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Tumor Necrosis Factor α-Induced E-selectin Expression Is Activated by the Nuclear Factor-κB and c-JUN N-terminal Kinase/p38 Mitogen-activated Protein Kinase Pathways*

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E-selectin expression by endothelium is crucial for leukocyte recruitment during inflammatory responses. Transcriptional regulation of the E-selectin promoter by tumor necrosis factor α (TNFα) requires multiple nuclear factor-κB (NF-κB) binding sites and a cAMP-responsive element/activating transcription factor-like binding site designated positive domain II (PDII). Here we characterize the role of the stress-activated family of mitogen-activated protein (MAP) kinases in induced expression of this adhesion molecule. By UV cross-linking and immunoprecipitation, we demonstrated that a heterodimer of transcription factors ATF-2 and c-JUN is constitutively bound to the PDII site. TNFα stimulation of endothelial cells induces transient phosphorylation of both ATF-2 and c-JUN and induces marked activation of the c-JUN N-terminal kinase (JNK1) and p38 but not extracellular signal-regulated kinase (ERK1). JNK and p38 are constitutively present in the nucleus, and DNA-bound c-JUN and ATF-2 are stably contacted by JNK and p38, respectively. MAP/ERK kinase kinase 1 (MEKK1), an upstream activator of MAP kinases, increases E-selectin promoter transcription and requires an intact PDII site for maximal induction. MEKK1 can also activate NF-κB -dependent gene expression. The effects of dominant interfering forms of the JNK/p38 signaling pathway demonstrate that activation of these kinases is critical for cytokine-induced E-selectin gene expression. Thus, TNFα activates two signaling pathways, NF-κB and JNK/p38, which are both required for maximal expression of E-selectin.

The recruitment of leukocytes from the circulation into the extravascular space is critical for inflammatory responses and repair of tissue injury. The process of leukocyte emigration involves several steps (reviewed in Refs. 1, 2). The initial interaction between leukocytes and endothelium appears to be transient, resulting in the rolling of leukocytes along the vessel wall. The rolling leukocytes then become activated by local factors generated by the endothelium, resulting in their arrest and firm adhesion to the vessel wall. Finally, the leukocyte transmigrates the endothelium. These complex processes are regulated in part by specific endothelial-leukocyte adhesion molecules. The initial rolling interactions are mediated by the selectins, while firm adhesion and diapedesis appear to be mediated by the interaction of integrins on the surface of leukocytes with immunoglobulin gene superfamily members expressed by endothelial cells.

Expression of some of the endothelial-leukocyte adhesion molecules is dynamically regulated at sites of leukocyte recruitment. These changes in surface proteins provide the endothelial cell with a mechanism of regulating cell-cell interactions during recruitment of specific types of leukocytes. For example, endothelial expression of E-selectin is dramatically induced at sites of inflammation. The E-selectin gene is transcriptionally silent in quiescent endothelial cells and is rapidly and transiently transcribed when endothelial cells are activated with tumor necrosis factor α (TNFα)[1] or interleukin-1β (3). Transcription of E-selectin peaks in 1–2 h and returns to base-line levels by 12 h post-induction. Previous studies of the E-selectin promoter identified several promoter elements or positive regulatory domains (PDs) necessary for TNFα responsiveness (4). DNA binding studies reveal a requirement for nuclear factor-κB (NF-κB) and a small group of other transcriptional activators (reviewed in Ref. 5). Three of the elements (PDI, -III, and -IV) contain NF-κB recognition sequences. One of these, PDI, is a consensus NF-κB site and has been shown to bind p50/p65 heterodimers (6). The other two κB elements (PDIII and PDIV) are immediately adjacent to each other, and at least one, and perhaps both, are also occupied by the p50/p65 heterodimer (4, 7, 8). The fourth required region, PDII, contains an element similar to the cAMP response element/activating transcription factor element (CRE/ATF). The sequence, TGA-

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[1] The abbreviations used are: TNFα, tumor necrosis factor-α; BAEC, bovine aortic endothelial cells; HUVEC, human umbilical vein endothelial cell; JNK, c-JUN N-terminal kinase, IκBα, inhibitor κB-α; MAP, mitogen activated protein kinase; MEKK, MAP kinase-kinase-kinase, MKK, MAP kinase-kinase; MKP, MAP kinase phosphatase; NF-κB, nuclear factor-κB; PD, positive regulatory domain; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; CRE/ATF, cAMP response element/activating transcription factor element; PP2A, protein phosphatase 2A.

CATCA, varies at one site (underlined) from the consensus CRE/ATF sequence (TGACGTCA) and is identical to a functional site in the c-*JUN* promoter (9, 10). Previous studies using recombinant proteins have shown that the PDII element can be occupied by a variety of members of the ATF family of transcription factors, including ATF-2, ATF-a, ATF-3, and c-JUN (11, 12). Although the nature of the ATF family members bound to this element in TNFα-activated endothelial cells is uncertain, ATF-2 appears to be essential for E-selectin expression in that ATF-2 homozygous null mice are defective in E-selectin induction (13). In addition to the transcriptional activators, cytokine-induced E-selectin expression depends upon the chromatin architectural protein, high mobility group protein I(Y) (HMGI(Y)), which binds specifically to several sites in the promoter and potentiates binding of NF-κB to either PDIII or PDIV and binding of ATF-2 to PDII (4, 8).

The best-studied signaling pathway involved in TNFα activation of E-selectin gene transcription is the NF-κB/IκBα system (reviewed in Refs. 14–17). Constitutively present in all cells, the p50/p65 subunits of NF-κB are held in the cytoplasm by one or more members of a family of inhibitors, including p100, p105, IκBα, and IκBβ. In endothelial cells, TNFα stimulation results in phosphorylation of IκBα, which targets the inhibitor for ubiquitination and degradation by the proteasome (18–20). Loss of IκBα results in nuclear translocation of the p50/p65 heterodimer, where it binds its recognition sequences and activates transcription.

In contrast to the NF-κB/Rel family of proteins, other classes of transcription factors reside constitutively in the nucleus but require an activation signal. The family of mitogen-activated protein (MAP) kinases are important mediators of signals transduced from the cell surface to transcription factors in the nucleus (for review see Ref. 21). Multiple MAP kinases with different substrate specificities are activated by distinct extracellular stimuli. Recent studies revealed two novel subgroups, the c-JUN N-terminal kinase (JNK) protein kinases (also called stress-activated protein kinases) and p38 kinases which are activated in response to TNFα, interleukin-1β, bacterial lipopolysaccharide, and UV light (22–30). The JNK protein kinases and the related p38 kinases can phosphorylate the N-terminal activation domain of c-JUN on serines 63 and 73 and ATF-2 on threonines 69 and 71 (27, 31, 32) as well as other substrates (33). Activation of the JNK/p38 protein kinases involves a kinase cascade in which the upstream activator MAP kinase kinase kinase (MEKK) phosphorylates and activates MAP kinase kinase 3, 4, and 6 (MKK 3, 4 and 6), which in turn phosphorylate and activate JNK and p38 MAP kinases (34–37). Ultimately, phosphorylation of c-JUN and ATF-2 by JNKs increases the transactivating properties of both of these proteins (31, 32, 36, 38–41).

To further understand how transcription of the E-selectin gene is induced in response to cytokines, we examined the role of the PDII element and its associated transcriptional activators to act as a signaling target for the JNK/p38 MAP kinase pathway. Herein we show that c-JUN/ATF-2 heterodimers are the preferred complex bound at the E-selectin PDII site. TNFα stimulation of endothelial cells results in transient enhanced phosphorylation of both c-JUN and ATF-2, and concomitant activation of JNK and p38 kinases. Moreover, an upstream activator of the JNK signaling pathway, MEKK1 (42, 43), can activate E-selectin as efficiently as TNFα, and activation requires that the PDII element be intact. Overexpression of phosphorylation-defective ATF-2 or kinase-inactive JNK can block TNFα and MEKK1 activation of the E-selectin promoter. Additionally, overexpression of MEKK1 activates κB-dependent reporter gene expression, an effect which is decreased by a dominant negative form of a member of the JNK signaling pathway and by MAP kinase phosphatase. Thus, TNFα and MEKK1 activate at least two signaling pathways, NF-κB/IκBα and JNK/p38 MAP kinases, which converge on the E-selectin promoter and confer maximal cytokine responsiveness to the gene.

EXPERIMENTAL PROCEDURES

Cell Culture and Cytokine Treatment—Human endothelial cells obtained from collagenase-digested umbilical veins (HUVEC) (44) were cultured in M199 with 20% fetal bovine serum, 100 μg/ml porcine intestinal heparin, 50 μg/ml endothelial mitogen, 50 units/ml penicillin, and 50 μg/ml streptomycin, and 25 mM HEPES in gelatin-coated plates. Bovine aortic endothelial cells (BAEC) were isolated and maintained in culture using previously described procedures (45). For experimental cytokine induction, confluent monolayers of endothelial cells were exposed to recombinant human TNFα (Genentech, San Francisco, CA) at a final concentration of 100 units/ml in complete media.

Nuclear and Cytoplasmic Extracts—Following experimental treatment of HUVECs, nuclear and cytosolic extracts were prepared as described previously (20) The resulting crude nuclear extracts were diluted 1:1 with buffer D (20 mM HEPES (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM DTT). All buffers additionally contained the following mixture of protease inhibitors and phosphatase inhibitors: 1.0 mM phenylmethanesulfonyl fluoride (PMSF), 10 μg/ml each leupeptin and aprotinin, 1.5 μg/ml pepstatin A, 1.0 mM sodium fluoride, and 1.0 mM sodium orthovanadate.

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 (Sigma) (6, 46). Probes were designed to incorporate 5-bromo-2′-deoxyuridine 5′-triphosphate into the CRE/ATF-like site within PDII. Oligonucleotides utilized were template (lower strand) 5′GTACAATGATGTCAGAAACTCTGTC3′ and primer (upper strand) 5′GACAGAGTTTC3′. Binding reactions (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), 0.5 μg of poly[dI·dC], 0.5 μg of salmon sperm DNA, and 10^6cpm of labeled DNA. Reactions were incubated at room temperature for 20 min. Samples were UV-irradiated for 15 min using a transilluminator (Fotodyne Inc, New Berlin, WI). For immunoprecipitation, radiolabeled adducts from four binding reactions were pooled and diluted to 200 μl with RIPA buffer (10 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.5% sodium dodecyl sulfate, 1% Nonidet P-40, and 1% deoxycholate supplemented with 200 μg/ml bovine serum albumin, 0.1 mg/ml salmon sperm DNA, 1.0 mM PMSF, 1 μg/ml each leupeptin, aprotinin, and pepstatin A, 1 mM sodium fluoride, and 1 mM sodium orthovanadate) or ELB (50 mM HEPES (pH 7.9), 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 0.5 mM DTT, supplemented with 200 μg/ml bovine serum albumin, 0.1 mg/ml salmon sperm DNA, 1 mM PMSF, 1 μg/ml each leupeptin, aprotinin, and pepstatin A, 1 mM sodium fluoride, and 1 mM sodium orthovanadate) and incubated with 2 μl of antisera (1 h on ice) followed by 20 μl Protein A-Sepharose (1 h at 4 °C with shaking). Immune complexes were washed three times with RIPA or ELB buffer and analyzed on 10% SDS-polyacrylamide gels. Duplicate sets of radiolabeled adducts were denatured prior to immunoprecipitation by heating at 100 °C for 3 min in 0.5% SDS. ATF-2 and c-JUN polyclonal rabbit antisera were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), p38 (HOG) rabbit antisera was purchased from Upstate Biotechnology Inc. (Lake Placid, NY), and JNK antisera was as described (31). JNK and p38 MAP kinases were expressed in the yeast *Pichia pastoris* using vectors purchased from Invitrogen Corp. JNK1 and p38 MAP kinase expression were induced with methanol using procedures recommended by the manufacturer (Invitrogen).

Immunoblotting of Proteins Bound to the Cytokine Response Region of the E-selectin Promoter—A biotinylated double-stranded oligonucleotide (Integrated DNA Technologies, Inc. Coralville, IA) spanning the cytokine response region of the E-selectin promoter (−156 to −78) was coupled to Dynabeads M-280 streptavidin (Dynal, Lake Success, NY) according to the manufacturer's recommendations. The E-selectin-coupled matrix (250 μg) was incubated with 25 μl of endothelial nuclear extract in binding buffer (50 mM NaCl, 5 mM $MgCl_2$, 10 mM Tris (pH 7.5), 1 mM DTT, 1 mM EDTA, 0.25 μg/ml poly[dI·dC], and 5% glycerol) for 20 min at room temperature with mixing every 5 min to keep the Dynabeads in suspension. The non-bound or flow-through fractions were collected, and the Dynabeads with bound extract proteins were

washed four times in 1 × binding buffer containing 0.5 μg/ml poly[dI·dC]. Proteins bound to the Dynabeads were solubilized in 1 × Laemmli sample buffer, boiled, and subjected to SDS-polyacrylamide gel electrophoresis, followed by transfer to nitrocellulose membranes. Membranes were probed with antisera to Sp1, p65, ATF-2, c-JUN (Santa Cruz Biotechnology, Santa Cruz, CA), and p50 (provided by Nancy Rice, NCI, Frederick, MD) at dilutions ranging from 1:2500–1:10,000, followed by enhanced chemiluminescent detection as described below.

Immune-complex Kinase Assays—Extracts were prepared from control and TNFα-treated HUVECs in which the media were replaced with media containing 1% fetal calf serum for 1 h prior to stimulation. Cells were solubilized with Triton lysis buffer (TLB, 20 mM Tris (pH 7.4), 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 2 mM pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin). Extracts were centrifuged at 14,000 × *g* for 15 min at 4 °C. The JNK, p38, or ERK protein kinases were immunoprecipitated by incubation for 1 h at 4 °C with specific rabbit polyclonal antibodies bound to Protein A-Sepharose (Pharmacia Biotech Inc.). The rabbit polyclonal JNK and p38 antibodies have been described (31) The immunoprecipitates were washed twice with TLB and twice with kinase buffer (20 mM HEPES (pH 7.4), 20 mM β-glycerophosphate, 20 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate). The kinase assays were initiated by the addition of 1 μg of substrate protein and 50 μM ([γ-^{32}P]ATP) (10 Ci/mmol) in a final volume of 22 μl. The reactions were terminated after 15 min at 30 °C by addition of Laemmli sample buffer. Control experiments demonstrated that the phosphorylation reaction was linear with time for at least 30 min under these conditions. The phosphorylation of the substrate proteins was examined by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

Immunoprecipitation and Phosphatase Treatment—HUVECs (approximately 1.5 × 10^6) were untreated or treated with TNFα for 15 min, rinsed three times in ice-cold phosphate-buffered saline, and harvested in RIPA lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% deoxycholate, 1% Triton X-100, 0.25% SDS, 1 mM PMSF, 0.1 mM DTT, 10 μg/ml each leupeptin and aprotinin, 1.5 μg/ml pepstatin, 1 mM each sodium vanadate and sodium fluoride). Both samples were divided equally to three tubes, and 5 μl of ATF-2 rabbit antiserum (Santa Cruz Biotechnology Inc.) was added to each. The samples were incubated overnight at 4 °C with rocking and precipitated with 50 μl of Protein A slurry for 4 h, and then washed three times in RIPA buffer. The pellets were then washed an additional two times in phosphatase buffer (50 mM HEPES (pH 7.5), 1 mM DTT, 1 mM $MgCl_2$, 1 mM PMSF, 10 μg/ml each leupeptin and aprotinin, 1.5 μg/ml pepstatin) to remove the phosphatase inhibitors. The Protein A pellet was resuspended in 50 μl phosphatase buffer, and 10 mM NaF was added to one tube to provide a negative control. Protein phosphatase 2A (PP2A, 0.5 units; UBI) or calf intestinal alkaline phosphatase (1 unit; Boehringer Mannheim) were then added, and the samples were incubated at 37 °C for 2 h. After incubation the samples were washed once in RIPA buffer, resuspended in 20 μl of SDS sample buffer, boiled, and fractionated by 8% SDS-polyacrylamide gel electrophoresis. The gel was transferred to nitrocellulose and immunoblotted with ATF-2 mouse monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) as described below.

Western Blot—Nuclear extracts from TNFα-treated human umbilical vein endothelial cells were electrophoresed on 8% SDS-polyacrylamide gels and transferred to nitrocellulose in 25 mM Tris, 192 mM glycine, 5% methanol at 100 V for 1 h. ATF-2 and c-JUN rabbit antisera (Santa Cruz Biotechnology Inc.) were used at 1:5,000 dilution. Antiserum specific for the phosphorylated form of c-JUN (Ser^{63}) was obtained from New England BioLabs (Beverly, MA) and used at 1:1000 dilution. Polyclonal antisera to MKK3 and MKK4 (Santa Cruz, Biotechnology Inc. Santa Cruz, CA) were used at 1:1000 dilution. Immunoreactive proteins were detected according to the enhanced chemiluminescent protocol (Amersham Corp.) using 1:10,000 horseradish peroxidase-linked donkey anti-rabbit secondary antiserum. Blots were exposed to film for 1–10 min.

Transfections and Measurements of Reporter Gene Expression—Bovine aortic endothelial cells (BAECs) were transfected, harvested, and assayed for reporter proteins as described previously (4). Relative transfection efficiency was determined by cotransfection with pTK-GH (Promega) (4 μg). For experimental cytokine induction, confluent monolayers of endothelial cells were exposed to recombinant human TNFα (Genentech, San Francisco, CA) at a final concentration of 200 units/ml in complete media. A fragment containing the region −578 to +35 of the E-selectin promoter was generated by polymerase chain reaction amplification as described previously (4), gel-purified, and subcloned into the *Sma*I site of the reporter plasmid pCAT3. The mPDII reporter includes a 4-base pair mutation in the PDII ATF-2 site (gtcaATCA) generated as described previously (4). The expression vectors for ATF-2 and ATF-2 [$Ala^{69/71}$] (32), MEKK1 (33, 42, 43), MKK4 (Ala) (36), and JNK1 [$Ala^{183}Phe^{185}$] (27) have been described. Transfection of Chinese hamster ovary cells was done as described previously (36). Transfection efficiency was monitored using a control plasmid (pCH110, Pharmacia LKB Biotech). Luciferase reporter and β-galactosidase activities were measured as described previously (32). The mouse MKP-1 expression plasmid was generously provided by Dr. N. K. Tonks (47).

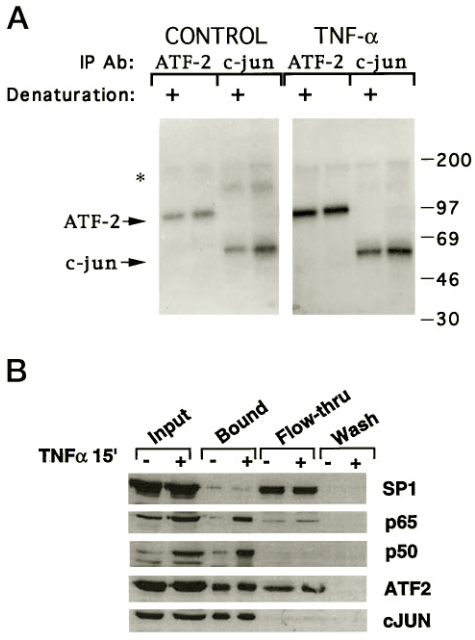

FIG. 1. **The E-selectin PDII site is constitutively bound by ATF-2/c-JUN heterodimers.** *A*, UV cross-linking of nuclear extracts with the PDII element of E-selectin reveals binding by ATF-2 and c-JUN. Human endothelial cells were obtained from collagenase-digested umbilical veins. Nuclear extracts from HUVECs untreated (*control*) or treated with TNFα for 12 h were incubated with a radiolabeled photoreactive probe spanning the PDII element, followed by immunoprecipitation with antisera to ATF-2 or c-JUN. *Arrows* indicate appropriately sized monomeric protein: DNA adducts. * indicates estimated size of heterodimeric adducts. Denaturation (+) indicates the sample was denatured by boiling in SDS prior to immunoprecipitation. *B*, immunoblotting of proteins bound to the cytokine response region of the E-selectin promoter. Nuclear extracts from control or TNFα-treated (15 min) HUVECs were incubated with a biotinylated oligonucleotide spanning the E-selectin cytokine response region. Bound proteins were identified by Western blotting using the indicated antibodies.

RESULTS

E-selectin PDII Contains a CRE-like Sequence That Is Bound in Vitro by ATF-2/c-JUN Heterodimers—The E-selectin promoter contains a functional CRE/ATF-like element which is necessary for cytokine responsiveness (4, 9, 13). DNA binding studies with recombinant proteins demonstrated that ATF-2, ATF-a, ATF-3, and a variety of heterodimers are all capable of binding the E-selectin CRE/ATF-like element (9). To fully define the preferential occupants of PDII in control and TNFα-activated endothelial cells, we used UV cross-linking followed by immunoprecipitation. Nuclear extracts from human umbilical vein endothelial cells (HUVECs) were subjected to UV irradiation to cross-link the bound proteins to a photoreactive PDII probe and then immunoprecipitated with antibodies to c-JUN and ATF proteins. Shown in Fig. 1*A* are the results with antiserum to ATF-2 and c-JUN using extracts from HUVECs untreated or treated with TNFα for 12 h. Under denaturing conditions, antiserum to ATF-2 immunoprecipitated predominantly ATF-2. Antisera to c-JUN immunoprecipitated predominantly c-JUN. Other antisera, including that raised to ATF-a,

ATF-3, or p65, failed to immunoprecipitate any photoreactive species.[2] This pattern of adducts was observed in nuclear extracts from untreated endothelial cells (Fig. 1*A*) and from cells treated with TNFα for 15 min and 1 h.[2] Thus, ATF-2 and c-JUN are constitutively associated with the promoter and are components of the cytokine-induced E-selectin enhancer complex.

Immunoblotting of Proteins Bound to the Cytokine Response Region of the E-selectin Promoter—The transcriptional activators binding the positive regulatory domains in the E-selectin promoter were defined by their ability to bind isolated promoter elements. To determine if the activators present in nuclear extracts would bind to the complete E-selectin cytokine response region, a biotinylated affinity matrix spanning the complete E-selectin enhancer was generated and coupled to streptavidin-coated magnetic beads. This biotinylated probe was incubated with nuclear extracts from control or TNFα-treated (15 min) HUVECs and washed extensively. Transcription factor complexes were released from the magnetic beads by boiling in SDS sample buffer and detected by immunoblotting. As shown in Fig. 1*B*, Sp1 binding was minimal, demonstrating that the association of transcriptional activators with the complex was specific. Additionally, binding of both the p65 and p50 subunits of NF-κB was TNFα-inducible. ATF-2 and c-JUN from both control and TNFα-treated cells were bound, confirming results obtained by UV cross-linking with the isolated PDII site (Fig. 1*A*). These results demonstrate that p50 and p65 are inducibly recruited to an intact E-selectin cytokine response region that contains constitutively bound ATF-2 and c-JUN. These findings are consistent with previous structural and functional studies characterizing the transcription factors bound to isolated positive regulatory domains (4, 6–8).

JNK and p38 MAP Kinases Are Rapidly Activated by TNFα Stimulation of HUVECs—Both JNK and p38 MAP kinases can phosphorylate ATF-2 while c-JUN is phosphorylated by JNK (27, 31, 32). To evaluate the activity of p38, JNK, and ERK MAP kinases in response to TNFα, kinase activity in immunoprecipitates was measured in whole cell extracts from control and TNFα-treated HUVECs using immune complex kinase assays. As shown in Fig. 2*A*, both p38 and JNK kinases were strongly and transiently activated within 15 min of TNFα stimulation. In contrast, ERK protein kinase was basally active and minimally affected by TNFα (Fig. 2*A*). These findings suggest that the TNF receptor preferentially initiates signaling events in endothelial cells leading to phosphorylation and activation of p38 and JNK kinases, rather than initiating the ERK MAP kinase system.

To determine whether these changes in kinase activity were due to alterations in levels or changes in subcellular location of p38 or JNK protein, Western blot analysis was performed on subcellular fractions from control and TNFα-treated HUVECs. As shown in Fig. 2*B*, p38 and JNK1 MAP kinases are constitutively present in both the cytosolic and nuclear compartments of fractionated cultured endothelial cells. Using the immunoprecipitation kinase assay, we have detected TNFα-induced JNK-1 activity in both cytosolic and nuclear extracts. Most of the JNK-1 activity was in the cytosol, and peak activity of the kinase in both the nucleus and cytosol was observed at 15 min after cytokine addition.[3] Thus the rapid changes in kinase activity seen in endothelial cells following TNFα treatment (Fig. 2*A*) were not due to alterations in levels or changes in subcellular location of p38 or JNK protein. The upstream activating kinases, MKK3 and MKK4, are also present in both the cytosol and nucleus of HUVECs (Fig. 2*B*). The distribution of

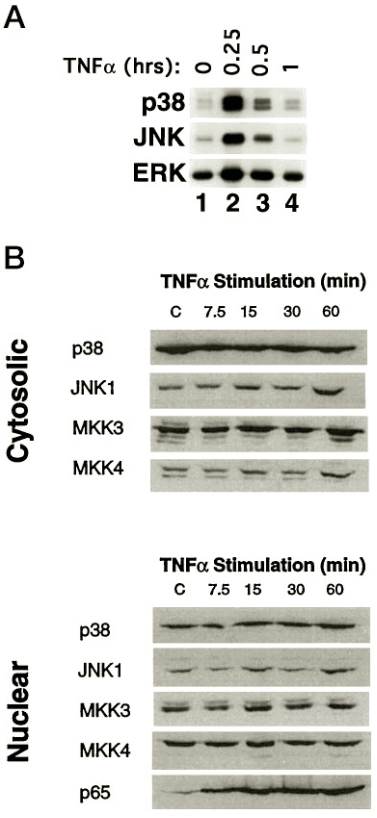

FIG. 2. **JNK and p38 MAP kinases are constitutively expressed in the nucleus of human endothelial cells and are rapidly activated by TNFα stimulation.** *A*, immune complex kinase assays were performed with whole cell extracts prepared from control and TNFα-treated HUVECs. p38, JNK, and ERK immunoprecipitates were incubated with GST-ATF-2 (for p38 and JNK) or GST-c-myc (for ERK) in the presence of [γ-^{32}P]ATP. *B*, Western blots of cytosolic (*upper panel*) and nuclear (*lower panel*) extracts from HUVECs treated with TNFα for the indicated times were probed with antisera to p38, JNK, MKK3 (MEK3), and MKK4 (MEK4) as indicated.

these kinases did not change with TNFα stimulation. In contrast to the findings with the kinases, dramatic nuclear accumulation of p65 was seen in response to TNFα stimulation (Fig. 2*B*), as reported previously (6). The constitutive presence of these kinases in the nuclear compartment indicates that their activation may occur in the nucleus of endothelial cells.

ATF-2 and c-JUN Are Rapidly and Transiently Phosphorylated upon TNFα Stimulation of Human Umbilical Vein Endothelial Cells—Selective activation of the JNK and p38 MAP kinases in response to TNFα suggests that the cytokine may increase phosphorylation of transcription factor targets of these protein kinases in endothelial cells. The phosphorylation of ATF-2 and c-JUN increases the ability of these proteins to act as transcriptional activators. To determine if ATF-2 and c-JUN were phosphorylated in endothelial cells in response to TNFα, cytosolic and nuclear extracts were prepared from control and TNFα-induced HUVECs and immunoblotted. ATF-2 was undetectable in the cytosolic fraction (data not shown) while constitutively expressed in the nucleus (Fig. 3*A*). ATF-2 from cells exposed to TNFα for 15 min exhibited a modest retardation in mobility when compared with ATF-2 from control cells (Fig. 3*A*, compare *lanes 1* and *2*). The time course of the ATF-2 mobility shift closely correlated with the kinase activation (Fig. 2*A*). To investigate whether this mobility shift was due to a change in phosphorylation status, extracts from HUVECs stimulated for 0 or 15 min with TNFα were immunoprecipitated with an ATF-2 antibody, treated with phosphatases, and immunoblotted with a second ATF-2 antiserum.

[2] M. Z. Whitley and T. Collins, unpublished observations.
[3] J. W. Pierce, J. Best, and T. Collins, unpublished data.

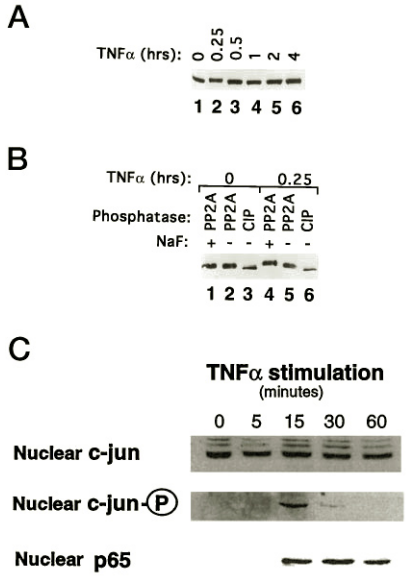

FIG. 3. **ATF-2 and c-JUN are rapidly and transiently phosphorylated in response to TNFα in HUVECs.** *A*, Western blot of nuclear extracts from HUVECs treated with TNFα for the indicated times, probed with antiserum to ATF-2 (Santa Cruz Biotechnology), and used at a 1:5,000 dilution. *B*, Western blot of control and TNFα-treated (0.25 h) HUVEC nuclear extracts that were first immunoprecipitated with antiserum to ATF-2 and treated with protein phosphatase 2A (*PP2A*) or calf intestinal phosphatase (*CIP*) in the presence or absence of 10 mM sodium fluoride (*NaF*) to specifically inhibit phosphatase activity (32). *C*, Western blots of nuclear extracts from HUVECs treated with TNFα for the indicated times were probed with antisera to c-JUN, phospho-c-JUN, and p65, as indicated.

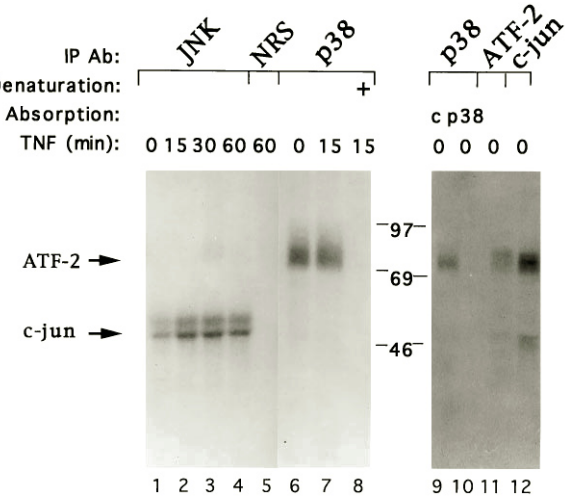

FIG. 4. **JNK and p38 MAP kinases are found in association with DNA-bound ATF-2 and c-JUN at the E-selectin PDII site.** UV cross-linking of HUVEC nuclear extracts with a photoreactive probe spanning the PDII element was followed by immunoprecipitation with the indicated antisera. Extracts were made from HUVECs treated with TNFα for the indicated time. Absorption indicates that the antibody was pre-absorbed with either control yeast extract (*C*), or p38 programmed yeast extract (*p38*) prior to immunoprecipitation. *Arrows* indicate appropriately sized monomeric protein: DNA adducts. *NRS*, normal rabbit sera.

In the presence of the phosphatase inhibitor, sodium fluoride (Fig. 3*B*, *lanes 1* and *4*), treatment with protein phosphatase-2A (PP2A), a phosphoserine/threonine-specific enzyme, did not alter the pattern of migration seen in the absence of PP2A (Fig. 3*A, lanes 1* and *2*). In the absence of sodium fluoride, PP2A treatment clearly resulted in a downward shift in the mobility of ATF-2 from TNFα-treated cells (*lane 5*) but did not affect ATF-2 mobility from control cells (*lane 2*). Treatment with calf intestinal phosphatase resulted in a downward shift of ATF-2 from both untreated and TNFα-stimulated cells (*lanes 3* and *6*), indicating that ATF-2 in cultured endothelial cells is basally phosphorylated. The differences in migration and the differences in sensitivity to the two phosphatases indicated that ATF-2 is hyperphosphorylated in response to TNFα stimulation of endothelial cells, consistent with findings obtained with IL-1 treated and UV-irradiated cells (32).

Similarly prepared nuclear extracts were analyzed for phosphorylation of c-JUN. A polyclonal antibody to a synthetic phosphopeptide corresponding to residues 59–67 of human c-JUN was obtained. This antibody detects only phosphorylated c-JUN (Fig. 3*C*) and is reported not to cross-react with the phosphorylated forms of JunD or JunB (New England BioLabs, Beverly, MA). As shown in Fig. 3*C*, c-JUN is constitutively present in HUVEC nuclei and, like ATF-2, is rapidly and transiently phosphorylated in response to TNFα. Shown for comparison, NF-κB p65 translocates into the nucleus following TNFα treatment. Translocation of p65 into the nucleus occurs simultaneously with phosphorylation of ATF-2 and c-JUN. Taken together, these data indicate that TNFα-activated JNK and p38 MAP kinases can phosphorylate the components of the transcription factor heterodimer associated with the E-selectin PDII regulatory domain.

JNK and p38 MAP Kinases Are Found in Association with DNA-bound ATF-2 and c-JUN at the E-selectin PDII Site—The ability of ATF-2 and c-JUN from unstimulated endothelial cells to bind to the PDII site, the constitutive presence of JNK and p38 MAP kinases and their upstream activators in the nucleus, and the transient activation of the kinases in response to TNFα suggested that phosphorylation of ATF-2 and c-JUN may take place while the heterodimer is actively bound to the E-selectin promoter. To explore this possibility, UV cross-linking and immunoprecipitation were employed. The radiolabeled photoreactive PDII probe was incubated with HUVEC nuclear extract and exposed to UV light to covalently couple ATF-2 and c-JUN to the labeled DNA (see Fig. 1). These extracts were subjected to immunoprecipitation with antibodies to JNK, p38, ATF-2, or c-JUN under less stringent conditions (ELB buffer) than those used in previous studies (RIPA buffer, Fig. 1). Immunoprecipitation with anti-JNK (Fig. 4, *lanes 1–4*) resulted in a doublet of apparent molecular mass of 45–55 kDa. The lower band of the doublet comigrates with the band produced by precipitation with anti-c-JUN (*lane 12*). The pattern of bands seen with anti-JNK did not change with TNFα stimulation, including up to 12 h (data not shown). Preimmune sera failed to precipitate any DNA-protein adducts (*lane 5*). Thus, results with anti-JNK show that JNK can interact with c-JUN, while bound to DNA.

A similar approach was used to explore whether p38 and ATF-2 are associated. When anti-p38 was used in the UV cross-linking and immunoprecipitation protocol, a broad band was observed (Fig. 4, *lanes 6, 7*) which comigrated with the band seen with precipitation of ATF-2 (*lane 11*). Again, this pattern did not change with various conditions of TNFα stimulation. When the cross-linked sample was boiled in SDS prior to addition of p38 antibody, the DNA-protein adduct was not precipitated (Fig. 4, *lane 8*), indicating that the immunoprecipitated species (*lanes 6, 7*) which co-migrates with ATF-2 (*lane 11*) is the result of protein-protein interactions. These results with anti-p38 show that p38 associates with ATF-2 while ATF-2 is bound to DNA. To further evaluate the specificity of the immunoprecipitation reaction, the kinase antibodies were incubated with yeast lysates, from either control yeast or yeast programmed to produce the respective kinase, prior to being

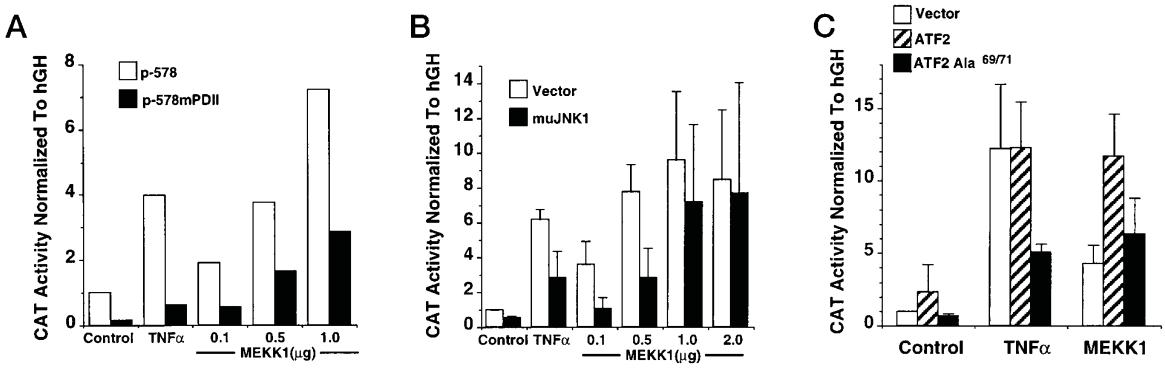

FIG. 5. **Phosphorylation-defective mutants of JNK and ATF-2 diminish TNFα and MEKK1 induction of E-selectin.** *A*, activation of the E-selectin promoter by TNFα or overexpressed MEKK1 is partially dependent upon PDII. BAECs were transfected with either the wild-type (*p-578*, *open bars*) or a mutant of the E-selectin promoter in pCAT3 that does not bind ATF-2 (*p-578mPDII, solid bars*). Cells were either untreated (*control*), treated with TNFα for 24 h, or co-transfected with the indicated amounts of an expression vector for MEKK1. Data shown are from one experiment that is representative of three experiments. *B*, BAECs were transfected with E-selectin p-578 in pCAT3 and either empty expression vector (*open bars*) or an expression vector containing a phosphorylation-defective form of JNK1 that is catalytically inactive (*muJNK1*) (*solid bars*). Cells were either untreated, treated with TNFα, or co-transfected with the indicated concentrations of an MEKK1 expression vector. Data represent the mean ± S.D. from three separate experiments. *C*, as in *A*, except BAECs were transfected with either 2 μg of empty vector (pBS) (*open bars*), or vector encoding ATF-2 (*striped bars*), or ATF-2 [$Ala^{69/71}$] (*solid bars*). Cells were either untreated (control), treated with TNFα for 24 h, or co-transfected with 0.5 μg of the MEKK1 expression vector. Data represent the mean ± S.D. from three separate experiments.

added to the cross-linked reaction. Strikingly, incubation of anti-p38 with p38 containing lysate completely abolished the ability of the antibody to precipitate the DNA-protein adduct (Fig. 4, compare *lanes 9* and *10*). Similarly, incubation of anti-JNK with JNK containing yeast lysate diminished the intensity of DNA-protein adduct compared with antibody incubated with control lysate (data not shown). These results indicated that the JNK and p38 kinases are capable of stable interactions with DNA-bound substrates, c-JUN and ATF-2, respectively. In addition, although both p38 and JNK kinases can both phosphorylate recombinant ATF-2, p38 appeared to be the preferred species associating with ATF-2 when the ATF-2/c-JUN heterodimer was bound to DNA.

A Phosphorylation-defective Mutant of JNK-1 Kinase Blocks TNFα and MEKK1 Induction of E-selectin—JNK kinases contain a Thr-Pro-Tyr motif and must be phosphorylated on Thr and Tyr for activation (27). Like the ERK group of MAP kinases, activation of the JNK and p38 kinases involves a kinase cascade in which the upstream kinase MAP kinase kinase kinase activates MAP kinase kinase 3, 4, and 6 (MKK3, 4 and 6) which in turn activate the JNK and p38 MAP kinases (31, 32, 34–37). To determine if activation of the JNK/p38 kinase pathway in response to TNFα is required for induction of E-selectin transcription, transient transfections were undertaken to overexpress members of this pathway in bovine aortic endothelial cells (BAEC). Increasing amounts of an expression plasmid coding for the upstream activator, MEKK1 (33, 42, 43), were transfected into BAECs with an E-selectin-promoter reporter plasmid (p-578), and the effects were compared with that of TNFα stimulation (Fig. 5*A*). As described previously (4), TNFα stimulation resulted in approximately 4-fold induction of E-selectin promoter activity and a plasmid bearing a mutation in the CRE/ATF sequence in PDII (p-578mPDII) was significantly less active (Fig. 5*A*). In addition, the lowest amount (100 ng) of MEKK1 was capable of stimulating expression from the wild-type E-selectin promoter, and induction occurred in a dose-dependent manner. The mutated E-selectin promoter responded less well to MEKK1 and only to the higher doses. These data indicate that an upstream activator of the JNK pathway can activate E-selectin transcription. In addition, we investigated whether a catalytically inactive form of JNK1 would act as a dominant inhibitor of TNFα and MEKK1 activation of the E-selectin promoter. Transient transfection assays were performed with a JNK1 mutant in which the sites of activating Thr and Tyr were replaced with Ala and Phe (27). As shown in Fig. 5*B*, the mutant JNK1 is capable of blocking a portion of the TNFα response and most of the response to the lowest dose of MEKK1. As increasing amounts of MEKK1 are expressed, the inhibition by mutant JNK1 is diminished. This may represent the activation of other signaling pathways (42, 43). The ability of catalytically inactive JNK1 to block TNFα and MEKK1 induction suggests that this kinase is important for TNFα induction of E-selectin transcription.

A Phosphorylation-defective Mutant of ATF-2 Blocks Cytokine-induced Expression of E-selectin—The JNK/p38 kinases phosphorylate the N-terminal activation domains of c-JUN and ATF-2. To determine the importance of ATF-2 phosphorylation in TNFα-induced expression of E-selectin, we tested whether a phosphorylation-defective mutant of ATF-2 inhibits E-selectin promoter activity. Plasmids expressing either wild-type ATF-2 or a phosphorylation-defective mutant of ATF-2 (ATF-2 [$Ala^{69, 71}$]) were transfected into BAECs with the p-578 reporter plasmid and challenged with TNFα. The cytokine activated the expression of the E-selectin promoter-reporter about 5-fold (Fig. 5*C*). The mutant ATF-2 [$Ala^{69, 71}$] diminished TNFα induction of the E-selectin promoter. The presence of wild-type ATF-2 did not affect the TNFα response of the E-selectin promoter (Fig. 5*C*). To determine the importance of ATF-2 phosphorylation in MEKK1-induced expression of E-selectin, cells were stimulated with MEKK1 at levels that do not activate a construct with a mutation in PDII (Fig. 5*A*). Under these conditions, activation in response to co-transfected MEKK1 was potentiated by wild-type ATF-2 but not by the mutant ATF-2 [$Ala^{69, 71}$] (Fig. 5*C*). Partial suppression by the dominant negative mutant may be due to the high levels of ATF-2 in endothelial cells. These data demonstrate that phosphorylation of ATF-2 is necessary for full activation of the E-selectin promoter by TNFα and MEKK1.

Overexpression of MEKK1 Activates NF-κB-dependent Gene Transcription—Maximal activation of the E-selectin promoter by MEKK1 involves phosphorylation of ATF-2 and requires an intact PDII site. However, like TNFα, MEKK1 still activates an E-selectin promoter reporter in which the PDII site is mutated but the NF-κB sites (PDI, III, and IV) are intact (Fig. 5*A*). These findings suggest that MEKK1 may also serve as an upstream activator of NF-κB. To directly test this hypothesis, NF-κB-dependent promoters which lack ATF-2 and c-JUN binding sites were evaluated for the ability to respond to

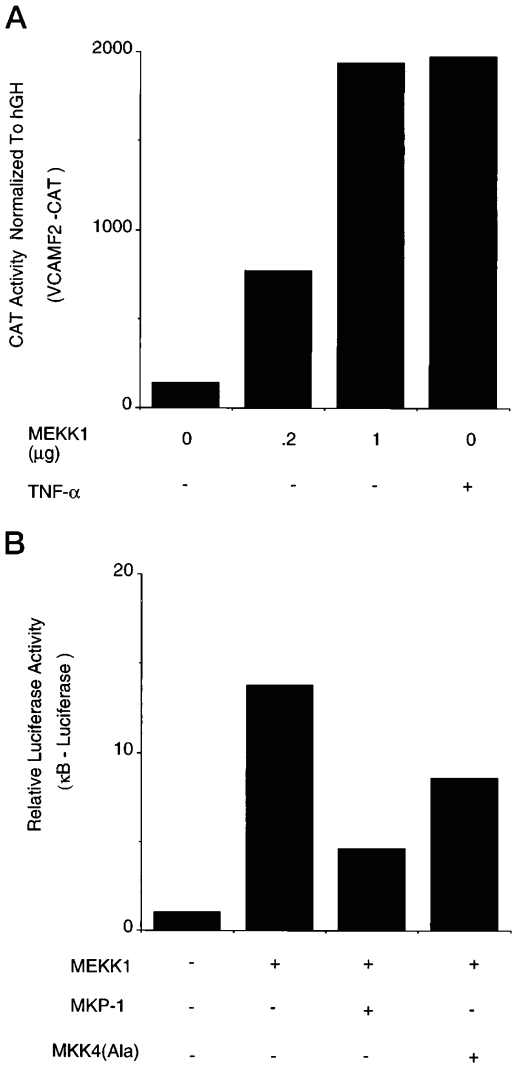

FIG. 6. **Expression of MEKK1 activates NF-κB dependent gene transcription.** *A*, activation of the VCAM-1 promoter with either TNFα or overexpressed MEKK1. BAECs were transfected with the VCAM-1 promoter in pCAT3, as in Fig. 5. Cells were either untreated (*control*), treated with TNFα for 24 h, or co-transfected with the indicated amounts of the expression vector for MEKK1. *B*, activation of NF-κB-dependent reporter gene expression by MEKK1 and inhibition with a dominant negative MKK4 (MKK4 Ala) or MKP. Chinese hamster ovary cells were transfected with a κB luciferase reporter (κb_2-luciferase) without or with co-expression of MEKK1. Co-expression of MKK4 (Ala) or MKP-1 inhibited MEKK1 activation of the κB-dependent reporter.

MEKK1 overexpression. VCAM-1 is a cell adhesion molecule which, like E-selectin, is highly inducible by TNFα in endothelial cells (1). The VCAM-1 promoter contains two tandem consensus NF-κB binding sites (45, 48). Transfection of BAECs with the MEKK1 expression vector resulted in increased activity of a promoter-reporter plasmid containing the two isolated κB elements from the VCAM-1 promoter. The level of activation of the reporter construct by MEKK1 was similar to that seen with TNFα (Fig. 6*A*). These findings are consistent with recent studies in which MEKK1-induced transcription from other κB-dependent reporters and co-expression of IκBα inhibited MEKK1-induced transcriptional activity (49, 50). Taken together, the results demonstrate that the MEKK kinase system can activate NF-κB-dependent gene expression but do not define the signaling pathway.

The activation state of NF-κB is determined by association with a cytoplasmic inhibitor, such as IκBα. Phosphorylation of IκBα results in the ubiquitination, dissociation, and subsequent degradation of the inhibitor by the proteasome proteolytic pathway (18–20, 51–53). The signaling pathways that couple MEKK1 to the activation of the IκBα kinase remain elusive. To explore these events, we utilized two approaches that build on the observation that MEKK1 activates κB-dependent transcription. Expression of MEKK1 increased activity of a κB-dependent reporter in a model system by about 14-fold (Fig. 6*B*), consistent with the kinase-dependent induction of the authentic E-selectin promoter (Fig. 5*A*) and other κB-dependent reporters (Fig. 6*A* and Refs. 49, 50). In the first approach to investigate the signaling pathways that couple MEKK1 to NF-κB activation, a dominant negative form of a MAP kinase kinase was used to block signaling from MEKK1. Cotransfection of a dominant negative mutant in the MEKK1 signaling pathway should inhibit MEKK1-activated κB-dependent reporter gene expression, if these events are coupled. MEKK1 activates MKK4 that phosphorylates both JNK and p38 (34, 35). A dominant negative mutant of MKK4, MKK4(Ala), inhibited the ability of MEKK1 to activate the κB-luciferase reporter (Fig. 6*B*). The partial inhibition may be due to an inefficient inhibitory effect of the MKK4 mutant and/or high levels of MKK4 activity in the transfected cells. Nevertheless, these findings substantiate MEKK1-activated NF-κB transcription and demonstrate a role of the components of the MEKK1 kinase cascade in NF-κB-mediated transcription. In the second approach to investigating the signaling pathways that couple MEKK1 to NF-κB activation, we examined whether the MAP kinase phosphatase, MKP-1, affected the ability of MEKK1 to activate the κB-luciferase reporter. MKP-1 has been reported to dephosphorylate ERK, JNK, and p38 kinases (30, 31, 47, 54). Coexpression of MKP-1 with MEKK1 resulted in a decrease in activation of the κB-reporter in this system (Fig. 6*B*). Taken together, these findings link MEKK1 activation and NF-κB gene expression.

DISCUSSION

The current study has identified an additional pathway of kinase activation in endothelial cells that occurs simultaneously with the previously described pathway of NF-κB activation (Fig. 7). Both pathways are required for full activation of E-selectin gene transcription in response to TNFα. In endothelial cells, TNFα interaction with a TNFα receptor (TNFR1) (55) may induce coupling with receptor-associated proteins and generation of various TNF-induced signals (56, 57). These initial events lead to activation of both JNK/p38 kinases and NF-κB. One signaling pathway results in phosphorylation, ubiquitination, and degradation of IκB-α by the proteasome (18–20, 51–53, 58) with nuclear accumulation of NF-κB. Concomitantly, the second set of TNFα-induced events leads to activation of the JNK and p38 kinases, resulting in phosphorylation of ATF-2 and c-JUN (22–32). These two pathways are rapidly activated and converge on the E-selectin promoter to result in full cytokine responsiveness of this gene (Fig. 7).

Although both pathways are activated simultaneously, there are notable differences in the duration of activation. Nuclear translocation of NF-κB in endothelial cells treated with TNFα occurs by 15 min and persists over many hours when TNFα is continuously present. E-selectin transcription requires continuous presence of the activating cytokine and the continuous presence of NF-κB in the nucleus (3, 59). As shown in the present study, activation of JNK/p38 MAP kinases and the subsequent phosphorylation of ATF-2 and c-JUN also occurs within 15 min. In contrast to NF-κB, both JNK and p38 kinase activity and phosphorylation of ATF-2 and c-JUN are transient events. Phosphorylation of c-JUN and ATF-2 by the kinases increases their ability to activate transcription without affect-

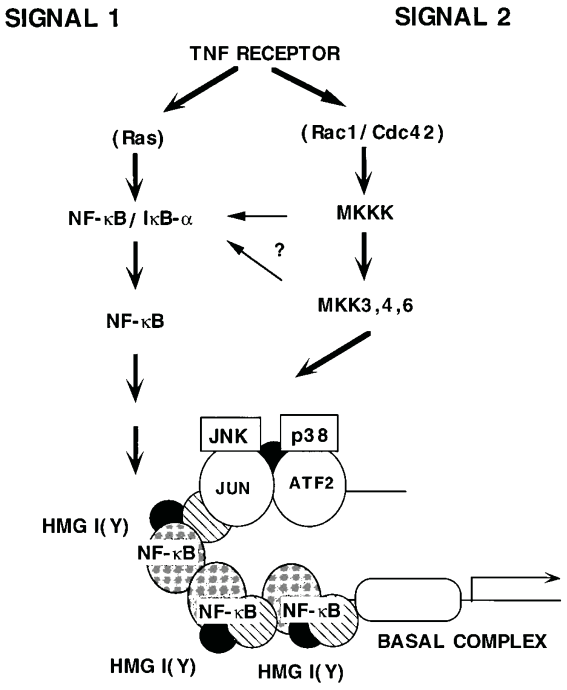

FIG. 7. **Cytokine-induced E-selectin gene expression is activated by the NF-κB and MAP kinase (JNK and p38) signal transduction pathways.** TNFα stimulation of endothelial cells results in the activation of at least two signaling pathways that converge during the assembly of the E-selectin enhanceosome. *Signal 1*, in response to TNFα, IκBα is phosphorylated, ubiquitinated, and targeted for degradation by the proteasome, allowing NF-κB to translocate to the nucleus (18–20, 51–53, 58). Upon activation, NF-κB in association with the architectural protein HMGI(Y) occupies several functional elements in the E-selectin cytokine-response region (4). *Signal 2*, a signaling cascade leads to activation of JNK and p38 MAP kinases. The Rho family of GTPases, Cdc42 and Rac 1, are upstream activators of this pathway (42, 73–76). Through the p21-activated kinase, activation of MAP kinase kinase kinase (*e.g.* MEKK1) and MAP kinase kinase (MKK3, MKK4, MKK6) leads to the activation of JNK and p38 MAP kinases, which phosphorylate c-JUN and ATF-2, respectively. JNK and p38 are constitutively present in the nucleus and are associated with the transcriptional activators. Phosphorylation of c-JUN and ATF-2 stimulates their ability to activate transcription (32, 38, 39). Phosphorylation of MAP kinase can also activate the NF-κB pathway, although the point where these pathways interact is not clear. Thus, the E-selectin cytokine-induced enhancer is a site of functional integration of MAP kinase (JNK and p38) and NF-κB signal transduction pathways.

ing their DNA binding or dimerization properties (10, 32, 38–40). The precise role of transient phosphorylation in transcriptional activation is unclear but may involve recruitment of co-activators such as p300 and the CRE-binding protein (60–62). Phosphorylation may be a prerequisite for recruitment of some components of the basal transcriptional apparatus. Once transcription is initiated, sustained phosphorylation of ATF-2 and c-JUN may no longer be required. Dephosphorylation may be accomplished by phosphatases, whose levels or activity may also be regulated by TNFα.

A key regulatory event in the ERK MAP kinase activation pathway is where the kinases are located within the cell. The ERKs are present in the cytoplasm of quiescent cells and translocate into the nucleus following activation (63–65). In contrast, the present study shows that in cultured endothelial cells, JNK and p38 MAP kinases, as well as two of the upstream activators, MKK3 and 4, are constitutively present in the nucleus. The constitutive presence of the JNK/p38 MAP kinases and the corresponding transcriptional activators in the nucleus of endothelial cells suggest that TNFα-induced phosphorylation events may occur in a DNA-bound substrate-kinase complex. We have demonstrated the presence of JNK and p38 kinases constitutively associated with the ATF-2 and c-JUN heterodimers bound to the E-selectin PDII element. These data are consistent with earlier reports that show that epitope-tagged JNK can interact with recombinant c-JUN, regardless of whether the JNK1 has been activated (22, 27, 30, 66). Our studies extend these observations and show that the authentic kinases present in endothelial nuclear extracts can associate with their intact transcription factor substrates while bound to DNA. Such associations have been postulated to occur and could serve to target the kinases to phosphorylate additional protein substrates bound to neighboring elements. These associations may be characteristic of certain types of inducible genes. Several other genes induced by inflammatory mediators have functional elements for ATF-2 or ATF-2-c-JUN heterodimers, including c-*JUN*, urokinase, and β-interferon (67–70). The similarities between the E-selectin promoter and the other promoters suggest that activation of the JNK/p38 families of MAP kinases in the nucleus may be a common theme in the regulation of genes involved in various inflammatory and ischemic responses associated with cardiovascular diseases (71).

The JNK/p38 MAP kinase and NF-κB signaling systems may represent two distinct but interactive signal transduction pathways. The JNK/p38 MAP kinase and NF-κB signaling systems may emanate directly from distinct TNF receptor-associated proteins (55–57). Our data supports the concept that cross-talk occurs between the pathways of NF-κB and JNK/p38 MAP kinase activation. MEKK1 was shown in other cell types to be activated by TNFα (72). The findings in this paper are consistent with recent studies that demonstrate MEKK1-induced transcription from other κB-dependent reporters and inhibition of MEKK1-induced transcriptional activity by co-expression of IκBα (49, 50). Taken together, these results demonstrate that the MEKK kinase system can activate NF-κB-dependent gene expression but do not define the signaling pathway. To examine the signaling pathways that couple MEKK1 to the activation of the IκBα kinase, we utilized two approaches that build on the observation that MEKK1 activates κB-dependent transcription. MEKK1 activation of NF-κB-dependent gene transcription was inhibited by a dominant negative form of MKK4 and by a phosphatase (MKP-1) that dephosphorylates ERK, JNK, and p38 kinases. These findings substantiate MEKK1-activated NF-κB transcription and demonstrate a role of the components of the MEKK1 cascade in NF-κB-mediated transcription. Moreover, MEKK1-driven phosphorylation of JNK MAP kinases may be involved in activation of NF-κB. However, a direct effect of these kinases on IκBα is unlikely, since the inhibitor is not a substrate for these MAP kinases.[4] Whether the component(s) of the NF-κB/IκBα signaling pathway are directly targeted by JNK or p38 is unknown. Nonetheless, activation of MEKK1 and subsequent downstream signaling events involving both the NF-κB system and the JNK/p38 MAP kinases may be important for TNFα-induced gene expression.

Various stimuli and cellular stresses that activate the JNK/p38 MAP kinases are also physiological activators of E-selectin expression. Our findings demonstrate the importance of this signaling pathway in the activation of the E-selectin gene. The acute induction of E-selectin expression at sites of inflammation may be attributed to the combination of these two rapidly activated kinase cascades, which converge on the E-selectin gene promoter to ensure immediate responsiveness. The identification of a second signaling pathway provides another mechanistic link between the endothelial environment, E-se-

[4] S. Gupta and R. Davis, unpublished observations.

lectin gene expression, and the early events in leukocyte adhesion and may be a means of generating specificity in the pattern of adhesion molecule expression at sites of inflammatory responses.

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