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Endothelial Interferon Regulatory Factor 1 Cooperates with NF-κB as a Transcriptional Activator of Vascular Cell Adhesion Molecule 1

ANDREW S. NEISH,[1] MARGARET A. READ,[1] DIMITRIS THANOS,[2]† RICHARD PINE,[3] TOM MANIATIS,[2] AND TUCKER COLLINS[1]*

Vascular Research Division, Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115[1]; Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138[2]; and Public Health Research Institute, New York, New York 10016[3]

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Transcription of the vascular cell adhesion molecule 1 (VCAM-1) gene in endothelial cells is induced by lipopolysaccharide and the inflammatory cytokines interleukin-1β and tumor necrosis factor alpha (TNF-α). Previous studies have demonstrated that tandem binding sites for the inducible transcription factor NF-κB are necessary but not sufficient for full cytokine-mediated transcriptional activation. Herein, we demonstrate that full cytokine-induced accumulation of *VCAM1* transcript requires protein synthesis. We report the definition of a functional regulatory element in the *VCAM1* promoter interacting with the transcriptional activator interferon regulatory factor 1 (IRF-1). DNA-protein binding studies with endothelial nuclear extracts revealed that IRF-1 is cytokine inducible and binds specifically to a consensus sequence motif located 3′ of the TATA element. We have identified heterodimeric p65 and p50 as the NF-κB species binding to the *VCAM1* promoter in TNF-α-activated endothelial cells. Experiments with recombinant proteins showed that p50/p65 and high-mobility-group I(Y) protein cooperatively facilitated the binding of IRF-1 to the *VCAM1* IRF binding site and that IRF-1 physically interacted with p50 and with high-mobility-group I(Y) protein. Transient transfection assays in endothelial cells showed that overexpressed IRF-1 resulted in superinduction of TNF-α-stimulated transcription. Site-directed mutations in the IRF binding element decreased TNF-α-induced activity and totally abolished superinduction. Cotransfection assays in P19 embryonal carcinoma cells revealed that IRF-1 synergized with p50/p65 NF-κB to activate the *VCAM1* promoter or heterologous promoter constructs bearing isolated *VCAM1* NF-κB and IRF binding motifs. Cytokine inducibility of *VCAM1* in endothelial cells utilizes the interaction of heterodimeric p50/p65 proteins with IRF-1.

Vascular cell adhesion molecule 1 (VCAM-1) is a 110-kDa member of the immunoglobulin gene superfamily first described as a cytokine-inducible endothelial adhesion molecule (49, 59). VCAM-1 binds circulating monocytes and lymphocytes expressing the integrins α4β1 (very late antigen 4) and α4β7 (4, 6, 7, 14, 40, 60, 71) and may participate in the recruitment of these chronic inflammatory cells from the bloodstream to sites of tissue injury. Vascular expression of VCAM-1 is found associated with a variety of inflammatory processes (61) and in atherosclerotic lesions (11, 47). VCAM-1 is present on synovium and mesothelium in chronic inflammatory conditions and may also mediate recruitment of monocytic leukocytes to these tissues (29, 45). VCAM-1 is constitutively expressed on follicular dendritic cells in the normal human lymph node (15, 33) and on bone marrow stromal cells in the mouse (43), possibly mediating immune cell interactions and B-cell development. VCAM-1 also binds tumor cells, suggesting a role in metastasis (59). Finally, VCAM-1 has been implicated in skeletal myogenesis and in the early development of extraembryonic tissues (23, 62).

VCAM-1 has a distinct pattern of cytokine induction in cultured endothelial cells. Naive cells do not express *VCAM-1* message; however, exposure to inflammatory mediators or cytokines [lipopolysaccharide (LPS), poly(I-C), interleukin-1β, and tumor necrosis factor alpha (TNF-α)] results in rapid upregulation (39, 49). *VCAM1* message levels reach a sustained high level by 2 to 3 h and then gradually diminish over several days (49, 57). In contrast, the neutrophil-specific adhesion molecule, E-selectin, is highly expressed by 1 h and is transcriptionally silent by 24 h (2). The relatively delayed and sustained VCAM-1 response to cytokine may correspond to the switch to preferential adhesion and infiltration of mononuclear leukocytes typical of chronic inflammatory processes (5, 48).

In an effort to understand the molecular mechanisms controlling the VCAM-1 response to cytokines, we characterized the structure of the VCAM-1 gene (10) and defined the *VCAM1* 5′ flanking region (46). Nuclear runoff assays revealed no *VCAM1* message in unstimulated endothelial cells and rapid upregulation within 2 h after TNF-α induction. Transient transfection experiments with segments of *VCAM1* 5′ flanking sequence coupled to a chloramphenicol acetyltransferase (CAT) reporter defined a −258-bp region capable of directing full cytokine-induced gene expression. By mutational analysis, we and others have found that activation of VCAM-1 requires two tandem binding sites for NF-κB located in the basal *VCAM1* promoter at positions −73 and −58, both of which are necessary for cytokine-mediated transcriptional response (27, 46, 66).

NF-κB is a ubiquitous cellular transcription factor variably found in both constitutively active and inducible forms (reviewed in reference 22). Endothelial cells utilize NF-κB as a rapidly inducible transcriptional activator (8). In quiescent cells, the factor is sequestered in the cytoplasm by a specific inhibitor, IκB-α (MAD3). Multiple stimuli, including inflam-

* Corresponding author. Mailing address: Department of Pathology, Brigham and Women's Hospital, 221 Longwood Ave., Boston, MA 02115. Phone: (617) 732-5990. Fax: (617) 732-5933.

† Present address: Department of Biochemistry and Molecular Biophysics, Columbia University, New York, N.Y.

matory cytokines, result in phosphorylation and/or degradation of IκB-α, which allows release and nuclear translocation of the active protein (20). The DNA binding form of NF-κB is a dimer, composed of protein species sharing the Rel homology domain. Originally described as a heterodimer of p50 (NF-κB1) and p65 (RelA), additional Rel family members, such as c-Rel, p52 (NF-κB2), and RelB, have been identified. Rel proteins form combinations of homo- and heterodimers, each with potentially different binding and activation specificities (17, 34, 53). Using transactivation assays with overexpressed Rel proteins, we have shown that p65 can activate the *VCAM1* promoter in a manner dependent on both NF-κB binding sites. The Rel protein p50 positively synergized with p65, while p52 acted as an inhibitor (66). The NF-κB species interacting with the *VCAM1* promoter in cytokine-activated endothelial cells are not known.

NF-κB has been implicated in the activation of many genes (reviewed in reference 22). In most of these inducible promoters, however, NF-κB is necessary but not sufficient for activation. Current models postulate that combinatorial interactions of multiple transcription factors are required for appropriate regulation of a given promoter. A well-studied example is the induction of the beta interferon (IFN-β) gene (reviewed in reference 37). *IFN*-β message is rapidly upregulated by virus, peaks within 6 to 12 h, and rapidly declines. This response is mediated, in part, by an NF-κB binding site at −66 (PRD II) in the basal promoter. Mapping of functional domains in the *IFN*-β promoter revealed additional discrete sequence motifs required for full activation. Immediately 5′ of the *IFN*-β NF-κB site are two tandem sites (PRD I and III) necessary for promoter activation. These binding motifs interact with interferon regulatory factor 1 (IRF-1), a phosphoprotein transcription factor implicated in the activation of *IFN*-β and other cytokine-inducible genes (16, 44, 54, 55). IRF-1, also known as IFN-stimulated gene factor 2 (ISGF2) (55), and its physiologic repressor, IRF-2 (24), bind to the same motif, IRF-E [G(A)AAANNGAAA(G/C)(T/C)] (32, 69). Because of the presence of this sequence motif in the *VCAM1* promoter, we sought to implicate IRF-1 in the regulation of VCAM-1.

This report characterizes the Rel species interacting with the NF-κB sites in the *VCAM1* promoter and describes a functional IRF-1 sequence motif in the *VCAM1* core promoter. We demonstrate that full cytokine-induced accumulation of *VCAM1* transcript requires protein synthesis. We show that TNF-α stimulates the endothelial expression of nuclear IRF-1 and demonstrate that IRF-1 can modulate the *VCAM1* response to cytokine. In addition, we provide evidence showing NF-κB and IRF-1 exhibit cooperative DNA binding and transactivational properties. The presence of interacting NF-κB and IRF-1 sites in the *VCAM1* and *IFN*-β promoters suggests a common theme in inducible gene expression.

MATERIALS AND METHODS

Cell culture and cytokine treatment. Human endothelial cells obtained from collagenase-digested umbilical veins (HUVEC) (21) were cultured in M199 with 20% fetal bovine serum, 100 μg of porcine intestinal heparin per ml, 50 μg of endothelial mitogen per ml, 50 U of penicillin per ml, and 50 μg of streptomycin per ml, and 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) in gelatin-coated plates. Bovine aortic endothelial cells (BAEC) were isolated and maintained in culture as previously described (46). P19 embryonal carcinoma cells were maintained in Dulbecco modified Eagle medium with 7.5% bovine serum, 2.5% fetal bovine serum, glutamine, penicillin, and streptomycin as specified above.

For experimental cytokine induction, confluent monolayers of endothelial cells were exposed to recombinant human TNF-α (Genentech, San Francisco, Calif.) at a final concentration of 200 U/ml in complete media.

Northern (RNA) blot. Confluent HUVEC monolayers were treated with cytokine as described above. Cycloheximide (10 μg/ml) was added directly to the media 20 min prior to cytokine stimulation. Total RNA was extracted with TRIzol reagent (Gibco BRL, Gaithersburg, Md.) and was analyzed by Northern hybridization analysis as described previously (57). Blots were hybridized with ^{32}P-labeled cDNA probes encoding VCAM-1 (10), E-selectin (2), and glyceraldehyde phosphate dehydrogenase.

Nuclear extracts. Following experimental treatment of HUVEC, nuclear extracts were prepared as described previously (72). Monolayers (3×10^7 to 5×10^7 cells) were harvested by scraping, washed in cold phosphate-buffered saline, and incubated in 2 packed cell volumes of buffer A (10 mM HEPES [pH 8.0], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol [DTT], 200 mM sucrose, 0.5 mM phenylmethanesulfonyl fluoride [PMSF], 1 μg each of leupeptin and aprotinin per ml, 0.5% Nonidet P-40) for 5 min at 4°C. The crude nuclei released by lysis were collected by microcentrifugation (15 s), rinsed once in buffer A, and resuspended in 2/3 packed cell volume of buffer C (20 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM PMSF, 1.0 mM DTT, 1.0 μg each of leupeptin and aprotinin per ml). Nuclei were incubated on a rocking platform at 4°C for 30 min and clarified by microcentrifugation for 5 min. The resulting supernatants were diluted 1:1 with buffer D (20 mM HEPES [pH 7.9], 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, 0.5 mM PMSF, 1 μg each of leupeptin and aprotinin per ml). Nuclear extracts were frozen on dry ice and stored at −80°C.

Electrophoretic mobility shift assay (EMSA). Double-stranded oligonucleotides were end labeled with [α-^{32}P]dCTP (50 μCi at 3,000 Ci/mmol; New England Nuclear, Boston, Mass.) and the Klenow fragment of *Escherichia coli* DNA polymerase I. Binding reaction mixtures in 20 μl contained 15 μg of nuclear extract protein, binding buffer [10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, 1 μg of poly(dI-dC), 1 μg of salmon sperm DNA, 50,000 cpm of labeled DNA]. Reaction mixtures were incubated at room temperature for 20 min and analyzed by electrophoresis on a 4% nondenaturing polyacrylamide gel at 180 V for 2 h in 45 mM Tris-borate-EDTA as described by Ausubel et al. (1). Following electrophoresis, gels were dried and DNA-protein complexes were localized by autoradiography for 18 h. Competition studies were performed by adding unlabeled double-stranded oligonucleotides to the binding reaction mixture for 10 min prior to the addition of labeled oligonucleotide. For supershift analysis, nuclear extracts from TNF-α-treated endothelial cells were incubated with 1 μl of antisera or nonimmune rabbit serum for 15 min at room temperature prior to addition of binding buffer containing labeled oligonucleotide. Samples were subjected to electrophoresis as described above.

The oligonucleotides used were VCAM-NF-κB (5′-TGCCCTGGGTTTCCCCTTGAAGGGATTTCCCTCC), VCAM-IRF (5′-gatcGGAGTGAAATAGAAAGTCTGTG), VCAM-mIRF1 (5′-gatcGGAGT**t**AAATAG**c**AAGTCTGTG), VCAM-mIRF2 (5′-gatcGGAGTGA**c**ATAGA**g**AGTCTGTG), IFN-β–IRF (5′-gatcAAGTGAAAGTGAAAGTGAAATGTA), and nonspecific (5′-aagcATCGTGGATAGGACAGAAGC). Mutated bases are in boldface lowercase. Reverse complement strands were designed to leave GATC overhangs on both ends. All oligonucleotides were polyacrylamide gel purified prior to annealing and labeling.

UV cross-linking. Photoreactive labeled DNA probes were prepared by annealing coding- or noncoding-strand templates with complementary primers and filling in with the Klenow fragment of DNA polymerase 1 in the presence of [α-^{32}P]dATP, [α-^{32}P]dCTP, dGTP, and 1:1 dTTP and 5-bromo-2′-deoxyuridine 5′-triphosphate (BrdU; Sigma Chemical Co., St. Louis, Mo.) (3). Two probes were designed to incorporate BrdU into the 5′ and 3′ κB sites, respectively: VCAM 5′, 5′-CCCTGGCTCTGCCCT-3′ and 5′-tcgaTTCAAGGGGAAACCCAGGGCAGAGCCAGGG-3′; and VCAM-3′, 5′-agctTTGAAGGGATTTCCCTCCGCCTCTGCAA-3′ and 5′-TTGCAGAGGCGGAGGG-3′. DNA binding reactions were performed in 20 μl as described above but with the BrdU-substituted probe. Samples were UV irradiated for 15 min with a transilluminator (Fotodyne Inc., New Berlin, Wis.). For immunoprecipitation, radiolabeled adducts were diluted in 180 μl of radioimmunoprecipitation assay buffer (10 mM Tris-Cl [pH 8.0], 150 mM NaCl, 0.5% sodium dodecyl sulfate [SDS], 1% Nonidet P-40, and 1% deoxycholate supplemented with 200 μg of bovine serum albumin [BSA] per ml, as well as 1 mM PMSF and 1 μg each of leupeptin, aprotinin, and pepstatin A per ml) and incubated with 1 μl of antiserum (1 h on ice) followed by 20 μl of protein A-Sepharose (1 h on ice). Immune complexes were washed three times with radioimmunoprecipitation assay buffer and analyzed on SDS–8% polyacrylamide gels. Duplicate sets of radiolabeled adducts were denatured prior to immunoprecipitation by heating at 100°C for 3 min in 0.5% SDS.

Expression and purification of bacterially expressed proteins. IRF-1, high-mobility-group I protein (HMGI), p65, and a region of the p105 precursor were expressed in bacteria and purified as described previously (13, 70). Binding reactions for recombinant proteins were performed in 10 mM Tris-Cl (pH 7.5), 50 mM NaCl, 1 mM DTT, 1 mg of BSA per ml, 5% glycerol, and 50,000 cpm of labeled oligonucleotide. For EMSA experiments with HMGI facilitation of IRF-1 binding, recombinant HMG1 was preincubated with probe and binding buffer for 5 min at room temperature prior to addition of recombinant IRF-1 (rIRF-1). Incubations and gel analysis were performed as described above.

DNase I footprinting. A *VCAM1* promoter fragment spanning −258 to +42 was single end labeled and incubated with the indicated proteins in 20 μl of binding buffer consisting of 10 mM Tris (pH 8), 15 mM HEPES (pH 7.9), 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, and 1 mg of BSA per ml on ice for 30 min.

DNase I (Worthington) was diluted in 50 mM $CaCl_2$–20 mM HEPES (pH 7.9) to 2 ng/ml, and 2 μg was added directly to the binding reaction mixture. Digestion for 5 min on ice was followed by the addition of 200 μl of stop buffer (2.5 M ammonium acetate, 25 μg of sonicated salmon sperm DNA per ml). The DNA was ethanol precipitated, dried, resuspended in 3 μl of formamide dye, and loaded on a 6% sequencing gel.

Plasmid constructions. A fusion gene carrying the region from −285 to +42 of the *VCAM1* promoter coupled to a CAT reporter gene (pF2) was described and characterized earlier (46). Constructs bearing internal point mutations in the IRF site, pF2-mIRF1 and pF2-mIRF2, were made from two separate PCR amplification products as previously described (46). The amplified products were gel purified and subcloned into the *Sma*I site of the reporter plasmid pCAT3. Mutagenizing oligonucleotides used for construct generation were VCAM-RP-IRF1 (CTTTATAAAGGGTCTTGTTGCAGAG), VCAM-FP-mIRF1 (CACA GACTTgCTATTTaACTCC), and VCAM-FP-mIRF2 (CACAGACTcTCTAT gTCACTCC). Mutated bases are in lowercase.

Reporter constructs containing *VCAM1* NF-κB and IRF sites were generated with double-stranded synthetic oligonucleotides. Cassettes were subcloned into a blunted *Bgl*II site upstream of a minimal simian virus 40 promoter-driven CAT reporter, pCAT-Promoter (Promega), either in isolation or in combination. Oligonucleotides used for construct generation were vNFx2 (TGCCCTGGGTT TCCCCTTGAAGGGATTTCCCTCCCGG) and v2IRF (GTGAAATAGAAA GTCGTGAAATAGAAAGTC). The introduction of desired mutations of all reporter constructs was confirmed by dideoxynucleotide sequencing.

cDNA encoding the full-length murine p65 and p105 were generously provided by Michael Leonardo. cDNAs were subcloned in the sense orientation in the eukaryotic expression plasmid pcDNA (Invitrogen, San Diego, Calif.). The cDNA encoding the full-length murine p105 was truncated at the *Xho*I site at bp 1781 before subcloning into pcDNA.

Human IRF-1 and IRF-2 cDNAs were subcloned into pcDNA as described previously (50).

DNA sequencing. Nucleotide sequence was determined by the dideoxynucleotide chain termination method with modified T7 polymerase (United States Biochemical, Cleveland, Ohio) and [α-^{35}S]dATP (New England Nuclear). Oligonucleotide primers were synthesized on a model 381A oligonucleotide synthesizer (Applied Biosystems, Foster City, Calif.).

Transfections and CAT assays. BAEC and P19 cells were transfected by a modified calcium phosphate technique (65). Cells were transfected with 10 μg of reporter plasmid and variable quantities of expression plasmids. All cotransfection reactions were balanced for total amount of activator DNA with pcDNA vector. Relative transfection efficiency was determined by cotransfection with pTK-GH (Promega) (5 μg). After transfection, cultures were washed twice with Hanks balanced salt solution and refed with complete Dulbecco modified Eagle medium with or without TNF-α. After 24 h of incubation, samples of the medium were collected and assayed for growth hormone by using a commercially available radioimmunoassay kit (Nichols Institute Diagnostics, San Juan Capistrano, Calif.), and cells were harvested for CAT assay. Confluent monolayers were collected by trypsin dissociation, centrifuged (650 × *g* for 5 min), washed once in phosphate-buffered saline, and resuspended in 0.2 ml of 0.25 M Tris-HCl (pH 7.8) with 10 μg of aprotinin (Sigma) per ml. Cells were lysed by sonication and one freeze-thaw cycle. Supernatants containing the cell extracts were obtained after centrifugation for 10 min at 14,000 × *g* and stored at −80°C. Conversion of radiolabeled acetyl coenzyme A to acetylated chloramphenicol was assayed by the two-phase fluor diffusion technique (65). For each sample, 30 to 50 μl of cell extract was incubated at 65°C for 15 min to inactivate endogenous transacetylases. The assay was performed in a reaction mixture containing 1.25 mM chloramphenicol (Sigma), 125 mM Tris HCl (pH 7.8), and 0.1 μCi of ^{3}H-labeled acetyl coenzyme A (New England Nuclear) in a reaction volume of 0.2 ml. The reaction mixture was overlaid with 5 ml of water-immiscible scintillation fluid (Econofluor; Dupont, Wilmington, Del.) and incubated at 37°C for 2 h. The activity of the ^{3}H-labeled acetylated chloramphenicol was measured on an LKB Rackbeta scintillation counter.

Transfection experiments were repeated at least three times, using at least two independent plasmid preparations. A negative control, the promoterless plasmid pCAT3, showed no inducible activity from any experimental manipulation.

Antisera. Antisera to the Rel proteins p65 (N and C termini), p50, and cRel were generously provided by Nancy Rice (National Cancer Institute, Frederick, Md.) and Warner Greene (University of California, San Francisco). Antisera to p52 and RelB were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.). Antiserum to IRF-1/ISGF2 was raised against protein purified from IFN-α-treated HeLa cells as described previously (55).

Western blotting (immunoblotting). Nuclear extracts from TNF-α-treated HUVEC were electrophoresed on SDS–8% polyacrylamide gels and transferred to nitrocellulose in 25 mM Tris–192 mM glycine–5% methanol at 100 V for 1 h. IRF-1/ISGF2 antiserum was used at a 1:20,000 dilution. Immunoreactive proteins were detected according to the enhanced chemiluminescence protocol (Amersham, Arlington Heights, Ill.), using 1:10,000 horseradish peroxidase-linked donkey anti-rabbit secondary antiserum. Blots were exposed to film for 1 min.

In vitro translation and protein-protein interaction. In vitro translation was carried out with nuclease-treated rabbit reticulocyte lysate as described by the supplier (Promega). Full-length cDNAs encoding p50, HMGI, and IRF-1 were cloned in frame to glutathione *S*-transferase (GST)-expressing plasmids (13). GST alone or its fusion derivatives immobilized to glutathione-agarose beads were incubated with in vitro-translated ^{35}S-labeled proteins for 2 h at 4°C in binding buffer (150 mM NaCl, 10 mM Tris [pH 8.0], 0.2% Nonidet P-40, 0.2% BSA) and then washed three times with binding buffer and once with binding buffer but without BSA. Interactions were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

RESULTS

The Rel proteins p50 and p65 interact with the *VCAM1* promoter in TNF-α-induced endothelial cells. To characterize the specific Rel protein(s) involved in TNF-α induction of *VCAM1*, we studied the interaction of nuclear proteins derived from induced HUVEC with the *VCAM1* NF-κB sites. Two modalities were used; first, EMSAs were performed with an oligonucleotide spanning both NF-κB sites (designated VCAM-NF-κB) (Fig. 1A). TNF-α induced two specific complexes (Fig. 1A; compare lanes 1 and 2). Specificity was determined by competition experiments with wild-type and mutant oligonucleotides (data not shown). One constitutive nonspecific complex also formed. Similar complexes from endothelial cell nuclear extracts have been observed with other NF-κB sites (57, 72). Extracts were incubated with 1 μl of antisera to p50, N- and C-terminal p65, and c-Rel and with normal rabbit serum before the EMSA. Anti-p50 caused a supershift of both induced complexes (lane 4), while anti-N- and C-terminal p65 supershifted only the upper complex (lanes 5 and 6). Anti-c-Rel (lane 7), anti-p52 (data not shown), and anti-RelB (data not shown) failed to affect complex formation.

To further characterize the VCAM-1 NF-κB-binding proteins induced by TNF-α, UV cross-linking analysis was performed with probes containing either the 5′ or 3′ NF-κB site (Fig. 1B and C). Photoreactive forms of the probes were incubated with nuclear extracts from TNF-α-treated endothelial cells. The resulting protein-DNA adducts were immunoprecipitated with antisera to p65, p50, p52, c-Rel, and RelB. With both the 5′ and 3′ probes, antisera to N- and C-terminal p65 identified a 70- to 75-kDa adduct which migrated as a doublet, corresponding to the predicted mobility of p65 covalently bound to the oligonucleotide (Fig. 1B and C, lanes 2 and 3). Anti-p65 coimmunoprecipitated 50- to 55-kDa adducts, which correspond to p50 covalently bound to the oligonucleotide. Denaturation prior to immunoprecipitation with anti-p65 eliminated the 50- to 55-kDa adducts, indicating that these were p65 associated (Fig. 1B and C, lanes 9 and 10). Similarly, immunoprecipitation with anti-p50 resulted in the expected 50- to 55-kDa adducts, as well as the 70- to 75-kDa adducts identified by anti-p65 (Fig. 1B and C, lanes 4). The p65 adducts coimmunoprecipitated with anti-p50 were eliminated with the denaturation step (Fig. 1B and C, lanes 11). With both probes, the intensity of the cross-linking signal observed with p50 was much greater than that seen with p65. This probably reflects higher cross-linking efficiency with p50, due either to the location of the photoreactive substitutions or to higher binding affinity of p50 than of p65. Consistent with the supershift data, no radiolabeled adducts were identified with anti-p52 (Fig. 1B and C, lanes 5). Surprisingly, anti-c-Rel and anti-RelB both coimmunoprecipitated small amounts of the p50 adducts (Fig. 1B and C, lanes 6 and 7), and with both antisera, the p50 adducts were eliminated by denaturation prior to immunoprecipitation (Fig. 1B and C, lanes 13 and 14). Radiolabeled adducts consistent with the molecular weights of c-Rel and RelB were not observed with either probe. Our ability to detect these adducts is probably limited because endothelial cells express very low nuclear levels of c-Rel (57) and RelB (57a).

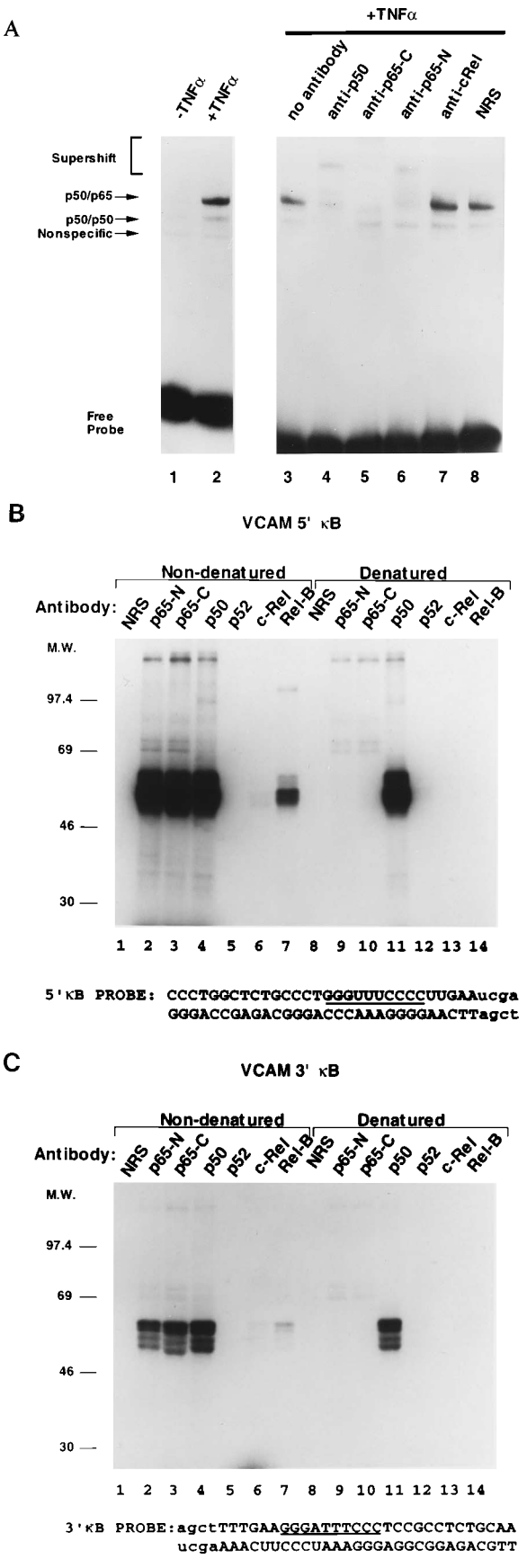

FIG. 1. Heterodimeric p50/p65 interacts with both of the *VCAM1* NF-κB sites in TNF-α-induced endothelial cells. (A) Supershift analysis of DNA-binding proteins. Nuclear extracts from control (lane 1) or TNF-α-treated (100 U/ml, 2 h) endothelial cells (lanes 2 to 8) were incubated with normal rabbit serum (NRS; lane 8) or antiserum to p50 (lane 4), p65 (lanes 5 and 6), or c-Rel (lane 7) before the assay for binding to an oligonucleotide containing the two *VCAM1* NF-κB sites. (B and C) UV cross-linking analysis of *VCAM1* NF-κB sites. Nuclear extracts from TNF-α-treated endothelial cells were incubated with photoreactive forms of the *VCAM1* 5′ and 3′ κB sites. Protein-DNA adducts were immunoprecipitated with normal rabbit serum (lanes 1 and 8) or antiserum to p65 (lanes 2, 3, 9, and 10), p50 (lanes 4 and 11), p52 (lanes 5 and 12), c-Rel (lanes 6 and 13), and RelB (lanes 7 and 14). In lanes 8 to 14, protein-DNA adducts were subjected to denaturation prior to immunoprecipitation. The resultant immune complexes were analyzed by SDS-PAGE (8% polyacrylamide gel).

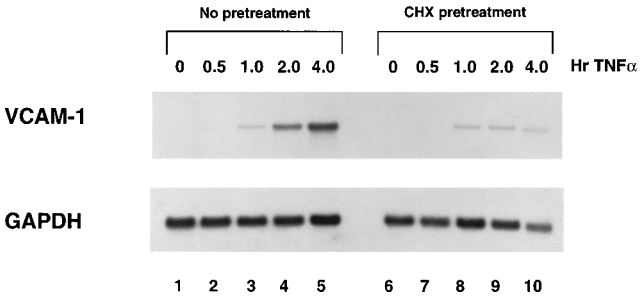

FIG. 2. Cycloheximide pretreatment diminishes TNF-α-induced *VCAM1* transcript accumulation. Shown is Northern analysis of total RNA from HUVEC stimulated with TNF-α (100 U/ml) for the indicated times. Cycloheximide (10 μg/ml) was added to cells 20 min prior to cytokine stimulation. GAPDH, glyceraldehyde phosphate dehydrogenase.

Likewise, because of the low levels present, supershift analysis did not identify complexes containing either of these proteins. From these data, we conclude that both the 5′ and 3′ *VCAM1* NF-κB sites bind predominantly heterodimeric p50/p65 and lesser amounts of p50 homodimer, p50/c-Rel, and p50/RelB during TNF-α-mediated endothelial cell activation.

Full cytokine induction of the *VCAM1* transcript requires protein synthesis. NF-κB is activated by posttranslational, protein synthesis-independent mechanisms. To determine whether the induction of the *VCAM1* transcript requires additional, protein synthesis-dependent factors, HUVEC were stimulated with TNF-α in the presence and absence of cycloheximide and the levels of *VCAM1* mRNA were analyzed by Northern blot analysis. In the absence of TNF-α, no endogenous *VCAM1* message is detected in unstimulated HUVEC (Fig. 2, lanes 1 and 6). Addition of TNF-α results in the appearance of the *VCAM1* transcript within 30 min, with progressive accumulation over 1, 2, and 4 h (lanes 2 to 5). In the presence of cycloheximide, lower levels of transcript accumulation were seen (lanes 7 to 10). Transcript levels of constitutively expressed glyceraldehyde phosphate dehydrogenase were not significantly affected by either TNF-α or cycloheximide treatment. From this experiment, we conclude that protein synthesis is required for full TNF-α induction of *VCAM1* mRNA. This observation is consistent with the presence of protein synthesis-dependent activators playing a role in *VCAM1* transcription.

IRF-1 specifically interacts with an element of the *VCAM1* promoter. Inspection of the human *VCAM1* promoter sequence revealed the sequence GAAATAGAAAGT located at −2 to −13, which fits the consensus (GAAANNGAAAGT) for an IRF binding element. This motif had 100% homology with the corresponding region of the murine *VCAM1* promoter (9). Table 1 shows the DNA sequence homology of the

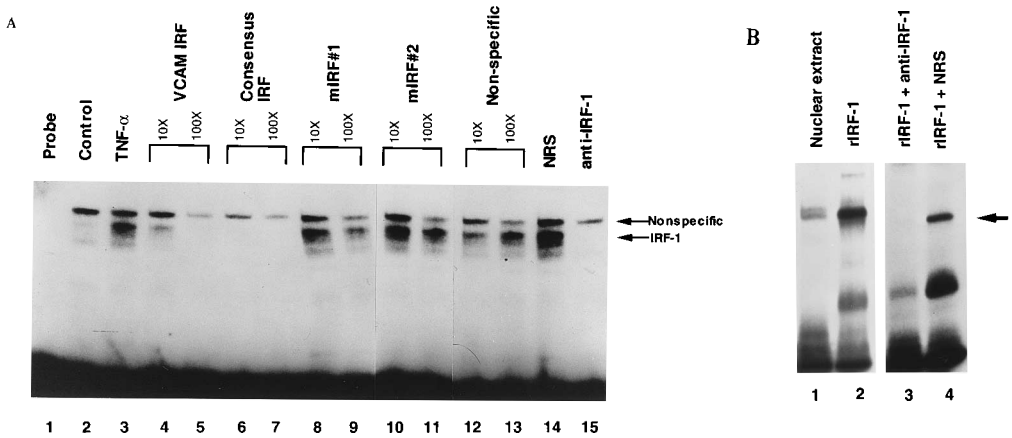

FIG. 3. Specific binding of IRF-1 to an element in the *VCAM1* promoter. (A) EMSA with labeled *VCAM1* IRF binding site and HUVEC nuclear extracts. Nuclear extracts were prepared from control (lane 2) or TNF-α-treated (100 U/ml for 2 h; lanes 3 to 15) HUVEC and assayed for binding to the *VCAM1* IRF element in the absence or presence of the indicated excess unlabeled competitor oligonucleotide or antiserum. Competition was seen with wild-type *VCAM1* IRF (lanes 4 and 5) and consensus IRF (lanes 6 and 7) but not with mutant oligonucleotides VCAM-mIRF1 (lanes 8 and 9) and VCAM-mIRF2 (lanes 10 and 11) or an irrelevant oligonucleotide (lanes 12 and 13). Normal rabbit serum (NRS; lane 14) did not affect complex formation, while antisera to IRF-1 abolished the specific complex labeled with an arrow and IRF-1 (lane 15). (B) EMSA with nuclear extracts and rIRF-1. *VCAM1* IRF binding sites probes were incubated with TNF-α-treated HUVEC nuclear extract (lane 1), rIRF-1 (lane 2), rIRF-1 preincubated with anti-IRF-1 (lane 3), or normal rabbit serum (NRS; lane 4).

VCAM1 IRF element to other functional IRF binding elements.

To demonstrate the presence of DNA binding activity interacting with the *VCAM1* IRF element in endothelial cells, EMSAs were performed with nuclear extracts derived from unstimulated and TNF-α-stimulated HUVEC. Nuclear extracts incubated with radiolabeled *VCAM1* IRF probes formed two complexes when visualized on a nondenaturing gel (Fig. 3A). The faster-mobility complex is present in trace amounts in uninduced nuclear extract and in greater amounts in TNF-α-induced extract (Fig. 3A; compare lanes 2 and 3). Competition experiments reveal that the lower band is effectively competed for by excess unlabeled probe (lanes 4 and 5) and competed for with greater affinity by a double-stranded oligonucleotide composed of two tandem consensus IRF binding sites (lanes 6 and 7). Double-stranded oligonucleotides bearing point mutations in the *VCAM1* IRF element, or an oligonucleotide with a totally unrelated sequence, do not significantly compete (lanes 8 to 13). The retarded band of slower mobility is not competed for by unlabeled probe and is therefore considered a nonspecific DNA-protein interaction.

To determine the identity of protein(s) interacting with the *VCAM1* IRF element, supershift analysis was performed. EMSA (Fig. 3A, lane 15) shows that the specifically bound nucleoprotein complex can be completely abolished by preincubation with a well-characterized anti-IRF-1/ISGF2 antiserum (51, 55), demonstrating the presence of an IRF-1 immunoreactivity bound to the *VCAM1* IRF element. In contrast, the nonspecific complex is not affected by this antibody. The lack of any residual complex suggests that IRF-1 alone is present under the conditions studied. Preincubation with nonimmune rabbit serum had no effect on the shifted band (lane 14). As controls, we show that recombinant IRF-1 comigrates with the specific nucleoprotein complex (Fig. 3B, lanes 1 and 2), and rIRF-1 is supershifted itself with the anti-IRF-1/ISGF2 antiserum but not with nonimmune rabbit serum (lanes 3 and 4). Anti-IRF-1/ISGF2 had no effect on the p50/p65 complexes bound to the VCAM1 NF-κB probe (data not shown), demonstrating that the supershift seen in Fig. 3 is not a nonspecific interference of protein binding. Taken together, these experiments demonstrate that the *VCAM1* IRF element is capable of interacting specifically with the IRF-1 found in HUVEC nuclear extracts.

IRF-1 is cytokine inducible in endothelial cells. EMSAs

TABLE 1. Comparison of IRF binding motifs in cytokine-responsive promoters[a]

Promoter	Sequence	Reference
VCAM-1 (nc)	GAAATAGAAAGT	
IFN-α (c)	GAAATGGAAAGT	56
IFN-β (c)	GAA-GTGAAAGT	37
iNOS (nc)	GAAAGTGAAATC	73
IRF-2 (c)	GAAAATGAAATT	25
HLA-B7 (nc)	GAA-GTGAAACT CC	28
Consensus	GAAANNGAAAGT	

[a] An IRF binding motif is present in the *VCAM1* promoter. c, coding strand; nc, noncoding strand.

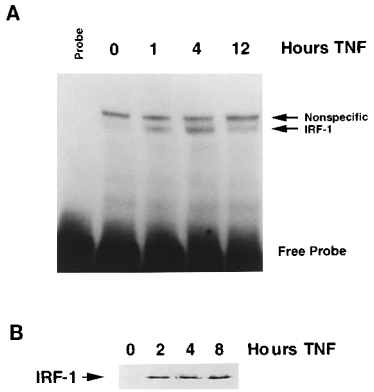

FIG. 4. IRF-1 is constitutively present in endothelial cells and is upregulated by TNF-α. (A) EMSA with the *VCAM1* IRF binding site; (B) Western blot analysis of nuclear extracts prepared from HUVEC stimulated with 100 U of TNF-α per ml for the indicated times. Nucleoprotein complexes as defined in Fig. 3 are indicated with arrows.

were performed with nuclear extracts prepared from HUVEC stimulated with TNF-α for various time periods. The results (Fig. 4A) indicate that the amount of IRF-1 present in HUVEC is increased by stimulation with TNF-α, with a peak occurring by 4 h. Western blot analysis of control and TNF-α-stimulated HUVEC nuclear extracts shows that IRF-1 is present in relatively low levels in uninduced cells and is upregulated by cytokine with kinetics consistent with the pattern observed in the EMSA experiments (Fig. 4B). The results obtained indicate that cultured human endothelial cells express low levels of IRF-1 in the uninduced state and that increased levels of the factor, peaking by 4 h, are found in the nuclei of cells stimulated with TNF-α.

The binding of IRF-1 to the *VCAM1* promoter is facilitated by NF-κB and HMGI(Y). DNA-protein interactions were further studied by DNase I footprinting with recombinant proteins (Fig. 5). rIRF-1, p50/p65 heterodimer, and HMGI(Y) were incubated in the indicated combinations with a single-end-labeled fragment of the *VCAM1* promoter spanning −258 to +21, which was subjected to partial DNase I digestion and then resolved on a denaturing acrylamide gel. The resulting footprints revealed that the NF-κB subunits bound to the region from −47 to −72 (Fig. 5, lane 2), completely in agreement with previous mutational studies (46). rIRF-1 protein protected a region +7 to −19 overlapping the putative IRF binding element (lanes 3 to 5). Interestingly, when IRF-1 and NF-κB were both allowed to interact with the probe, binding of IRF-1 was modestly facilitated (lanes 6 to 8). Previous work with the *IFN*-β promoter (13) and the E-selectin gene promoter (36, 72) has established that HMGI(Y) facilitates binding of ATF-2 and NF-κB to the promoters of these genes and that HMGI(Y) can physically interact with both NF-κB and ATF-2. To determine if HMGI(Y) could affect IRF-1 binding to the *VCAM1* promoter, footprint experiments were performed with recombinant HMGI(Y). As shown in Fig. 5 (lanes 9 to 11), addition of HMGI(Y) markedly increased the binding of IRF-1 (compare lanes 3 to 5 and 6 to 8). When fixed amounts of both NF-κB and HMGI(Y) were coincubated with IRF-1, striking facilitation of binding was observed (lanes 12 to 14). Thus, it appears that in the presence of both NF-κB and HMGI, IRF-1 is recruited to the promoter with high affinity.

To further study this phenomenon, EMSA experiments were performed. At low concentrations of rIRF-1, binding to the IRF element in the *VCAM1* or *IFN*-β promoter was barely detectable (Fig. 6, lanes 1, 2, 5, and 6). However, simultaneous addition of a fixed amount of HMGI(Y) resulted in a significant increase in IRF-1 binding to the *VCAM1* element (compare lanes 1 and 2 with lanes 3 and 4) and a similar but lesser effect on the IFN-β probe. These findings demonstrate that the *VCAM1* IRF element located at −2 to −13 can specifically interact with rIRF-1 and that this DNA-protein interaction is facilitated by the presence of NF-κB and HMGI.

IRF-1 physically interacts with p50 and HMGI(Y). Since NF-κB and HMGI(Y) can both facilitate the binding of IRF-1, it is possible that the three factors physically interact. To investigate this possibility, in vitro binding experiments were performed with p50 and HMGI fusion protein immobilized on glutathione-agarose beads. In vitro-translated IRF-1 was retained on both the GST-p50 beads (Fig. 7, lane 3) and the GST-HMGI beads (lane 2) but not on glutathione-agarose beads containing only GST (lane 1), indicating IRF-1 is capable of in vitro interaction with p50 and HMGI.

IRF-1 is a transcriptional activator of the *VCAM1* promoter. The results presented above demonstrate that the *VCAM1* IRF element specifically binds IRF-1. To determine if this DNA-protein interaction is functionally significant in cytokine-induced *VCAM1* expression, specific mutations which inactivated binding in EMSA experiments (with both nuclear extracts and rIRF-1) were placed in this element, and the effects of these changes were investigated in transfected endothelial and embryonal carcinoma cells.

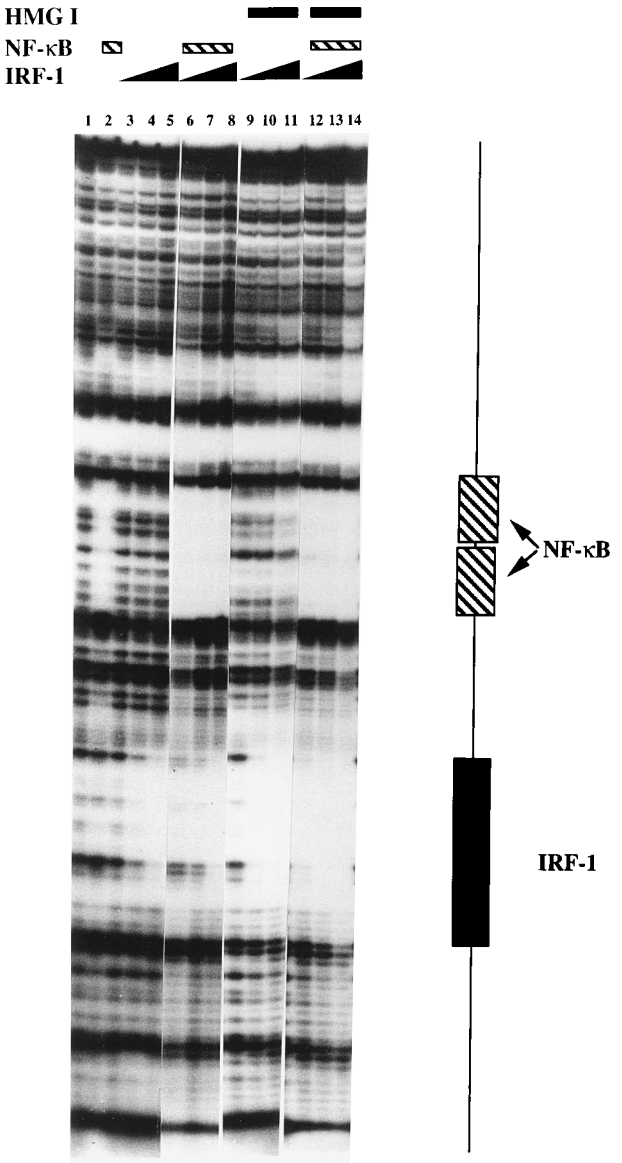

FIG. 5. IRF-1 binding is facilitated by NF-κB and HMGI(Y). DNase I footprint with recombinant proteins. A single-end-labeled probe spanning the basal *VCAM1* promoter was incubated with recombinant NF-κB (lane 2), increasing amounts IRF-1 (lanes 3 to 5), a fixed quantity of NF-κB with increasing amounts of IRF-1 (lanes 6 to 8), a fixed quantity of HMGI(Y) with increasing quantities of IRF-1 (lanes 9 to 11), and fixed quantities of NF-κB and HMGI(Y) with increasing quantities of IRF-1. A DNase digestion pattern without protein is shown in lane 1. The locations of the NF-κB and IRF binding sites are indicated on the right.

Mutations were introduced in the basal *VCAM1* promoter/CAT construct F2-pCAT3, which contains a fragment of the *VCAM1* promoter spanning −258 to +24 (46). The mutant reporter genes were designated F2-mIRF1-pCAT3 and F2-mIRF2-pCAT3 (Fig. 8A). Constructs were transfected into low-passage (<3) primary BAEC by a modified calcium phosphate technique. After recovery and replenishment of fresh

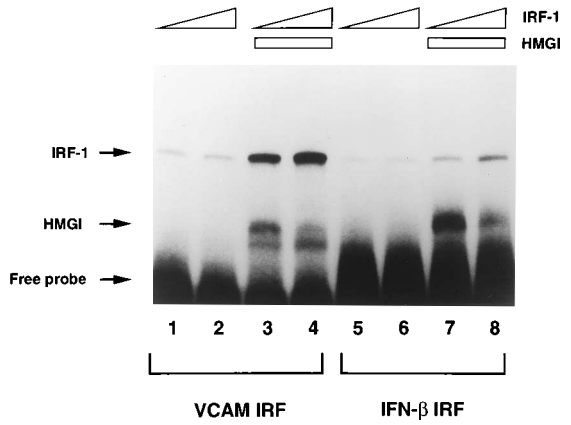

FIG. 6. HMGI(Y) facilitates binding of IRF-1 to the *VCAM1* IRF binding element. EMSA was performed with recombinant proteins. rIRF-1 in the absence (lanes 1, 2, 5, and 6) or presence (lanes 3, 4, 7, and 8) of HMGI(Y) was incubated with labeled *VCAM1* IRF (lanes 1 to 4) or IFN-β PRD I (lanes 5 to 8) oligonucleotide and assayed by EMSA. rIRF-1 and HMGI(Y) DNA-protein complexes are indicated by arrows.

media, cells were incubated with or without TNF-α for the times indicated. As previously reported (46), the wild-type construct, F2, shows minimal basal activity and is strongly induced by TNF-α (Fig. 8B, bars 1 and 2). In contrast, mutations in the *VCAM1* IRF element have little effect on basal expression (bars 5 and 9) but result in decreased cytokine inducibility (bars 6 and 10). These results suggest that a positively acting transcriptional activator binding to this element is necessary for full cytokine inducibility.

To further investigate whether IRF-1, acting through the IRF element, could act as a positive regulator of transcription, transactivation assays were performed in endothelial cells. Eukaryotic expression plasmids bearing a full-length cDNA encoding IRF-1 were cotransfected into BAEC along with wild-type and mutant reporter genes, and the cells were cytokine stimulated as described above. *VCAM1* constructs in unstimulated cells showed slight but reproducible transactivation with overexpressed IRF-1 (Fig. 8B, bar 3). The TNF-α-induced activity of wild-type constructs is superinduced by overexpressed IRF-1 (bar 4). The IRF-1-mediated superinduction is completely abolished by point mutations within the IRF element (bars 8 and 12), demonstrating that induction is dependent on an intact IRF binding site. IRF-1 overexpression had no effect on promoterless pCAT3 reporter vector, and expression vector alone had no effects on any of the reporter constructs used (data not shown).

IRF-1 functionally synergizes with NF-κB on the *VCAM1* promoter. We have demonstrated that NF-κB can physically interact with IRF-1 and can influence its binding. To determine whether IRF-1 functionally interacts with NF-κB, two approaches were taken. First, wild-type and mutant *VCAM1* promoter/reporter constructs were challenged with overexpressed IRF-1 and the NF-κB components p50 and p65. Second, *VCAM1* NF-κB and IRF binding elements subcloned upstream of a basal heterologous promoter, both in isolation and in combination, were subjected to similar cotransactivation assays. For these transactivation studies, p50 and p65 were used together to reproduce the endothelial heterodimer interacting with the *VCAM1* NF-κB sites. P19 embryonal carcinoma cells were used as host cells because they are readily transfectable and have no detectable endogenous IRF-1 and NF-κB activity (26).

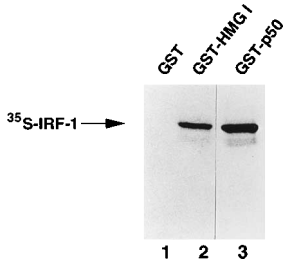

FIG. 7. p50 and HMGI physically interact with IRF-1 in vitro. In vitro-translated ^{35}S-labeled IRF-1 was incubated with GST (lane 1), GST-HMGI (lane 2), and GST-p50 (lane 3). Protein-protein complexes are indicated by an arrow.

As shown in Fig. 9, relatively low doses of p50/p65 strongly activated the *VCAM1* promoter (Fig. 9, bar 3), while IRF-1 overexpression alone weakly activated (bar 2). When IRF-1 was cotransfected with p50/p65, superinduction of the F2 reporter was observed (bar 4). A reporter construct with a mutant IRF-1 site was equally induced by over expressed p50/p65 (bar 7) but showed no response to over expressed IRF-1, either alone or in combination with p50/p65 (bars 6 and 8). An *IFN*-β promoter/CAT reporter containing two functional IRF sites (PRD III and I) and one NF-κB site (PRD II) exhibited a similar response to the transactivators (bars 9 to 12), though at greater concentrations of p50/p65. The larger degree of IRF-1-induced transcriptional synergy seen with the *IFN*-β promoter may reflect the action of two tandem IRF binding sites, in contrast to the single site present in the *VCAM1* promoter.

The previous experiments demonstrate that the *VCAM1* promoter responds synergistically to overexpressed p50/p65 and IRF-1. To obtain further evidence implicating a functional interaction between NF-κB and IRF-1, we sought to define the contribution of the respective *VCAM1* binding motifs in the context of a heterologous promoter. Constructs bearing the *VCAM1* NF-κB and IRF binding elements subcloned upstream of a constitutively active promoter are diagrammed in Fig. 10A. Cotransfection experiments were performed in P19 cells as described above. An IRF-2 expression construct was included as a negative control. IRF-2 binds to the same recognition site as IRF-1 but is not a transcriptional activator (24). The basally active promoter by itself was not influenced by any combination of activators (Fig. 10B, bars 1 to 5). A heterologous promoter construct containing the isolated *VCAM1* NF-κB sites could be transactivated fourfold by overexpressed p50/p65 (bar 7). IRF-1 and IRF-2 expression had no effect on either basal expression or p50/p65-stimulated expression (compare bars 8 to 10). A third construct, containing two copies of the *VCAM1* IRF site, exhibited no significant induction in the presence of overexpressed p50/p65 and/or IRF-1 (bars 11 to 15). However, when both *VCAM1* NF-κB and IRF binding sites were present, the construct was fully inducible (fourfold) by transfected p50/p65 (bar 17) and was superinduced ninefold by the addition of overexpressed IRF-1 (bar 19). Overexpressed IRF-2 did not superinduce p50/p65-mediated induction (compare bars 17 and 20). Expression vector alone had no transactivational properties (data not shown). Taken together, these results demonstrate that overexpressed IRF-1 alone is a weak transactivator, even in promoters with functionally defined IRF binding elements. However, overexpressed IRF-1 acts in a synergistic manner with p50/p65, and this effect is absolutely dependent on an intact IRF element.

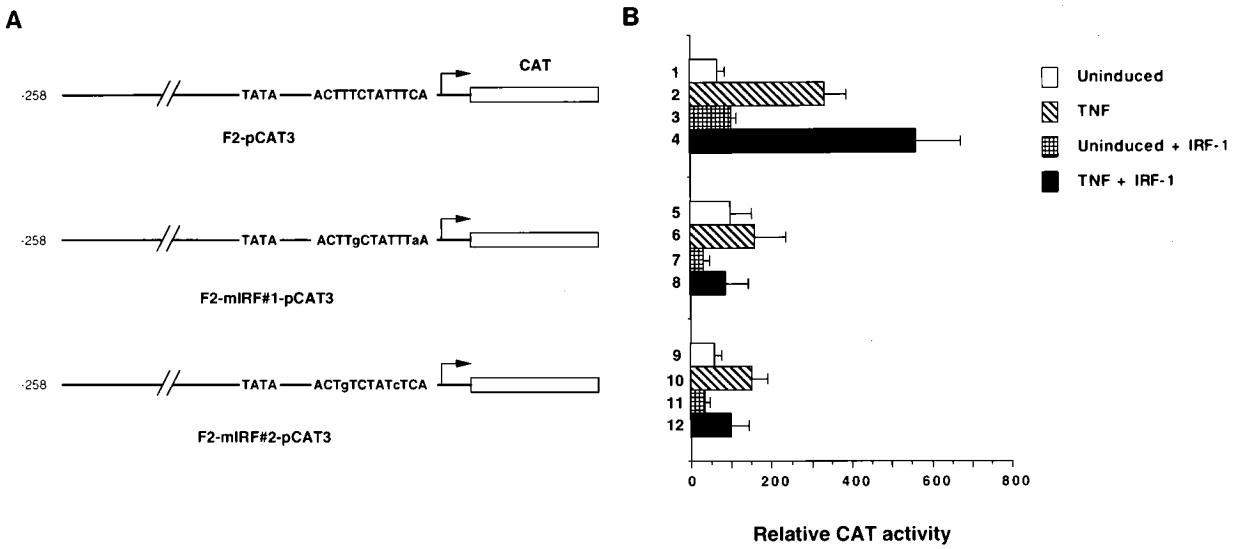

FIG. 8. IRF-1 is a transcriptional activator of *VCAM1* in endothelial cells. (A) Diagram of wild-type (F2-pCAT3) and mutant (F2-mIRF1-pCAT3 and F2-mIRF2-pCAT3) reporter constructs. (B) Functional activity of reporter constructs. BAEC were transfected with 10 μg of VCAM-CAT reporter and 5 μg of pTK-GH as an internal reference for transfection efficiency. Plates received either 1 μg of IRF-1 expression vector (bars 3, 4, 7, 8, 11, and 12) or 1 μg of control vector (bars 1, 2, 5, 6, 9, and 10). Cells posttransfection were treated for 24 h with complete medium (bars 1, 3, 5, 7, 9, and 11) or complete medium containing 200 U of TNF-α per ml (bars 2, 4, 6, 8, 10, and 12). Values are displayed as CAT activities relative to that of human growth hormone.

DISCUSSION

In this report, we have identified and characterized a novel positively acting functional domain in the *VCAM1* promoter. This element, defined by the sequence GAAATAGAAA, binds the transcription factor IRF-1. We demonstrate that IRF-1 is present in cultured endothelial cells and is upregulated by TNF-α stimulation. Previously reported work has shown that the *VCAM1* NF-κB binding motifs are necessary for cytokine induction (27, 46). Herein, we characterize these binding proteins in endothelial cells as heterodimeric p50 and p65. We demonstrate that the binding of IRF-1 is facilitated by the presence of HMGI(Y) and bound NF-κB. We also show a physical interaction between IRF-1 and both p50 and HMGI(Y). Mutational analysis and transactivation assays show that IRF-1 is a positively acting transcription factor necessary for full responsiveness to TNF-α. We show that exogenous p50/p65 and IRF-1 functionally synergize to activate both the *VCAM1* promoter and heterologous promoters containing subcloned NF-κB and IRF binding sites.

Although heterodimeric p50 and p65 are the major Rel species interacting with both NF-κB sites in TNF-α-stimulated HUVEC, we did detect trace amounts of c-Rel and somewhat greater amounts of RelB interacting with p50-DNA photoadducts under these conditions. RelB alone has no intrinsic DNA binding activity (64) and has been described only as a heterodimer with p50 or p52 (12). Interestingly, RelB/p50 heterodimers can activate transcription of the *VCAM1* promoter (45a). Homodimeric p50 (KBF1) can also be detected bound

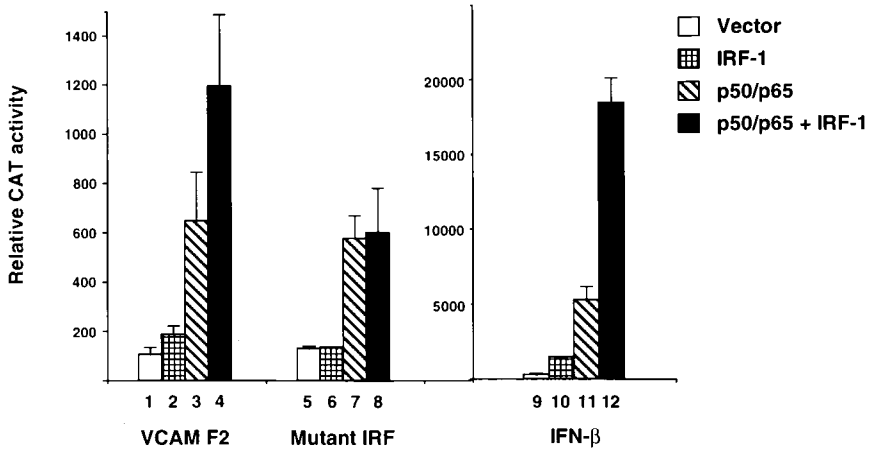

FIG. 9. Overexpressed IRF-1 synergizes with p50/p65 to activate the *VCAM1* promoter. P19 embryonal carcinoma cells were transfected with 10 μg of wild-type *VCAM1* reporter (VCAM F2-pCAT3; bars 1 to 4), mutant IRF reporter (F2-mIRF1-pCAT3; bars 5 to 8), or IFN-β–CAT reporter (lanes 9 to 12) construct and 5 μg of pTK-GH as an internal standard. VCAM1 wild-type and mutant reporters were activated with 25 ng of p50 and p65 expression plasmid and 250 ng of IRF-1 expression plasmid, as indicated. The IFN-β reporter was activated with 1 μg of p50, p65, and IRF-1 expression vectors, as indicated. Various quantities of activator construct are balanced for total DNA with empty activator plasmid (pcDNA).

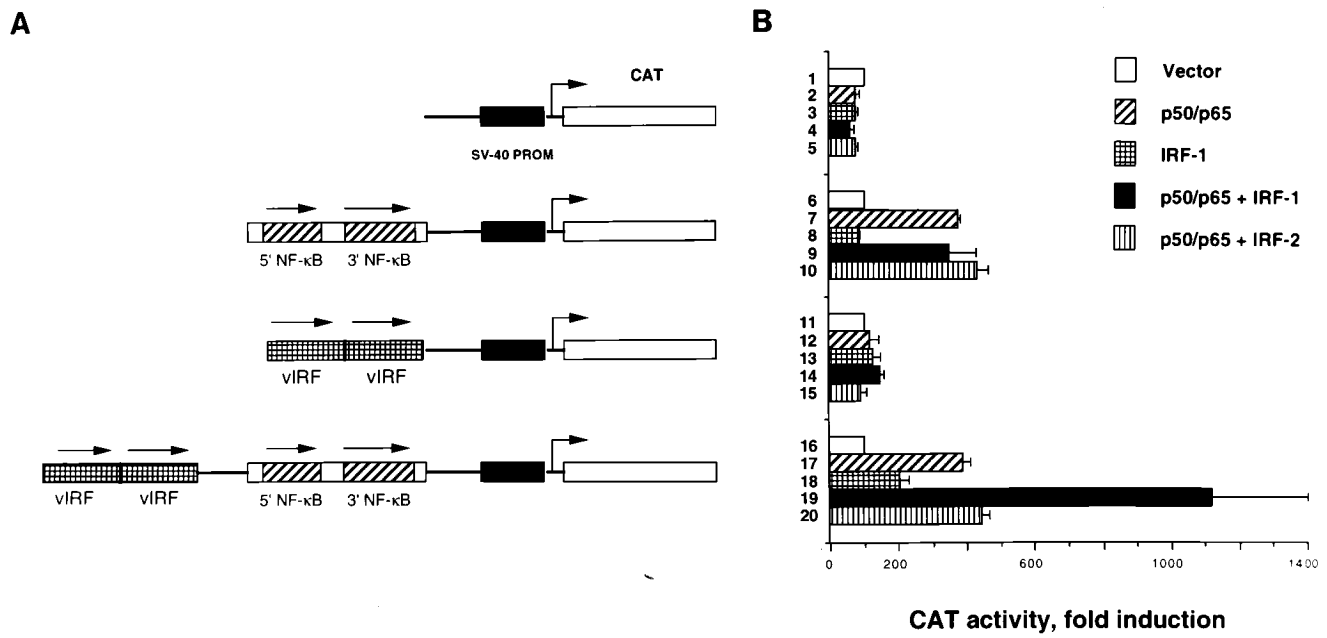

FIG. 10. Transcriptional synergy can be mediated through *VCAM1* NF-κB and IRF sites in heterologous promoters. (A) Diagram of heterologous reporter constructs. (B) Relative activities of reporter constructs. Constructs were transfected into P19 embryonal carcinoma cells as described for Fig. 9 except that 100 ng of p50 and p65 and 1 μg of IRF-1 and IRF-2 were transfected, as indicated. Results are displayed as fold induction, with baseline (100) defined as reporter plasmid activity transfected with an insertless expression vector alone.

to the *VCAM1* NF-κB sites in unstimulated endothelial cells. Whereas overexpressed p50 can synergize with p65 at low concentrations, higher amounts can suppress p65-mediated transactivation (45a). This latter observation is consistent with reports implicating homodimeric p50 as a transcriptional repressor (31). Potentially, Rel species other than heterodimeric p50/p65 may modulate VCAM-1 expression in other cell types or in different functional states.

We show that the in vitro binding of IRF-1 to the *VCAM1* IRF binding motif is facilitated by the presence of HMGI(Y). Previous work has demonstrated that HMGI(Y) enhances the binding of NF-κB and ATF-2 to their cognate sites in the promoters of the IFN-β and E-selectin genes (13, 36, 72). The mechanism by which HMGI(Y) facilitates IRF-1 binding on the *VCAM1* promoter may be due to extensive protein-protein and/or protein-DNA interactions. In these previously published experiments, facilitated binding could be abolished by mutating the AT-rich sequences recognized by HMGI(Y). Thus, HMGI(Y) may bind to the AT region of the *VCAM1* IRF binding site (G**AAATA**GAAA) and facilitate binding by affecting the structure of the DNA. This finding is consistent with the fact that the IFN-β IRF element lacks an AT-rich region and may account for the reduced facilitation seen with this probe (Fig. 6; compare lanes 3 and 4 with lanes 7 and 8). Regardless of specific mechanism, these findings place IRF-1 among the transcription factors whose binding activities are influenced by HMGI(Y).

Recent work has indicated that Rel family members may interact with other transcription factors, greatly expanding the possibilities for combinatorial control of any given NF-κB-dependent promoter beyond variable homo- and heterodimerization within the Rel family. The transcription factors SP-1 (52), C/EBPα (68), C/EBPβ (NF-IL6) (41), AP-1 (Jun, Fos) (67), and ATF-2 (13) can physically interact with NF-κB, as assayed by in vitro protein interaction assays, and can transcriptionally synergize in cotransfection experiments. Functional sequence motifs interacting with Rel family members and IRF-1 have been characterized in the major histocompatibility class 1 β-chain gene (28). While no evidence for physical or functional interaction was described, the NF-κB and IRF-1 sites mediated transcriptional response to TNF-α and IFN-γ, respectively, and both were necessary for the synergistic effects of these cytokines. The inducible nitric oxide synthetase (iNOS) gene promoter also contains NF-κB and IRF-1 binding sites which are necessary for synergistic transcriptional response to LPS and IFN-γ (38, 73). In this study, we used cotransfection assays to demonstrate that members of the Rel and IRF families of transcription factors can activate the *VCAM1* promoter in a synergistic manner. Similar experiments showing functional interactions between IRF-1 and NF-κB in the context of the IFN-β promoter have been reported (19). Additionally, we have presented evidence that NF-κB subunits can physically interact with IRF-1 in solution and facilitate the binding of IRF-1 when bound to DNA. Taken together, these data provide the first in vitro evidence of physical interactions between Rel and IRF family members. Finally, we have shown that the combination of NF-κB and HMGI(Y) stimulates IRF-1 binding to a degree greater than either protein by itself. This phenomenon may represent cooperative physical interactions of three proteins involved in preinitiation complex assembly.

IRF-1 expression in endothelial cells is regulated by cytokines. Low but detectable amounts of IRF-1 immunoreactivity and DNA binding activity are present in unstimulated cells. There is marked upregulation in the level of nuclear IRF-1 by 4 h after TNF-α stimulation. This is in contrast to the non-protein synthesis-dependent activation of p50/p65 NF-κB, which is almost undetectable in unstimulated endothelial cells but is maximally present in the nucleus within 15 min after cytokine induction (57). NF-κB itself may mediate transcrip-

tional control of IRF-1. The *IRF1* promoter contains a functional NF-κB (GGGGAATCCC) motif (25), suggesting a possible positive autoregulatory loop. Protein synthesis-dependent induction of IRF-1 activity by IFN-α and IFN-γ has been observed in other cell types (55). Although TNF-α-mediated induction of *IRF-1* mRNA has been shown (18), functional consequences (induced DNA binding activity and regulation of target gene expression) in response to TNF-α have not been previously reported.

While the kinetics of IRF-1 induction in endothelial cells is not consistent with this factor playing a role in the initial upregulation of *VCAM1* message, a potential physiologic role for IRF-1 may be in modulating the intensity and duration of promoter activation. This hypothesis is consistent with our experiments demonstrating a reduction of cytokine-induced *VCAM1* transcript accumulation with protein synthesis blockade (Fig. 2). Several other lines of evidence implicate IRF-1 as a positive transcriptional activator of *VCAM1*. In our experiments, the *VCAM1* promoter specifically bound IRF-1, and mutational analysis revealed that a disrupted IRF site resulted in decreased TNF-α-induced reporter gene activity. Similarly, point mutations in the IRF-1 binding element of the iNOS gene promoter has been shown to diminish the transcriptional response to IFN-γ (38). Overexpressed IRF-1 activated intact *VCAM1* promoter constructs, though weakly. Constructs bearing single or multimerized IRF binding elements derived from the promoters of IFN-β, guanylate-binding protein, and ISG15 can be transactivated by overexpressed IRF-1 (54, 55a). We also demonstrated that exogenous IRF-1 superinduced TNF-α-mediated transcriptional activation of the *VCAM1* reporter. This finding is consistent with other observations showing that overexpressed IRF-1 can superinduce target gene expression induced by exogenous stimuli. Leblanc et al. (35) demonstrated that IRF-1 could superinduce phorbol ester-mediated activation of an *IFN*-β promoter/reporter construct. Reis et al. (58) studied cells stably overexpressing IRF-1 and observed superinduced levels of endogenous IFN-β when transfected cells were stimulated by either poly(I)-poly(C) or virus. In our experiments and in these two studies, the degree of superinduction mediated by overexpressed IRF-1 was greater than the inductive effect of IRF-1 alone. Together, these findings suggest a synergistic interaction between IRF-1 and a separate transcriptional activator. In the case of *VCAM1*, these observations are consistent with an interaction between NF-κB and IRF-1.

IRF-1-null mice have been generated and analyzed. Significantly, these mice are devoid of iNOS activity, implicating IRF-1 as a critical component of one inducible promoter (30). However, experiments also showed that IRF-1 is not a crucial regulator of the IFN-β gene or IFN-α/β-stimulated genes (42, 63). This finding is consistent with earlier studies based on the expression of endogenous genes (55) but contrasts with conclusions based on transfected constructs (24, 58). Thus, while it is clear from our experiments that IRF-1 can participate in regulation of *VCAM1* expression in the systems studied, further experiments are needed to confirm the physiologic role of IRF-1 in the regulation of VCAM-1.

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