAM-1

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¶¶ The abbreviations used are: ICAM-1, intercellular adhesion molecule-1; TNF-α, tumor necrosis factor α; NF-κB, nuclear factor κB; CAT, chloramphenicol acetyltransferase; PMSF, phenylmethanesulfonyl fluoride; EMSA, electrophoretic mobility shift assay; HDMEC, human dermal microvascular endothelial cells; HUVEC, human umbilical vein endothelial cells; VCAM-1, vascular cell adhesion molecule-1; TK, thymidine kinase; IFN-γ, interferon-γ; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; BrdUrd, bromodeoxyuridine.
gene expression by TNF-\(\alpha\) (11, 15, 16), it is now apparent that this transcriptional regulatory process is more complex than initially described. By deletional and mutational analysis, we show that a variant \(\kappa B\) site plus the 5'- and 3'-flanking regions are necessary and sufficient for induction of ICAM-1 by TNF-\(\alpha\), demonstrating the requirement of specific flanking nucleotides to a \(\kappa B\) site. Thus, these specific flanking sequences may be necessary for stabilizing Rel protein binding to this sub-optimal \(\kappa B\) site. Further, these data implicate DNA sequences surrounding nonconsensus \(\kappa B\) sites in directing specific Rel family member binding and may, thus, participate in directing gene specific transactivation.

MATERIALS AND METHODS

Cell Culture—

C32 melanoma cells (ATCC) were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 3 mM l-glutamine, 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 0.25 mg/ml amphotericin B, and 10 \(\mu\)g/ml streptomycin (all from Life Technologies, Inc.). As described previously (17, 18), human dermal microvascular endothelial cells (HDMEC) were isolated from neonatal foreskins and cultured in MCDB131 (Life Technologies, Inc.) supplemented with 10% normal human serum (Irvine Scientific), dibutyryl cAMP (5 \(\times\) \(10^{-5}\) M Sigma), 100 units \(\mu\)g/ml amphotericin B, and 10 \(\mu\)g/ml streptomycin (all from Life Technologies, Inc.). Cultures were maintained at 37°C in humidified 5% CO₂. Experiments with HDMEC had been conducted with cells in passage 3–5.

Production of ICAM-1-based CAT Reporter Gene Constructs and Oligonucleotides for Electrophoretic Mobility Shift Assay (EMSA)—

ICAM-1 based heterologous promoter/reporter plasmids were designed and constructed using techniques and strategies as described previously (19). Briefly, various portions of the ICAM-1 5'-flanking region were isolated by appropriate restriction enzyme digestion of the ICAM-1 genomic sub-clone pG6G-2.05 (20), by polymerase chain reaction using primers that incorporated convenient restriction enzyme sites at their 5' ends and were specific for areas of interest within the ICAM-1 gene or, for smaller fragments, by annealing of complementary synthesized oligonucleotides (Emory University Microchemical Facility). Cold double-stranded DNA was made identically, except that unlabeled dCTP was substituted for labeled dCTP and the final oligonucleotides were synthesized by the Emory University Microchemical Facility. Double-stranded DNA was made identically, except that unlabeled dCTP was substituted for labeled dCTP and the final oligonucleotide concentration was 100-fold higher. The oligonucleotides used as probes and cold competition, with \(\kappa B\) sites underlined and primer sites double underlined, were as follows: ICAM-1 \(\kappa B\) with ICAM-1 flanking DNA (IC/IC, representing the region −191 to −172 upstream of the transcription start site), 5'-TTAGTTGGAATTCCGAGCTCGAGATCTCATG-3'; ICAM-1 \(\kappa B\) with random flanking sequences (IC/RT), 5'-TTAGTTGGAATTCCGAGCTCGAGATCTTGTTGGC-3'; ICAM-1 \(\kappa B\) with ICAM-1 flanking sequences (IC/R), 5'-TTAGTTGGAATTCCGAGCTCGAGATCTATG-3'; consensus \(\kappa B\) with random flanking sequences (C/R), 5'-AACCCCAAAGGAAAATTCCGATCTGCTGTG-3' (as shown schematically in Fig. 4A).

Transfections and CAT Assays—Subconfluent C32 cell cultures (5 \(\times\) 10⁵ cells/100-mm tissue culture dish) were transfected with 15 \(\mu\)g of plasmid DNA by the calcium phosphate precipitation technique (29). After exposure to precipitated plasmids for 16 h, cells were washed and medium replenished. At 48 h post-transfection, cells were either left untreated or treated with TNF-\(\alpha\) (300 units/ml, R&D Systems) for an additional 16 h. Cells were then harvested and lysates prepared as described previously (30). Assays for transfection efficiency, normalization of protein, and CAT expression were performed as described previously (19, 20). HDMEC were transfected with 20 \(\mu\)g of plasmid DNA (plus 2 \(\mu\)g of pG6S expression vector for cotransfection studies) in the presence of 500 ng/ml DEAE-dextran (Sigma) at 85% confluence for 30 min at 37°C, 5% CO₂. After 30 min, medium containing 8 \(\mu\)g chloroquine was added, and the transfection was continued for an additional 2 h. After exposure to plasmids for 2.5 h, the medium was replenished. At 24 h post-transfection, cells were left untreated or treated with TNF-\(\alpha\) (300 units/ml) for an additional 16 h. Cells were then harvested, and lysates were prepared as above.

Nuclear Extract Preparation—Nuclear extracts were prepared as described previously by Schreiber et al. (31) with modification by Stahl et al. (32). Briefly, after treatment with TNF-\(\alpha\) (300 units/ml), cells were harvested and washed two times with ice-cold PBS. They were then washed once with 400 \(\mu\)l of buffer A (10 mM Tris, pH 8.0, 10 mM KCl, 2 mM MgCl₂, 1 mM DTT, 0.1 mM PMSF, 2 \(\mu g/ml\) aprotinin) and incubated on ice for 10 min with 400 \(\mu\)l of buffer A supplemented with 0.1% Nonidet P-40. Nuclei were pelleted by centrifugation (4000 rpm, 2 min), the supernatant was discarded, and the pellet was resuspended in 400 \(\mu\)l of buffer A without Nonidet P-40. Nuclei were resuspended in 150 \(\mu\)l of buffer C (150 mM HEPES, pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10% glycerol, 2.2 \(\mu g/ml\) aprotinin, 2 \(\mu g/ml\) leupeptin, 1 \(\mu g/ml\) pepstatin A) and incubated on ice for 30 min. Extracts were harvested by centrifugation (14,000 rpm, 15 min, 4°C). The supernatant was collected and stored at −70°C for protein assay and EMSA.

In Vitro Transcription/Translation—Proteins for the transfection factors p50, p65, RelB, c-Jun, ATF2, C/EBPα, C/EBPβ, and C/EBPδ, and the Rel inhibitory protein IκB-α were generated by in vitro transcription/translation of the mammalian expression vectors described above in the T7 Coupled TNT Rabbit Reticulocyte Lysate System (Promega) according to the manufacturer protocol. 3\(-(2\textsuperscript{35}P)\)Sine labeling and autoradiography or Western blotting were used to verify the integrity of the proteins generated (data not shown). For EMSA and UV cross-linking, SBS-PAGE analysis, 2 \(\mu,l\) of a 50\(\mu\)l transcription/translation reaction was used per condition shown.

EMSA—The method for EMSA has been described previously (33, 34). In brief, the binding reactions were performed with 5 \(\mu\g of nuclear extract in binding buffer (12% glycerol, 12 mM HEPES, pH 7.9, 4 mM Tris-Cl, pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF) containing 2 \(\mu g\) of poly(dI-dC) and 5 \(\mu g\) of bovine serum albumin in a
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RESULTS

The ICAM-1 Decameric Modified κB Site Is Insufficient to Confer TNF-α Inducibility—Our previous work has focused on the identification and characterization of the ICAM-1 5′ regulatory region (20). Using ICAM-1-based 5′ deletion CAT reporter gene constructs, we observed that inducibility by TNF-α in C32 cells is conferred by DNA downstream of position −199 (data not shown). These data are in agreement with the results of others (11, 15, 16). Further, a construct with a 5′ deletion to −182 retained no TNF-α inducibility (data not shown). These results demonstrate that the region below −199 and perhaps surrounding −182 is necessary for TNF-α induction of ICAM-1 expression.

Sequence analysis of the ICAM-1 −199/−170 promoter region identifies a modified decameric κB site between positions −186 and −177, on the coding strand in reverse orientation. This sequence differs from the traditional κB consensus sequence in that it contains a p65 binding site but not the requisite OGG for p50 binding. To test whether the region bounded by −199/−182 or a region extending further 3′ to −199 is sufficient to confer TNF-α inducibility upon a heterologous promoter, we cloned these fragments into a thymidine kinase enhancer trap expression vector (19, 21) and assayed their responsiveness to TNF-α in C32 melanoma cells. Fig. 1A demonstrates that the region bounded by −199/−170 is sufficient to confer responsiveness to TNF-α, but the region −199/−182 does not confer TNF-α inducibility. Since the −199/−182 construct includes the modified κB site, it is not surprising that it has no ability to respond to TNF-α (Fig. 1A). Interestingly, the construct containing the modified κB site alone (−182/−177) shows no induction by TNF-α (Fig. 1A) while the construct −191/−172 retains TNF-α responsiveness (although to a lower magnitude than −199/−170 because of its loss of a 5′ C/EBP binding site necessary for maximal induction (35)).

Mutation Analysis of the Minimal ICAM-1 TNF-α-responsive Region Indicates a Requirement for Specific κB Flanking Nucleotides—Since these results suggest that specific flanking sequences surrounding the modified κB site are necessary for TNF-α induction of transcription through this element, a series of single base pair mutations within the modified κB site and the surrounding flanking regions were made. As shown in Fig. 1B, mutations affecting the decameric κB site abolish TNF-α induction of CAT activity and partially repress constitutive CAT activity. Most interestingly, however, mutations affecting either the 5′- or 3′-flanking regions also significantly abrogate or abolish TNF-α-induced CAT activity. These results demonstrate that these specific flanking sequences are necessary for TNF-α induction of ICAM-1 gene expression. Further, these specific flanking sequences, together with the modified κB site, represent the total sequence that is necessary and sufficient for induction of ICAM-1 expression by TNF-α. We have demonstrated similar results in HDMEC for both the minimal region and precise nucleotide sequence (data not shown), indicating that these results are not cell type specific and that induction of ICAM-1 by TNF-α within the context of endothelial cells also requires these flanking regions.

Conversion of the Decameric ICAM-1 Modified κB Site to a Consensus κB Site Confers the Ability to Transactivate Reporter Gene Expression in Response to TNF-α—To test whether the wild-type ICAM-1 modified κB site, which deviates from a
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Fig. 2. Conversion of wild-type ICAM-1 decameric κB site to a consensus κB site restores its ability to mediate transactivation in response to TNF-α. HDMEC were transiently transfected with heterologous promoter CAT reporter gene constructs (−186/−177 TK-CAT, −186/−177 Cmut TK-CAT, −199/−170 TK-CAT, −199/−170 Cmut TK-CAT) and either cotransfected with an expression vector for p65 or treated with TNF-α (300 units/ml for 16 h). Cells were then harvested and assayed for CAT activity. Values are expressed as -fold induction normalized to percent conversion in mock cotransfected or untreated cells. Results shown are the average of duplicate plates and are representative of two experiments.

consensus κB site by a single G→C transition, is unable to drive transcriptional activation in response to TNF-α because of this single nucleotide difference, we undertook site-directed mutagenesis of this base in the heterologous promoter constructs (−186/−177 TK-CAT and −199/−170 TK-CAT). We then tested the ability of these constructs transfected into HDMEC to respond to either TNF-α or p65 cotransfection. Fig. 2 shows that the wild-type −186/−177 TK construct does not respond to either TNF-α or p65 cotransfection. However, when a single base C→G mutation is created at position −177, the resulting −186/−177 Cmut TK-CAT construct, which converts the wild-type decameric ICAM-1 modified κB site to a consensus κB site, is fully capable of responding to TNF-α and, at a somewhat lesser extent than the wild-type construct, to p65 cotransfection. Further, when the same change is made within the context of the −199/−170 construct that is maximally inducible by TNF-α, TNF-α responsiveness is maintained, but induction by p65 cotransfection is also decreased in comparison with the wild-type construct. These data support a transactivation pathway with the wild-type ICAM-1 TNF-α-response region that is optimally driven by p65 homodimers, and conversion of the wild-type ICAM-1 decameric κB site to a consensus κB site results in an element that likely is best driven by p50/p65 heterodimers. These results are also supported by those of Ledebur and Parks (11) who demonstrate the formation of a specific DNA-protein complex, the ICAM-1 TNF-α-response element (−191/−172) was used in an EMSA. Two DNA-protein complexes were formed when labeled probe was incubated with nuclear extracts from TNF-α-treated HDMEC (Fig. 3, bottom complexes). The specificity of these complexes is demonstrated by their competition with excess cold identical oligonucleotide but not with excess cold irrelevant oligonucleotide (data not shown). Both anti-p65 antibody (Fig. 3, lane 5) and anti-p50 antibody (lane 3) supershift these complexes, and irrelevant antibody (lane 8) and antibodies to c-Rel, RelB, and p52 (lanes 4, 6, 7) have no effect. A similar set of results was obtained using untreated or TNF-α-treated nuclear extracts from HUVEC and C32 melanoma cells (data not shown). These results show that a specific DNA-protein complex is formed between TNF-α-treated HDMEC, HUVEC, or C32 nuclear extract proteins NF-κB p65 and NF-κB p50 and the identified TNF-α-response element (−191/−172) of the ICAM-1 gene. Thus, although cotransfection data by us and others (11) strongly suggest that p65 alone best drives expression through the wild-type ICAM-1 TNF-α-responsive region and that p50 serves to inhibit expression through this element, these in vitro binding data indicate that both p65 and p50 can bind to the complete TNF-α-responsive region.

To further explore the ability of Rel proteins to bind both the ICAM-1 TNF-α-responsive region and the comparable consensus probe, we generated in vitro transcription/translation products for p50, p65, RelB, and p52, the non-Rel TNF-α-inducible transcription factors ATF2 and c-Jun, the Rel inhibitor molecule IkB-α, and the high mobility group protein HMG-I(Y). We then used these proteins in EMSA and UV cross-linking/SDS-PAGE studies. When the wild-type ICAM-1 TNF-α-responsive region (−191/−172) was used as a labeled probe in an EMSA, HDMEC nuclear extracts showed two inducible bands when cells had been treated with TNF-α (Fig. 4A, lane 2). These responsive to p65 cotransfection alone and is probably driven optimally by a combination of p65 and p50. What appears to be unique concerning transactivation via the full ICAM-1 TNF-α-responsive region is that specific flanking regions surrounding the wild-type decameric modified κB site are required in order for TNF-α or p65 to drive expression, whereas conversion to a consensus κB site by mutation of a single base removes the necessity of these specific flanking sequences.

The ICAM-1 TNF-α-responsive Element Binds p65—To demonstrate the formation of a specific DNA-protein complex, the newly identified TNF-α-response element (−191/−172) was used in an EMSA. Two DNA-protein complexes were formed when labeled probe was incubated with nuclear extracts from TNF-α-treated HDMEC (Fig. 3, bottom complexes). The specificity of these complexes is demonstrated by their competition with excess cold identical oligonucleotide but not with excess cold irrelevant oligonucleotide (data not shown). Both anti-p65 antibody (Fig. 3, lane 5) and anti-p50 antibody (lane 3) supershift these complexes, and irrelevant antibody (lane 8) and antibodies to c-Rel, RelB, and p52 (lanes 4, 6, 7) have no effect. A similar set of results was obtained using untreated or TNF-α-treated nuclear extracts from HUVEC and C32 melanoma cells (data not shown). These results show that a specific DNA-protein complex is formed between TNF-α-treated HDMEC, HUVEC, or C32 nuclear extract proteins NF-κB p65 and NF-κB p50 and the identified TNF-α-response element (−191/−172) of the ICAM-1 gene. Thus, although cotransfection data by us and others (11) strongly suggest that p65 alone best drives expression through the wild-type ICAM-1 TNF-α-responsive region and that p50 serves to inhibit expression through this element, these in vitro binding data indicate that both p65 and p50 can bind to the complete TNF-α-responsive region.
bands are present in low amounts in untreated cells (lane 1) and are specifically competed by the addition of excess unlabeled DNA (lane 3). In addition, these baseline complexes, as well as TNF-α-induced complexes, are competed by the addition of in vitro-generated IxB-α (lane 4). Of all of the transcription factor proteins generated, only p65 is capable of forming a distinct DNA-protein complex (lane 6) with the wild-type ICAM-1 TNF-α-responsive region, and binding of in vitro-generated p65 is also inhibited by addition of IxB-α (lanes 13 and 14). All other in vitro generated proteins show essentially baseline complex levels formed by the apparent presence of background amounts of a p65-like protein in the rabbit reticulocyte lysate, as indicated by the ability of IxB-α to inhibit formation of this basal complex either alone (lane 17) or when added with other proteins (lanes 12–14). Furthermore, addition of rabbit reticulocyte lysate alone forms the same basal complex that is also competed away with the addition of IxB-α (lane 17). Arrows indicate migration locations of specific p65/probe complexes, and arrowsheads indicate complexes formed with probe and p50. Results shown are representative of three experiments for each panel.

other Rel family members or other TNF-α-inducible transcription factors. Again, IxB-α alone (lane 17) or in combination with other proteins (lanes 12–14) inhibits p65 present in the reticulocyte lysate from forming DNA-protein complexes. In combination with the p65 cotransfection data and EMSA supershift analysis, these data strongly suggest that complex formation is dependent upon the ability of NF-κB, particularly p65, to bind to DNA. They further suggest that the wild-type ICAM-1 TNF-α-responsive region preferentially binds a p65 homodimer while a consensus κB site, created by a single base mutation, binds both p50 and p65 (i.e. a classical NF-κB complex).
flanking sequences (C/R). The sequences for the random flanking nucleotides used in these studies were designed to include a maximum number of mutations relative to the wild-type ICAM-1 flanking sequences. Similar results have been obtained with two additional sets of arbitrarily random flanking sequences (data not shown). When each of these four oligonucleotides was labeled and used as a probe in an EMSA, only IC/R failed to form any TNF-α-induced complexes (Fig. 5B, lanes 4–6). In addition, although IC/IC and the two consensus oligonucleotides (C/IC and C/R) formed TNF-α-induced complexes, the composition of complexes formed with the wild-type ICAM-1 modified kB sequence versus the consensus kB sequence was different, as indicated by their different mobilities (Fig. 5B, lanes 1–3 versus lanes 7–9 and 10–12, arrows). These differences in complex mobility cannot be attributed to differences in oligonucleotide size. Indeed, the IC/IC and C/IC probes are identical in length and differ in sequence only by a single nucleotide. Furthermore, complex mobility is identical with both consensus kB oligonucleotides (C/IC and C/R) though the C/R probe is five nucleotides shorter. These results show that the ICAM-1 specific flanking sequences are required for DNA-protein complex formation to occur. They also suggest that the nature of the DNA-protein complexes that form with the ICAM-1 kB site differs from that which binds to the consensus kB site.

To study the nature of the proteins that interact with these oligonucleotides, each of these four oligonucleotides was labeled and used as probe in UV cross-linking/SDS-PAGE. Similar to our EMSA results, while the ICAM-1 wild-type (IC/IC) and kB consensus (C/IC and C/R) oligonucleotides all demonstrate cross-linking of specific proteins, the IC/R oligonucleotide is incapable of cross-linking any proteins (Fig. 5C, lanes 4–6). Although the exposure shown here is light, all DNA-protein bands present using the consensus probes (lanes 7–9 and 10–12) are also seen with the ICAM-1 wild-type sequence, IC/IC (lanes 1–3). All detected bands are also specific, as shown by incubation of the samples with a mixture of micrococcal nuclease/DNase I after UV cross-linking and before addition of SDS-PAGE loading buffer (data not shown).

Coupled with our reporter assays, these analyses of TNF-α-induced DNA-protein complex formation indicate that specific flanking sequences are required for the ICAM-1 kB site to be biologically functional for both transcription factor optimal binding and activation of transcription. However, these flanking sequences that are required for the ICAM-1 modified kB site to be functionally active become irrelevant when the kB site is changed by one nucleotide to form a classic consensus kB site. Our data also support a model in which specific flanking sequences, coupled to the wild-type ICAM-1 modified kB site, preferentially form a binding site for p65 homodimers, with minimal involvement of p50, while a consensus kB site binds, regardless of flanking sequences, traditional p65/p50 heterodimer (i.e. NF-κB) complexes.

DISCUSSION

The present study identifies the TNF-α-responsive region of the ICAM-1 gene as −191/−172 upstream of the transcription start site by deletional mutation of the ICAM-1 5'-flanking region. Previous studies (11, 15, 16) have also identified this region and have further suggested that a modified kB site contained within the region is sufficient for up-regulation of ICAM-1 expression, as has been shown for the TNF-α responsiveness of other adhesion molecules (36–39). Through mutational analysis, cotransfection studies, supershift EMSA, and in vitro transcription/translation, our results confirm the importance of this modified kB site and demonstrate the involvement of p65 in the DNA binding complex. However, in addition to previous studies, we find that specific 5'- and 3'-flanking sequences are necessary for TNF-α induction and that the wild-type ICAM-1 decameric modified kB site alone is insufficient. Further, conversion of the wild-type ICAM-1 decameric modified kB site to a consensus kB site results in restoration of the ability of the 10-base pair kB site alone to respond to TNF-α but decreases its ability to respond to p65 transfection. The requirement of these specific kB flanking regions for TNF-α responsiveness represents a novel finding for kB-mediated gene transactivation.

The NF-κB proteins p65 and p50, first described in the transcriptional activation of the B cell immunoglobulin gene, form the classical NF-κB complex that binds to the consensus sequence GGGGRNNYYYCC (10). In the promoter of the ICAM-1 gene, however, the identified TNF-α-response element contains a modified kB sequence CGGAAATTC, differing at the first position by a G→C transition. This transition, although relatively uncommon, has also been shown to occur in the promoters of GM-CSF, G-CSF, tissue factor, urokinase, and interleukin-8 genes (40–44). It has been suggested that this modified sequence is capable of selectively binding members of the Rel family other than the classical p65/p50 heterodimer (45). Previous investigators have shown that one complex forming with this ICAM-1 region on EMSA consists of either p65 homodimers (11) or p65/c-Rel heterodimers (15), whereas the other complex consists of the classical p65/p50 heterodimers (11, 15). The present study confirms the preferential involvement of p65, and to a lesser extent p50, in these in vitro complexes by EMSA but fails to demonstrate involvement of c-Rel in either complex using lysates from C32 melanoma cells, HDMEC, and HUVEC. In addition, only p65 in vitro transcription/translation product, and not other Rel protein products including p50, is capable of binding the ICAM-1 minimal TNF-α-responsive region. Further, the ability of p65 co-expression to drive transcriptional activation through the TNF-α-response element supports the involvement of p65 homodimers in TNF-α-induced transcriptional activation.

Our results demonstrate that the molecular requirements for induction of ICAM-1 gene expression by TNF-α are more complex than initially described (11, 15, 16). Ledebur and Parks (11) described a TNF-α-responsive region of −227 to −136, while both van de Stolpe et al. (16) and Jahnke and Johnson (15) further limit the TNF-α-responsive region to −227 to −173 in transcriptional activation studies (CAT assays). All three groups proceed to show that a shorter fragment (−200 to −163 (11) or −190/−173 (15, 16)) is capable of forming protein-DNA complexes on EMSA, but they do not address the ability of these shorter fragments to activate transcription. Most interestingly, when van de Stolpe et al. (16) used a shorter fragment (−189/−174) containing the wild-type modified kB site but deleting portions of the flanking sequences in transcriptional activation studies, these investigators could not demonstrate any TNF-α-inducible increase in reporter gene expression (16). However, an engineered construct containing a tri-tandem repeat of the wild-type ICAM-1 kB site was shown to display TNF-α inducibility (16). Relevant to our present studies, however, this engineered tri-tandem construct coincidentally restored the wild-type specific flanking sequences around the central repeated decameric element, though the contribution of these specific sequences was not further explored (16).

These data, as well as our own initial reporter gene analyses, certainly suggested that the wild-type modified kB site alone may be capable of forming complexes with NF-κB proteins in TNF-α-activated nuclear extracts. However, our subsequent data definitively show that, in the absence of the specific ICAM-1 flanking sequences, the ICAM-1 modified kB site is
incapable of forming DNA-protein complexes (EMSA) or binding any proteins (UV cross-linking/SDS-PAGE). Significantly, conversion of this ICAM-1 modified \( \kappa B \) site to a consensus decameric \( \kappa B \) site restores its ability to mediate transactivation in response to TNF-\( \alpha \), to form DNA-protein complexes even in the absence of any specific flanking DNA sequence, and to bind p50 protein in addition to p65.

The data demonstrating the involvement of specific flanking sequences for the ICAM-1 modified \( \kappa B \) site in TNF-\( \alpha \)-induced ICAM-1 transcriptional activation suggested a potential involvement of additional nuclear proteins or transcription factors. However, our results show that, potentially, five proteins of similar molecular weight and with similar intensity are bound and UV cross-linked to both the full wild-type ICAM-1 probe and both consensus \( \kappa B \) probes, either with ICAM-1-specific or random flanking sequences. While these results suggest that the proteins bound to these probes are the same, they cannot rule out the involvement of additional proteins in the ICAM-1 complex for several reasons. First, the mobility of the complexes formed with the ICAM-1 probe differs from that of the consensus probes on EMSA, which suggests that different proteins may be involved in binding to these probes. Also, UV cross-linking requires protein proximity to DNA and cross-linking to BrdUrd. Thus, additional proteins present in the ICAM-1 complex may not lie in close enough proximity to the DNA to be cross-linked, may not lie near BrdUrd-containing stretches of DNA, or may be sterically prevented from cross-linking by other members of the complex.

The interaction of NF-\( \kappa B \) proteins with other nuclear proteins and transcriptional activators (and repressors) has recently become evident. Precedent for direct protein-protein interaction between transcription factors comes from the discovery of the interaction of AP-1 binding proteins with the glucocorticoid receptor, which down-regulates expression of the individual target genes of both transcription factors (46–48). A similar interaction was proposed for TNF-\( \alpha \)-NF-\( \kappa B \)-mediated gene expression on the basis of a similar down-regulation (42, 49, 50). Recently, Leclair et al. (51) have shown a direct protein-protein interaction of NF-\( \kappa B \), specifically p50, with the interleukin-1-activated transcription factor NF-IL6, and Matsusaka et al. (52) have shown that such an interaction is important in TNF-\( \alpha \) and IL-1 regulation of IL-8 gene expression. In addition, Neish et al. (53) have shown that cooperation of NF-\( \kappa B \) with interferon regulatory factor 1 is necessary for maximal TNF-\( \alpha \) induction of VCAM-1 expression. Furthermore, binding of the high mobility group I(\( Y \)) (HMG-I(\( Y \))) protein in the minor groove of the NF-\( \kappa B \) sequence of the human interferon-\( \beta \) gene (54) and the E-selectin gene (55) is necessary for optimal activation of these genes.

We have explored the involvement of candidate transcription factors in TNF-\( \alpha \)-induced complex formation and transcriptional activation of ICAM-1. HMG I(\( Y \)) binds to AT-rich regions of DNA and has no specific consensus sequence for binding. We have ruled out a possible role for HMG I(\( Y \)) in ICAM-1 transcription regulation in both supershift EMSA and in \textit{vitro} transcription/translation and EMSA. Supershift EMSA does not detect any immunoreactive HMG I(\( Y \)) in the TNF-\( \alpha \)-induced complexes in HDMEC (data not shown). An \textit{in vitro} transcription/translation product for HMG I(\( Y \)) does not bind to the ICAM-1 TNF-\( \alpha \)-responsive region either alone or in combination with other Rel family members (Fig. 4), nor does it enhance Rel protein binding to this region as has been the case for IFN-\( \beta \) and E-selectin (54–55). In addition, a C/EBP binding site located upstream of the modified \( \kappa B \) site in the ICAM-1 5′ regulatory region and binding the factors C/EBP\( \alpha \) or C/EBP\( \beta \) in various homo- or heterodimers has been shown to be necessary for maximal inducibility of ICAM-1 by TNF-\( \alpha \) (56). However, the requirement for flanking sequences described in this manuscript does not rely on interaction of Rel proteins with C/EBP family members since antibodies to these C/EBP proteins (\( \alpha \), \( \beta \), and \( \delta \)) do not supershift EMSA complexes formed with the ICAM-1 TNF-\( \alpha \) minimal response region nor do a consensus C/EBP oligonucleotide or the ICAM-1 C/EBP oligonucleotide compete for TNF-\( \alpha \)-induced complex formation with this probe (data not shown). Finally, the sequences 5′ and 3′ of the ICAM-1 NF-\( \kappa B \) binding site do not resemble any known transcription factor consensus sequence.

Hansen et al. (57) have shown that flanking sequences for the HIV-1 and urokinase promoter NF-\( \kappa B \) binding sites are important in determining the specificity of NF-\( \kappa B \) interactions at those sites. Specifically, these flanking sequences dictate c-Rel/RelA binding and thus control transcriptional activation through these specific Rel family members. Our results support the results of that study in that specific flanking sequences for the ICAM-1 modified \( \kappa B \) site allow p65 (RelA) homodimer binding. They further demonstrate that these specific flanking sequences are absolutely required for DNA-protein complex formation with the modified \( \kappa B \) site and transcription activation through the ICAM-1 TNF-\( \alpha \)-responsive region. Unlike the study by Hansen et al. (57), however, in the context of a consensus \( \kappa B \) site (like that present in HIV-1), flanking sequences are dispensable both for DNA-protein complex formation and transcriptional activation in response to TNF-\( \alpha \). Further, in either the presence or absence of the ICAM-1 flanking sequences, the consensus \( \kappa B \) site bound p65/p50 heterodimers (classic NF-\( \kappa B \)) with high affinity. Thus, specific flanking sequences for the ICAM-1 TNF-\( \alpha \)-responsive region are unable to confer p65 homodimer binding on a consensus \( \kappa B \) site.

The present study has established a TNF-\( \alpha \)-responsive region for the ICAM-1 gene, which contains a modified \( \kappa B \) site and interacts with the Rel family member p65. However, this modified \( \kappa B \) site, while necessary, is not sufficient for TNF-\( \alpha \)-mediated up-regulation of gene expression. We demonstrate a novel requirement for specific 5′- and 3′-flanking sequences surrounding this modified \( \kappa B \) site. Further, we demonstrate that the G→C transition present in the ICAM-1 \( \kappa B \) site dictates binding of p65 homodimers. The requirement for specific 5′- and 3′-flanking sequences and the single-base modification make ICAM-1 a member of a unique subset of \( \kappa B \)-regulated genes. Because ICAM-1 is an attractive target for therapeutic intervention as a result of its involvement in the pathogenesis of various inflammatory processes, understanding the precise mechanisms by which its expression is regulated is important. The present study further refines the current understanding of the subtleties of gene-specific transcriptional activation by the pleiotropic and rather promiscuous group of transcriptional activators, the Rel family, possibly allowing for the development of highly specific therapeutics for intervention in inflammatory processes.

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