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Cyclic AMP-Independent ATF Family Members Interact with NF-κB and Function in the Activation of the E-Selectin Promoter in Response to Cytokines

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We previously reported that NF-κB and a complex we referred to as NF-ELAM1 play a central role in cytokine-induced expression of the E-selectin gene. In this study we identify cyclic AMP (cAMP)-independent members of the ATF family binding specifically to the NF-ELAM1 promoter element. The NF-ELAM1 element (TGACATCA) differs by a single nucleotide substitution from the cAMP-responsive element consensus sequence. We demonstrate that this sequence operates in a cAMP-independent manner to induce transcription and thus define it as a non-cAMP-responsive element (NCRE). We show that ATFa is a component of the NF-ELAM1 complex and its overexpression activates the E-selectin promoter. In addition, ATFa, ATF2, and ATF3 interact directly with NF-κB in vitro, linking two unrelated families of transcription factors in a novel protein-protein interaction. Furthermore, we demonstrate that the ability of overexpressed NF-κB to transactivate the E-selectin promoter in vivo is dependent on the NF-ELAM1 complex. Our results suggest that a direct interaction between ATFs and NF-κB is, at least in part, the mechanism by which these factors specifically regulate E-selectin promoter activity.

E-selectin (previously called ELAM-1) is a member of the selectin family of endothelial cell adhesion proteins which recognize carbohydrate ligands on circulating immune cells (6). E-selectin plays a central role in the binding and extravasation of neutrophils and a subset of leukocytes from the bloodstream into sites of inflammation (for a review, see reference 35). Expression of E-selectin is both cell specific and stimulus specific, as it is expressed only on endothelial cells in response to induction by the cytokines interleukin-1 (IL-1) and tumor necrosis factor alpha as well as lipopolysaccharide and phorbol myristate acetate (7). In addition, E-selectin gene activity is transient. Expression is maximal 2 to 4 h following cytokine induction and returns to the basal level by 24 h (7, 21, 56). This tight regulation of gene activity is likely to require complex control mechanisms.

We have some evidence that DNA methylation plays a role in the tissue-specific expression of the E-selectin gene (51). On the other hand, we and others have defined several proximal promoter elements involved in control of cytokine-induced expression of the human E-selectin gene (12, 31, 42, 56). One of these elements (−94 to −85) is a binding site for the ubiquitous transcription factor NF-κB, which is involved in control of cytokine-induced expression of many immune- and inflammatory-response genes (for reviews, see references 2 and 37). In most cell types NF-κB is retained in the cytoplasm in an inactive complex with the inhibitor IκB (3, 4). Inducing agents, for example, cytokines (45) and mitogens (9), cause the dissociation of NF-κB from IκB. NF-κB is then translocated to the nucleus, where it binds to its recognition sites within the promoters of responsive genes (22). The predominant NF-κB species is composed of two subunits, p50 and p65. The N-terminal 300 amino acids of both of these proteins are highly homologous to the *rel* oncogene product. This family of proteins, which also includes the *Drosophila* morphogen dorsal, RelB, and p49, is referred to as the Rel family (for reviews, see references 8 and 23).

Another adhesion protein expressed on endothelial cells following IL-1 and tumor necrosis factor alpha treatment is vascular cell adhesion molecule 1 (VCAM-1) (44). Like E-selectin, VCAM-1 expression is regulated by NF-κB (33). However, the temporal expression of VCAM-1 on endothelial cells is significantly longer compared with that of E-selectin. In addition, VCAM-1 is constitutively expressed on several other cells types (44). Therefore, factors in addition to NF-κB appear to play a central role in determining the specific expression of these two genes in response to cytokines. In support of this, we have shown that NF-κB alone, although essential, is not sufficient to mediate IL-1-induced activation of the E-selectin gene (56). We identified two additional factors, which we referred to as NF-ELAM1 and NF-ELAM2 (binding at positions −153 to −144 and −104 to −100, respectively), that play critical roles in controlling cytokine-induced expression of the E-selectin gene. Mutation of the binding sites for either of these factors results in an almost complete loss of IL-1 inducibility of the E-selectin promoter. While neither of these elements alone is sufficient to confer enhancer activity on a heterologous promoter, NF-ELAM1 was shown to cooperate with NF-κB to augment cytokine-induced expression to levels significantly above that observed with NF-κB alone (31). These results demonstrated that NF-ELAM1 functionally cooperates with NF-κB in IL-1 induction of the E-selectin gene. The specificity in the activation of other NF-κB-regulated genes, such as those for angiotensinogen and IL-8, also appears to be

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conferred by a combinatorial interaction between NF-κB and other factors (10, 18, 38, 43).

In this study we identify several proteins binding at the NF-ELAM1 site and examine how interactions between these factors and NF-κB control E-selectin expression. We demonstrate that cyclic AMP (cAMP)-independent members of the ATF/CREB family of transcription factors bind specifically to the NF-ELAM1 site. We show that a novel protein-protein interaction occurs between the p50 and p65 subunits of NF-κB and certain ATFs. Furthermore, our results demonstrate that NF-κB is dependent on NF-ELAM1 for strong induction of E-selectin expression. These results describe a novel mechanism operating to specifically control E-selectin gene transcription in response to cytokines.

MATERIALS AND METHODS

Isolation of recombinant clones encoding NF-ELAM1 binding proteins. Recombinant human umbilical vein endothelial cell (HUVEC) and HeLa cell cDNA expression libraries (Clontech, Palo Alto, Calif.) were screened as described elsewhere (50, 55). Approximately 10^6 recombinant plaques were screened with a multimerized, 5′-end-labeled double-stranded oligonucleotide probe containing the NF-ELAM1 binding site (−157 to −140) (GATCTTCTGACATCATTG TAAT). One positive plaque from the HUVEC cDNA library and two positive plaques from the HeLa cDNA library were purified, lambda DNA was isolated, and cDNA inserts were sequenced by standard procedures (1, 49).

Cell lines. HUVECs were extracted from human umbilical cords by collagenase treatment. The cells were cultured in medium MCDB 131 supplemented with epidermal growth factor (10 ng/ml), hydrocortisone (1 ng/ml), 2% fetal bovine serum, 0.4% bovine brain extract (Clonetics, San Diego, Calif.). HeLa cells were grown in Dulbecco modified Eagle medium containing fetal calf serum (10% [vol/vol]) and penicillin and streptomycin (50 μg/ml each).

Plasmid constructs. The E-selectin promoter–chloramphenical acetyltransferase (CAT) reporter vectors have been described previously (31, 56), except for the point mutant and CRE/κB(×2) reporter vectors. The point mutant vectors were generated by oligonucleotide-directed in vitro mutagenesis as described by Amersham. The CRE/κB(×2) vector was constructed by inserting a double-stranded oligonucleotide, ATG CATTGACGTCATTGTTGGGGATTTCCT, into the *Bgl*II restriction site of the plasmid pCAT-promoter (Promega). The p50 eukaryotic expression plasmid was made by subcloning the *Eco*RI-*Xba*I cDNA fragment of p105 (34) from the Bluescript vector into expression plasmid containing the simian virus 40 promoter pSG5 (26). This expression plasmid encodes the N-terminal 502 amino acids of p50. The cytomegalovirus promoter-containing eukaryotic expression plasmid soCMIN was used for expression of full-length p65 (48). The ATFa eukaryotic expression plasmid was constructed by subcloning the full-length ATFa cDNA into the pSG5 expression vector.

Expression and purification of recombinant proteins. Bacteriophage lysogen extracts were prepared from the bacteriophage λ expressing the ATF fusion proteins by standard procedures (49).

Partial or full-length ATF1, ATF3, and ATF4 were cloned into a modified pET vector (53) containing six contiguous histidines. Full-length ATF2 cDNA was cloned in the pRSET vector (with six histidines). All histidine-tagged proteins were purified by a nickle chelating column (Ni-nitrilotriacetic acid-agarose) essentially according to the protocol provided by the manufacturer (Qiagen). Briefly, BL21(DE3)LysS cells harboring the expression vectors were grown to mid-log phase (optical density at 600 nm, approximately 0.6) and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h. Cells (1 liter) were spun down and lysed in 30 ml of buffer A (100 mM sodium phosphate, 10 mM Tris-HCl [pH 8], and 6 M guanidine-HCl). Cell debris was removed by centrifugation, and the whole-cell lysate was loaded on a 5- to 10-ml nickle chelating column and washed with 5 column volumes of buffer A and then in buffer B (same as buffer A except 6 M guanidine-HCl was replaced by 8 M urea) and buffer C (same as buffer B except pH is 6.4 instead of 8). The protein was eluted in buffer D (same as buffer B except pH is 5) and renatured as follows. The protein was dialyzed in buffer H (5% glycerol, 100 mM NaCl, 50 mM sodium phosphate [pH 7], 50 mM β-mercaptoethanol, and 0.5 mM phenylmethylsulfonyl fluoride) containing 1 M urea for 3 h, then in buffer H containing 0.1 M urea for 3 h, and finally in buffer H overnight.

Nuclear extract preparation and band shift analysis. HUVEC nuclear extracts were prepared as described previously (14) with modifications as described elsewhere (15). Extracts were then stored at −70°C. The oligonucleotide used in the library screening, containing the NF-ELAM1 binding site, was 5′ end labeled and used as a probe in the band shift assay except for assays with ATF4, for which a cAMP-responsive element (CRE) consensus site was used. Heterodimer formation was carried out by incubating the proteins at 37°C for 30 min prior to addition of buffer and probe. Binding reaction mixtures contained 50 mM NaCl, 3 mM MgCl, 1 mM dithiothreitol, 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5), 0.05 μg of poly(dI-dC) per μl, 10% (vol/vol) glycerol, and approximately 1 to 5 μg of protein. Each 20-μl reaction mixture was incubated at room temperature for 20 to 30 min following addition of approximately 10,000 cpm of ^{32}P-labeled oligonucleotide probe. Competitions were carried out by preincubating the reaction mixture with excess unlabeled oligonucleotides for 10 min at room temperature prior to addition of labeled probe. Samples were then subjected to electrophoretic separation on a 4% nondenaturing polyacrylamide gel. The gels were dried and subjected to autoradiography.

DNA transfection and CAT analysis. Approximately 12 h prior to transfection HUVECs and HeLa cells were plated at densities of 1×10^6/60-mm-diameter plate and 0.5×10^5/100-mm-diameter plate, respectively. Transfection of plasmid DNA into HUVECs was carried out by the DEAE-dextran method as described elsewhere (13). Transfection of plasmid DNA into HeLa cells was carried out by the calcium phosphate coprecipitation method as described previously (57). CAT assays were carried out 48 h posttransfection essentially as described elsewhere (25). CAT enzymatic activity was quantitated by measuring conversion of chloramphenicol to its acetylated forms by real-time radiation imaging using an Ambis radioanalytic imaging system according to the manufacturer's instructions.

In vitro translation. Proteins were synthesized in vitro in the presence of ^{35}S-labeled methionine by using a coupled transcription-translation reticulocyte lysate system according to the manufacturer's instructions (Promega). The products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and quantitated by using a radioanalytic imaging system (Ambis). Equal amounts of labeled proteins were used in the immunoprecipitation or glutathione *S*-transferase (GST) fusion protein binding assays.

GST fusion protein binding assay. The p50 (amino acids [aa] 35 to 381) and p65 (aa 12 to 317) subunits of NF-κB were constructed as fusions with GST in plasmid pGEX-2T. The purification of GST, GST-p50, and GST-p65 was as described previously (41) except that the proteins were not eluted from glutathione-Sepharose beads (Pharmacia). ^{35}S-labeled ATFs and c-Jun of equal counts per minute (1 to 5 μl) were diluted to 100 μl with buffer containing 20 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, 10 mg of bovine serum albumin (BSA) per ml, and 0.1 M NaCl. The affinity beads carrying 12.5 μg of GST, GST-p50, or GST-p65 (25 μl of a 1:1 dilution) were added, and the slurry was incubated for from 2 h to overnight on a rotary shaker at 4°C. The resin was washed with 500-μl aliquots of the same buffer until the unbound proteins were removed (1.5 to 2.5 ml). The bound proteins were then eluted with 10 mM reduced glutathione in 50 mM Tris-HCl (pH 8.0) and 0.5 M NaCl by rotating the slurry for 30 min at 4°C. The proteins were subjected to SDS-PAGE. The gel was fixed in 40% methanol and 10% acetic acid, washed in Amplify (Amersham) to reduce the background, dried, and autoradiographed.

Immunoprecipitation. ^{35}S-labeled proteins containing equal counts per minute (1 to 5 μl) were incubated with 24 μg of bacterially purified p50 (41) in 100 μl of buffer containing 20 mM Tris-HCl (pH 8.0), 0.1% (vol/vol) Nonidet P-40, and 50 mM NaCl for 2.5 h on a rotary shaker at 4°C. Rabbit anti-p50 serum (5 μl) was added to the mixture, and the incubation was continued overnight. Protein A-Sepharose (50 μl of a 1:1 mixture) was added, and the slurry was incubated with rotation for another 1.5 h at 4°C. The beads were washed with 500-μl aliquots of the incubation buffer containing 0.1 mM NaCl until the free labeled proteins were removed (2 ml). The complexes were reduced by boiling in SDS-PAGE sample buffer and subjected to electrophoresis. The gel was fixed in 40% methanol and 10% acetic acid, washed in Amplify, dried, and autoradiographed.

RESULTS

ATF-encoding cDNAs isolated by expression cloning with an NF-ELAM1 probe. We previously identified an E-selectin promoter element, which we called NF-ELAM1, that is necessary for the cytokine induction of gene transcription. To isolate a cDNA(s) encoding the NF-ELAM1 binding protein(s), we screened λgt11 expression libraries from both endothelial and HeLa cells with a radiolabeled NF-ELAM1 oligonucleotide probe. From approximately 10^6 recombinants in the endothelial and HeLa libraries, one and two independent clones, respectively, to which the NF-ELAM1 probe bound specifically were isolated (Table 1). The endothelial cell cDNA is identical to a portion of the previously identified ATF3 transcription factor (itself a partial clone). The two HeLa cDNAs contain sequences of the ATF2 and ATFa transcription factors. All three factors identified are members of the ATF/CREB family of transcription factors. These proteins have a high degree of sequence similarity in their basic/leucine zipper domains. They were originally believed to bind specifically to the CRE (TGACGTC/AA/G) (for reviews, see references 27 and 58). The NF-ELAM1 site, however, differs from this consensus sequence at a single position (TGAC**A**TCA). Interestingly, as described below, the ATFs which we found to bind the NF-ELAM1 site appear to be activated by a cAMP-independent mechanism(s), in contrast to some other members of this family of factors which are cAMP dependent. This led us to hypothesize that the NF-ELAM1 site may specifically bind a subset of the cAMP-independent members of the ATF/CREB family.

TABLE 1. Recombinant clones that bind specifically to the NF-ELAM1 site identified by screening of λgt11 cDNA expression libraries

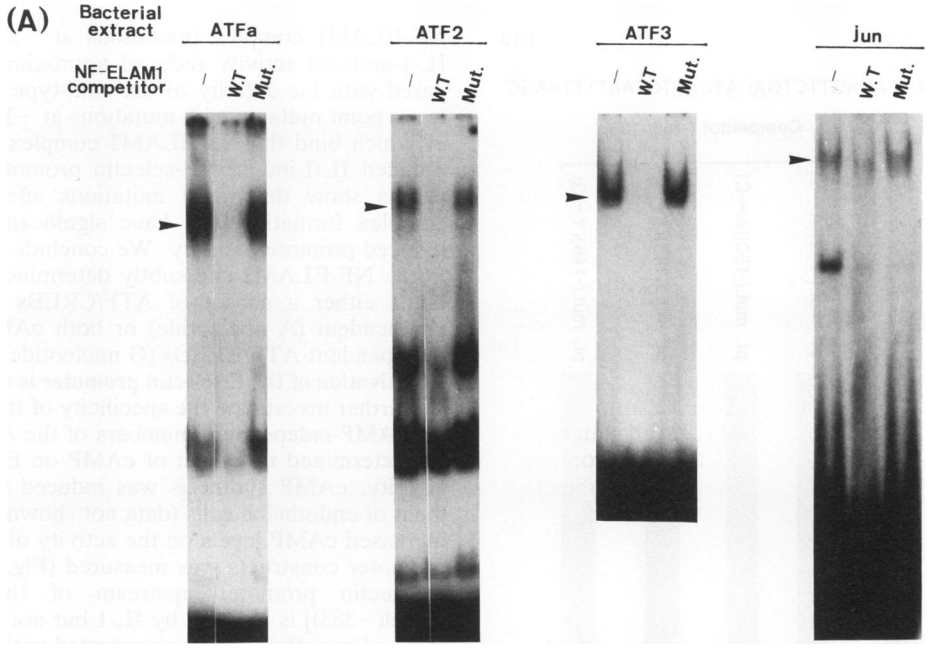
cDNA library	Insert size (kb)	Insert sequence
Endothelial (HUVEC)	1.8	ATF3
HeLa	1.4	ATF2
HeLa	1.6	ATFa

The NF-ELAM1 site specifically binds cAMP-independent ATF factors. To determine the binding specificities of different ATF proteins to the NF-ELAM1 site, band shift assays were performed. Lysates prepared from bacteria expressing ATFa, ATF2, and ATF3 or purified ATF1, ATF2, ATF3, and ATF4 proteins were used in this analysis. In addition, we examined the ability of recombinantly produced pure c-Jun to interact with the NF-ELAM1 site. Although c-Jun is not classified as a member of the ATF/CREB family, it binds specifically to the CRE as a homodimer and as a heterodimer complex with ATF2 and ATF3 (28).

As homodimers, ATFa, ATF2, and ATF3 all bind specifically to the NF-ELAM1 probe, as illustrated by the ability of wild-type but not mutant unlabeled oligomers to compete for complex formation (Fig. 1A). Homodimers of c-Jun also interact specifically with the NF-ELAM1 site, but with significantly lower affinity than the ATF factors. The weaker affinity of c-Jun for the NF-ELAM1 site can be deduced from the low intensity of the complex formed compared with that of the specific ATF complexes with approximately equivalent amounts of the purified proteins (Fig. 1B, lanes 1 to 3). In contrast, we were unable to demonstrate any specific binding of ATF1 or ATF4 to the NF-ELAM1 probe (data not shown).

We next examined the ability of various heterodimers to interact specifically with the NF-ELAM1 site. Previously, ATF2, ATF3, and c-Jun have been shown to heterodimerize with each other and bind specifically to a CRE binding site (28). We found that ATF2-Jun, ATF3-Jun, and ATF2-ATF3 heterodimers all bind specifically and with high affinity to the NF-ELAM1 site (Fig. 1B). The heterodimer complexes (lanes 4, 7, and 10) can be distinguished by their intermediate gel mobility compared with the mobility of the homodimer complexes (lanes 1 to 3). The specificity of heterodimer binding is again demonstrated by the competition for complex formation with an unlabeled wild-type oligonucleotide (lanes 5, 8, and 11) but not with an unlabeled mutant oligonucleotide (lanes 6, 9, and 12). Heterodimer complexes were not observed when ATF2, ATF3, or c-Jun was mixed with ATF1 (data not shown).

These results indicate that the NF-ELAM1 site, which differs at a single nucleotide from the CRE, has an affinity for the ATF-Jun homo- and heterodimers identical to that of the CRE, with one important exception—no specific binding of the ATF1 factor to the NF-ELAM1 site is observed. ATF1 is a cAMP-dependent member of this family of transcription factors. It contains a cAMP-dependent protein kinase (kinase A) consensus site, and phosphorylation of this site has been demonstrated to be important for its function (47). In contrast, ATFa, ATF2, and ATF3 do not have kinase A consensus sites, and thus far there is no evidence indicating that their functions are regulated by kinase A (27a). Therefore, these proteins may represent a subgroup of ATF/CREB



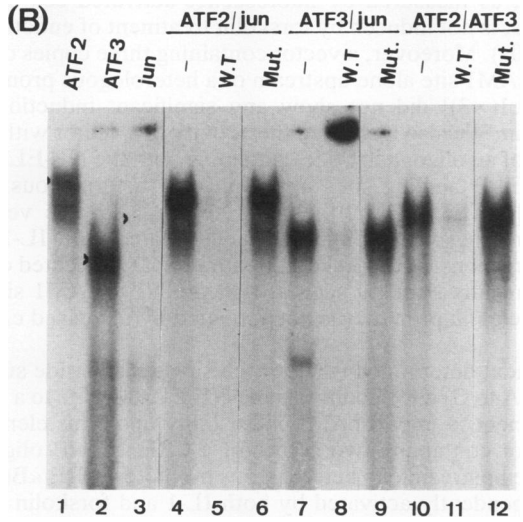


FIG. 1. Specific binding of leucine zipper-containing homodimers and heterodimers to the NF-ELAM1 site. (A) Bacterial extracts prepared from *Escherichia coli* Y1089 infected with the λATF clones or bacterially expressed pure Jun protein was used in the band shift analysis. An end-labeled oligomer incorporating the NF-ELAM1 site (−157 to −140) (see Materials and Methods) was used as a probe. An unlabeled probe oligomer (W.T) or a corresponding unlabeled oligomer in which the nucleotides of the NF-ELAM1 site are mutated (Mut.) (TTCTTCACGACGGTTAA) was used as the competitor (75-fold molar excess) to assess specificity of binding. (B) Bacterially expressed pure ATF and Jun proteins alone or mixed to allow heterodimer formation were used in the band shift analysis with the labeled NF-ELAM1 probe. Specificity of heterodimer binding was assessed by competition with unlabeled wild-type (W.T) or mutant (Mut.) oligomers. Arrowheads indicate positions of specific protein-DNA complexes.

transcription factors that do not respond to cAMP. Our results suggest that the NF-ELAM1 site which binds ATFa, ATF2, and ATF3 but does not appear to bind ATF1 may interact only with cAMP-independent members of the ATF/CREB transcription factor family. This is supported by the fact that screening of the HeLa cell λgt11 expression library with the NF-ELAM1 probe identified two independent clones of ATF2 as well as two independent clones of ATFa while not identifying any clones expressing either ATF1 or CREB. ATF1 and CREB mRNAs are present at 20-fold-higher levels than ATFa and 4-fold-higher levels than ATF2 in HeLa cells (11). If the NF-ELAM1 site was capable of binding either ATF1 or CREB they should have been identified as positive clones in the library screening.

A single nucleotide distinguishes the NF-ELAM1 binding site from a CRE. We determined the importance of individual nucleotides within the NF-ELAM1 site by assaying the effect of point mutations on both factor binding and the functional activity of the element. We examined the ability of oligonucleotides with point mutations in the NF-ELAM1 site to compete with wild-type oligonucleotide for binding of the endothelial cell NF-ELAM1 complex in the band shift assay (Fig. 2A). Four unlabeled competitor oligonucleotides, each carrying a single point mutation at position −152, −150, −147, or −146, were tested. Mutations at nucleotides conserved between the NF-ELAM1 element and the CRE (−152 and −147) failed to compete for binding of the NF-ELAM1 complex. These nucleotides are therefore important for the formation of the complex at both the CRE and the NF-ELAM1 element. In contrast, the mutant competitor carrying a substitution at the residue that distinguishes the NF-ELAM1 site from a CRE site was able to compete (an A-to-C transversion at −150). This result demonstrates that the precise nucleotide at position −150 is not essential for NF-ELAM1 complex binding. As a control, we show that an oligonucleotide with a mutation outside the binding site (point mutation at −146) competes equally well as the wild-type oligomer for binding of the NF-ELAM1 complex. The lack of stringency at nucleotide −150 for NF-ELAM1 complex formation contrasts with the strict conservation of a G residue at this position in virtually all published CRE sites and indicates a distinction between the NF-ELAM1 and CRE sites.

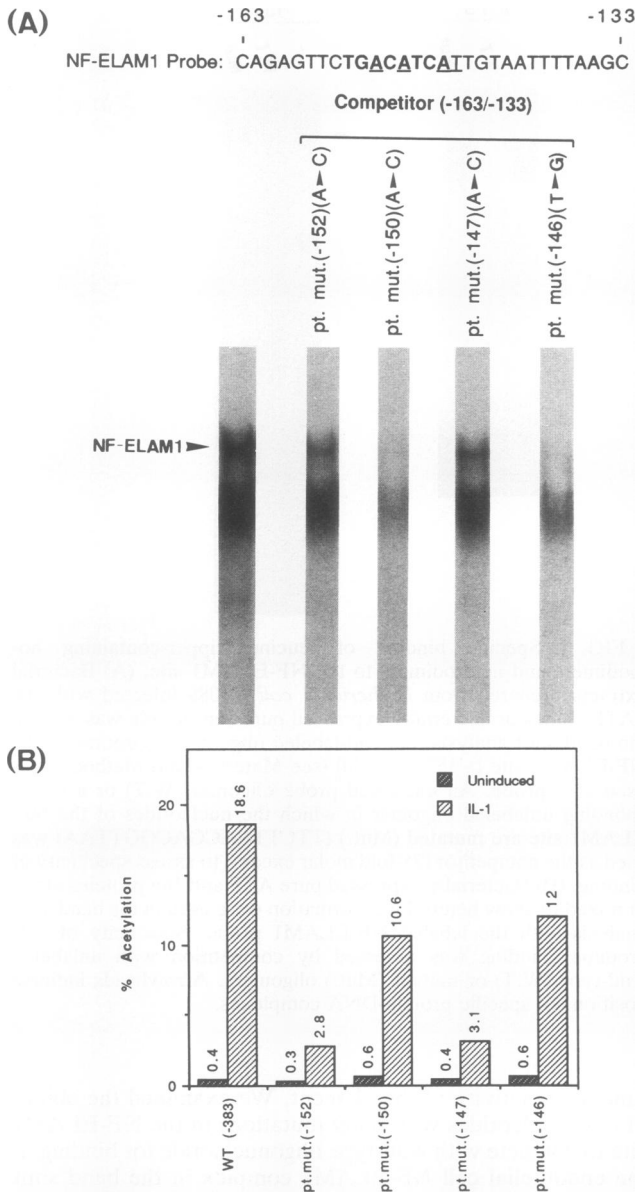


FIG. 2. Effect of point mutations within the NF-ELAM1 site on complex formation and promoter activity. (A) Band shift analysis was carried out using the end-labeled NF-ELAM1 oligomer shown and HUVEC nuclear extract. The importance of the indicated nucleotides within the NF-ELAM1 site (underlined) in complex formation was assessed by competition with an excess of the unlabeled probe oligomer containing the indicated point mutations (pt. mut.). (B) The importance of the indicated nucleotides in IL-1-induced activity was determined by transient transfection of HUVECs with the E-selectin promoter (−383 to +80) mutant-CAT constructs (10 μg) followed by analysis of IL-1 (20 U/ml)-induced CAT enzyme activity. Similar results were obtained in at least three separate experiments. Data shown are from one representative experiment. WT, wild type.

We also examined the functional importance of the same point mutations on E-selectin promoter activity. The IL-1-induced activity of promoters bearing these point mutations was compared with that of the wild-type promoter (Fig. 2B). The two point mutants which were unable to bind the NF-ELAM1 complex (mutations at −152 and −147) had IL-1-induced activity reduced approximately sixfold compared with the activity of the wild-type promoter. In contrast, point mutants with mutations at −150 and −146, both of which bind the NF-ELAM1 complex, had only slightly reduced IL-1-induced E-selectin promoter activity. These results show that point mutations affecting NF-ELAM1 complex formation also have significant effects on IL-1-induced promoter activity. We conclude that position −150 in the NF-ELAM1 site subtly determines whether the site binds either a subset of ATF/CREBs which are cAMP independent (A nucleotide) or both cAMP-dependent and -independent ATF/CREBs (G nucleotide).

Activation of the E-selectin promoter is cAMP independent. To further investigate the specificity of the NF-ELAM1 site for cAMP-independent members of the ATF/CREB family, we determined the effect of cAMP on E-selectin promoter activity. cAMP synthesis was induced by forskolin treatment of endothelial cells (data not shown). The effect of the increased cAMP levels on the activity of various E-selectin promoter constructs was measured (Fig. 3). The wild-type E-selectin promoter upstream of the CAT reporter [E-sel(−383)] is induced by IL-1 but not by forskolin treatment of endothelial cells transfected with the reporter construct. In addition, in vivo expression of the E-selectin protein, as measured by fluorescence-activated cell sorter analysis, is not induced by forskolin treatment of endothelial cells (20a). Moreover, a vector containing three copies of the NF-ELAM1 site alone upstream of a heterologous promoter [NF.EL1(×3)] did not show any significant induction by forskolin. We also measured the activity of a vector with two copies of an oligonucleotide containing both the NF-ELAM1 and NF-κB binding sites upstream of a heterologous promoter [NF.EL1/κB(×2)]. Similar to the wild-type vector, this construct was inducible in cells treated with IL-1 but was unresponsive to forskolin treatment of transfected cells. These results strongly suggest that the NF-ELAM1 site of the E-selectin promoter is not activated by increased cAMP levels.

We next determined whether the single nucleotide substitution (A to G) which converts the NF-ELAM1 site to a CRE is sufficient to impart cAMP inducibility upon this element. A vector containing two copies of a CRE–NF-κB oligonucleotide upstream of a heterologous promoter [CRE/κB(×2)] is independently activated by both IL-1 and forskolin (Fig. 3). Thus, the single base pair change, converting the NF-ELAM1 site to a CRE, is sufficient to render the promoter cAMP inducible. Therefore, the A-to-G substitution between NF-ELAM1 and CRE binding sites clearly distinguishes between the non-cAMP-responsive elements and CREs. We conclude that the ATF/CREB factor(s) within the NF-ELAM1 complex is activated in a cAMP-independent manner.

ATFa can modulate E-selectin promoter activity and is a component of the NF-ELAM1 complex. To determine which ATF family member(s) composes the NF-ELAM1 complex, we assayed the effect of overexpressing different ATFs on E-selectin promoter activity. When ATFa, ATF1, ATF2, and ATF3 were each expressed in combination with a wild-type E-selectin–CAT reporter (−233), only ATFa demonstrated an ability to activate the E-selectin promoter (Fig. 4A and data not shown). This activation is mediated by the NF-ELAM1 binding site, as shown by the inability of ATFa to activate the NF-ELAM1 mutant promoter (NF.EL1mut.). Thus, ATFa is capable of activating the E-selectin promoter in a DNA binding-dependent manner.

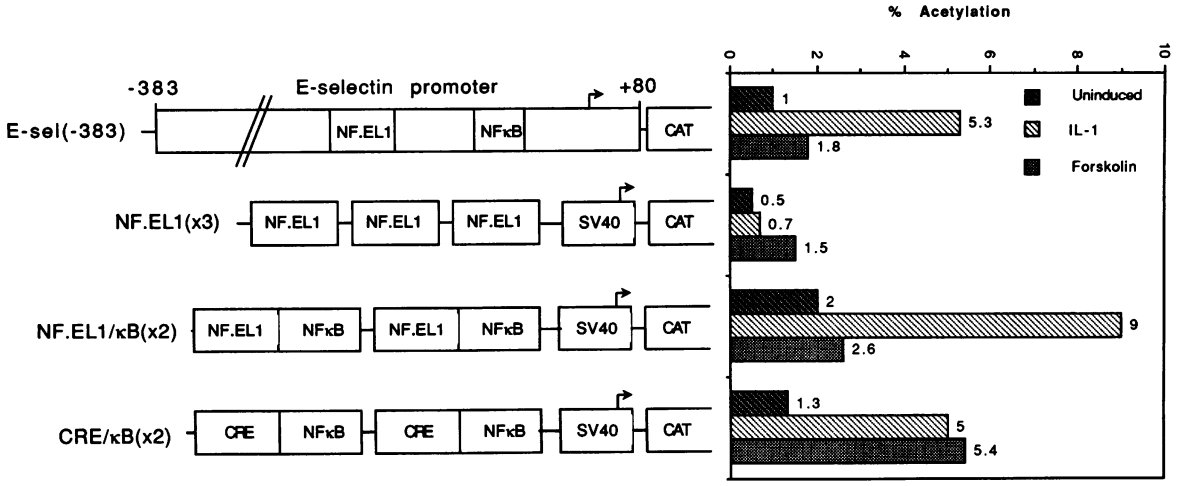


FIG. 3. Effect of forskolin on promoter constructs containing the NF-ELAM1 or CRE binding sites. The CAT enzymatic activity of the various NF-ELAM1- or CRE-containing promoter–CAT constructs (10 μg) was assayed following transient transfection in endothelial cells (HUVECs). The cells were treated as indicated with IL-1 (20 U/ml) and forskolin (100 μM) 24 h prior to extract preparation. Similar results were obtained in at least three separate experiments. Data shown are from one representative experiment. SV40, simian virus 40.

To assess whether ATFa is a component of the endogenous NF-ELAM1 complex, we examined the effect of an ATFa peptide-specific antiserum on NF-ELAM1 complex formation in band shift analysis. This antiserum does not cross-react with other ATF/CREB factors (data not shown). Incubation of HUVEC nuclear extract with the ATFa-specific antiserum resulted in a supershift of the NF-ELAM1 complex, while preimmune serum had no effect on the complex formation. The supershift we observed using endothelial nuclear extracts is similar to that observed when bacterial lysates containing cloned ATFa were used (Fig. 4B). Therefore, it appears that the NF-ELAM1 complex contains ATFa or an ATFa-like protein.

Since we have shown previously that the NF-ELAM1 site alone does not have enhancer activity in the absence of the NF-κB site (31), the endogenous NF-ELAM1 complex is unlikely to be exclusively composed of ATFa. Promoter activation by overexpression of ATFa may be a consequence of disruption or displacement of the in vivo NF-ELAM1 complex normally interacting at this site. Alternatively, activation by overexpressed ATFa alone may result from loss of its normal regulation when present at high concentrations. However, the effect of ATFa antiserum on NF-ELAM1 complex formation clearly indicates that ATFa is, at least in part, a component of this complex.

Direct protein-protein interaction occurs between leucine zipper-containing factors and NF-κB. Since both the NF-ELAM1 and NF-κB elements are essential for activation of the E-selectin promoter, we asked whether direct physical interaction occurs between the proteins binding to these elements. Using affinity chromatography, we assayed the ability of cAMP-independent ATF/CREB family members and c-Jun to associate with the p50 and p65 subunits of NF-κB. In addition, ATF4, which does not bind to NF-ELAM1, was also tested in this assay. A bacterially expressed fusion of GST and either the p50 (aa 35 to 381) or p65 (aa 12 to 317) subunit of NF-κB was immobilized on glutathione-Sepharose. In vitro-translated, radiolabeled ATFa, ATF2, ATF3, ATF4, and c-Jun (Fig. 5A, lanes 1, 5, 9, 17, and 13, respectively) were incubated with GST-p50 and GST-p65 beads or GST control resin. Bound proteins were eluted from the beads with free glutathione and analyzed by SDS-PAGE.

ATFa and ATF2 (aa 107 to 505), the most homologous members of the family, bound to both GST-p50- and GST-p65-Sepharose, as is shown by the presence of labeled bands in the eluate (Fig. 5A, lanes 3, 4, 7, and 8). The partial clone of ATF2 gave rise to two protein products presumably due to premature termination. ATF3 also bound to GST-p50 but not to GST-p65 (Fig. 5A, lanes 11, 11a, and 12). The other leucine zipper-containing factor, c-Jun, bound to GST-p50 and GST-p65 (Fig. 5A, lanes 15, 16, and 16a). In contrast, ATF4 did not bind to either GST-p50 or GST-p65 (Fig. 5A, lanes 19 and 20). None of the radiolabeled proteins interacted with the control GST beads (Fig. 5A, lanes 2, 6, 10, 14, and 18). Moreover, all of the in vitro-translated proteins were shown to retain native activity by binding to their respective DNA sites (data shown only for ATF4 in Fig. 5C). We conclude that the proteins which we found to bind the NF-ELAM1 site also specifically associate with NF-κB in vitro. Differences in the strength of these interactions may account for the differences in the intensity of the bands observed. However, given the qualitative nature of this experiment, differences in band intensities should be interpreted with caution.

We also carried out a series of immunoprecipitations as an alternative method for demonstrating direct protein-protein interactions between leucine zipper-containing factors and the p50 subunit of NF-κB. In vitro-translated, radiolabeled ATFa, ATF2, ATF3, ATF4, and c-Jun (Fig. 5B, lanes 1, 4, 7, 13, and 10) alone, or following incubation with unlabeled pure p50 (aa 35 to 381), were immunoprecipitated with p50-specific antiserum. None of the labeled proteins were immunoprecipitated in the absence of p50 (Fig. 5B, lanes 2, 5, 8, 11, and 14). ATFa, ATF2, ATF3, and c-Jun coprecipitated with p50 (Fig. 5B, lanes 3, 6, 9, 12, and 12a), suggesting a complex formation between these proteins. Again there was no detectable association between ATF4 and p50 (Fig. 5B, lane 15). These results correlate well with those obtained by the GST fusion protein binding assay. We conclude that the ATF/CREB family members and c-Jun which bind to the NF-ELAM1 site can directly interact with

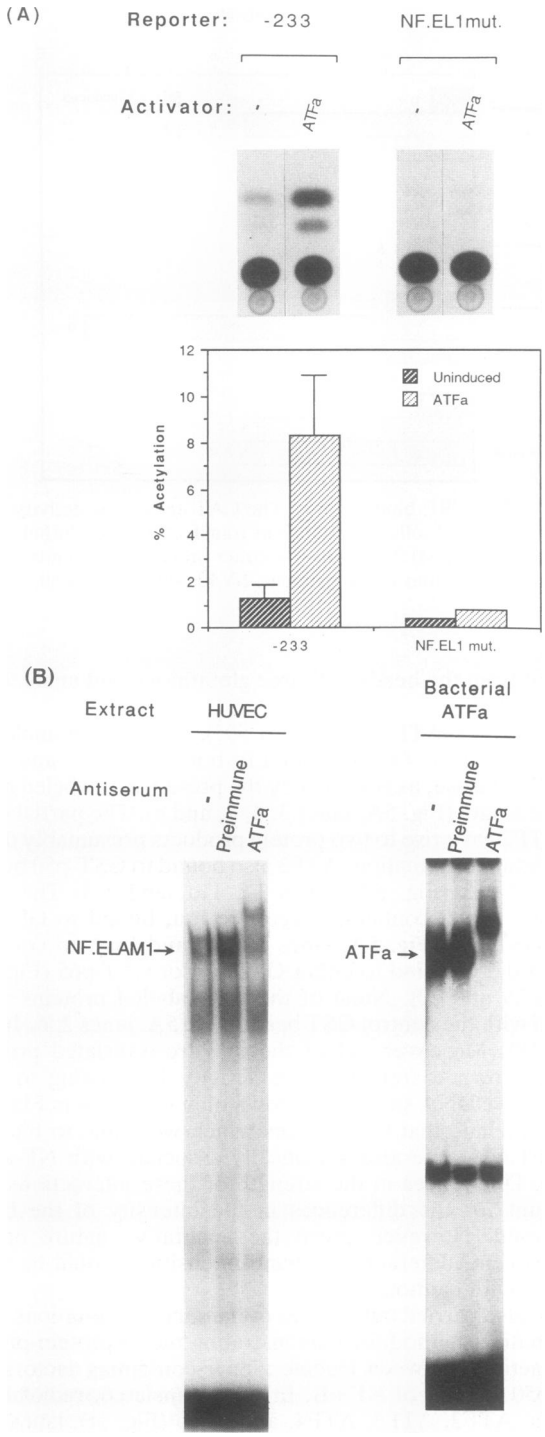


FIG. 4. ATFa activity and binding at the NF-ELAM1 site. (A) HeLa cells were transfected with either the wild-type (−233) (containing nucleotides −233 to +80) or NF-ELAM1 mutant (NF.EL1 mut.) (containing mutations of nucleotides −153 to −144) E-selectin promoter–CAT reporter plasmids (5 μg) in combination with either pSG5 alone (6 μg) (uninduced; lanes −) or the expression plasmid for ATFa (2 μg) (final DNA concentration made up to 11 μg with pSG5). Cells were harvested 48 h posttransfection, and CAT analysis was performed. Results with the wild-type vector are the means ± standard errors of the mean from three independent experiments. Results with the NF.EL1mut. vector are the means from two independent experiments. A representative CAT assay is shown. (B) Band shift analysis using an end-labeled NF-ELAM1 oligomer examining the effect of preimmune serum or ATFa peptide antiserum on NF-ELAM1 and ATFa complexes.

the p50 and p65 subunits of NF-κB. The most probable site of interaction is between the basic/leucine zipper region common to the ATF/CREB and c-Jun factors and the Rel homology region common to both p50 and p65.

Activation of the E-selectin promoter in vivo requires interaction between the NF-ELAM1 complex and NF-κB. We have previously demonstrated that in the uninduced state NF-κB is not bound to the E-selectin promoter, presumably being retained in the cytoplasm in a complex with IκB. IL-1 treatment induces NF-κB binding to the E-selectin promoter (56). On the other hand, we have shown that the NF-ELAM1 complex is constitutively bound to its site within the E-selectin promoter independent of IL-1 treatment of the cells (31). To determine the functional importance of the interaction between NF-κB and the constitutively bound NF-ELAM1 complex in vivo, we examined the effect of overexpressing the NF-κB subunits (p50 and p65) on activation of the E-selectin promoter (Fig. 6). The level of endogenous IκB is not sufficient to retain overexpressed NF-κB in the cytoplasm, thereby allowing it to translocate to the nucleus and bind to the E-selectin promoter.

Overexpression of the NF-κB subunits resulted in a strong induction (18-fold) in the activity of the wild-type E-selectin promoter (−233). In order to address the role of the constitutively bound NF-ELAM1 complex in this activation by NF-κB, we examined the ability of expressed NF-κB to activate the E-selectin promoter containing a mutated NF-ELAM1 binding site. In the absence of the NF-ELAM1 binding site the ability of overexpressed NF-κB to activate the E-selectin promoter was significantly reduced (NF.EL1 mut). An E-selectin promoter deletion mutant containing only 35 bp of upstream sequence (−35) is not activated by overexpressed NF-κB, confirming that NF-κB activation is mediated by the E-selectin promoter. We conclude from these results that the interaction between NF-κB and the NF-ELAM1 complex plays an important role in activation of the E-selectin promoter.

DISCUSSION

In this study we have shown that NF-ELAM1, a complex essential for cytokine induction of E-selectin transcription, is composed of cAMP-independent leucine zipper factors, one of which is ATFa. We have also demonstrated direct protein-protein interaction between specific ATFs and the two subunits of NF-κB in vitro. Moreover, we provided in vivo evidence which demonstrates that strong activation of the E-selectin promoter by NF-κB is dependent on interaction with the NF-ELAM1 complex. This novel protein-protein interaction between members of two different transcription factor families, leucine zipper and Rel, most likely plays a central role in the specific activation of the E-selectin gene by cytokines.

The ATF/CREB family can be divided into two subgroups based on their ability to be induced by cAMP and their ability to heterodimerize. To date, CREB and ATF1 form one subgroup based on their overall amino acid homology, ability to heterodimerize, and cAMP dependence for activity (24, 29, 30, 47). The cAMP-independent subgroup contains factors ATFa, ATF2, and ATF3 (17, 19, 29, 39, 40). ATF2 and ATF3 heterodimerize with each other and also with

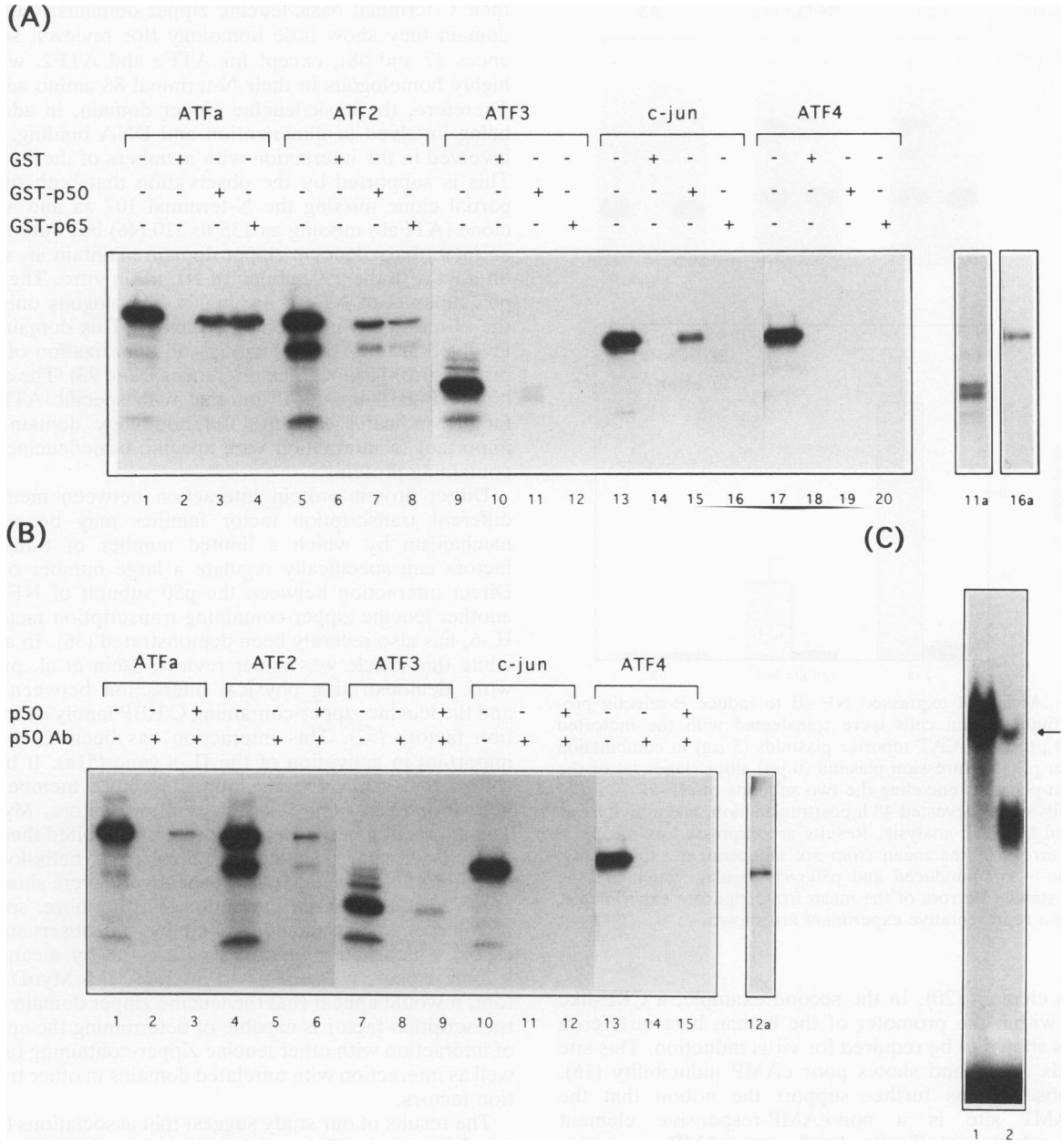


FIG. 5. Physical association between leucine zipper-containing proteins and NF-κB. (A) GST fusion protein binding assay. The indicated ^{35}S-labeled proteins were incubated with GST, GST-p50, and GST-p65 immobilized on glutathione-Sepharose. Lanes 11a and 16a are longer exposures of lanes 11 and 16. (B) Coimmunoprecipitation. The indicated ^{35}S-labeled proteins alone or incubated with homogeneous p50 were precipitated with p50 antiserum (Ab). Lane 12a is a longer exposure of lane 12. (C) Band shift assay using 1 μg of bacterially purified ATF4 (lane 1) and in vitro-translated ATF4 (lane 2) with a ^{32}P-labeled oligonucleotide containing the CRE consensus site. The arrow indicates the ATF4-DNA complex. The lower-molecular-weight band in lane 2 corresponds to a background complex obtained with the reticulocyte lysate alone.

c-Jun (28, 32). We have shown these heterodimers to bind the NF-ELAM1 site in this study. The NF-ELAM1 site differs from the CRE consensus sequence at a single position. We demonstrate that this single nucleotide alters both the DNA binding specificity and activity of the NF-ELAM1 site compared with those of the classical CRE. First, we isolated only non-cAMP-responsive ATF/CREB factors interacting specifically with the NF-ELAM1 element. Second, this element, either in single or multiple copies, is unable to impart cAMP inducibility upon a heterologous promoter. We conclude that the NF-ELAM1 complex is composed of cAMP-independent members of the ATF/CREB family.

Recently, two other CRE-like enhancer elements with the same G-to-A substitution as is found in the NF-ELAM1 site have been described. The δA enhancer element of the CD3δ gene, like the NF-ELAM1 site, is unresponsive to increased levels of cAMP. In addition, three isoforms of ATF2 were identified in a T-cell library which interacted specifically

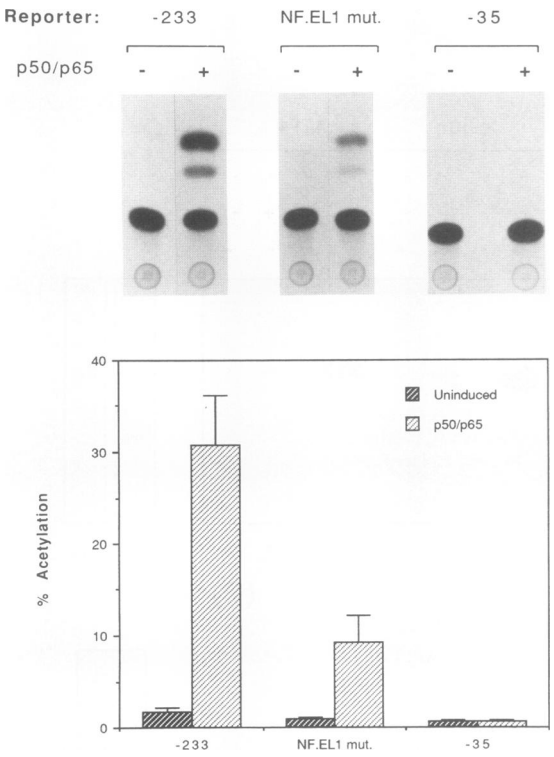


FIG. 6. Ability of expressed NF-κB to induce E-selectin promoter activity. HeLa cells were transfected with the indicated E-selectin promoter-CAT reporter plasmids (5 μg) in combination with either pSG5 expression plasmid (6 μg) alone (lanes −) or the expression plasmids encoding the two subunits of NF-κB (2 μg of each). Cells were harvested 48 h posttransfection, and activity was determined by CAT analysis. Results are expressed as means ± standard errors of the mean from six independent experiments, except the −35 (uninduced and p50/p65) results, which are the means ± standard errors of the mean from triplicate experiments. Results of a representative experiment are shown.

with this element (20). In the second example, a CRE-like element within the promoter of the human beta interferon gene was shown to be required for virus induction. This site also binds ATF2 and shows poor cAMP inducibility (16). These observations further support the notion that the NF-ELAM1 site is a non-cAMP-responsive element (NCRE) which specifically binds non-cAMP-responsive members of the ATF/CREB family. A recent publication has shown that a variant of the AP-1 binding site (TTACCTCA) within the c-Jun promoter appears to specifically bind a heterodimer of c-Jun and ATF2 (54). Therefore, it appears that variants of both the CRE and AP-1 consensus binding sites subtly determine the binding specificity of these sites for members of both the ATF/CREB and Jun/Fos family of factors.

We show direct physical interaction between specific members of the ATF/CREB family and the two subunits of NF-κB in vitro. Protein-protein interaction between these promoter-bound factors may result in specific activation of the E-selectin promoter. An important role for such Rel-basic/leucine zipper domain interactions is supported by our results in vivo which demonstrate that the ability of expressed NF-κB to strongly activate the E-selectin promoter is dependent on the NF-ELAM1 complex. Members of the ATF/CREB family are homologous to each other within their C-terminal basic/leucine zipper domains; outside this domain they show little homology (for reviews, see references 27 and 58), except for ATFa and ATF2, which are highly homologous in their N-terminal 88 amino acids (19). Therefore, the basic/leucine zipper domain, in addition to being involved in dimerization and DNA binding, may be involved in the interaction with members of the Rel family. This is supported by the observation that both the ATF2 partial clone missing the N-terminal 107 aa and an ATFa clone (ATFa0) missing aa 135 to 310 (46) but both retaining an intact basic/leucine zipper domain maintain an ability to interact with the p50 subunit of NF-κB in vitro. The p50 and p65 subunits of NF-κB are highly homologous only within the N-terminal Rel homology domain. This domain is also involved in both DNA binding and dimerization of the Rel proteins (for reviews, see references 8 and 23). The ability of both NF-κB subunits to interact with specific ATF/CREB factors indicates that the Rel homology domain is also important in interaction with specific basic/leucine zipper-containing proteins.

Direct protein-protein interaction between members of different transcription factor families may be a general mechanism by which a limited number of transcription factors can specifically regulate a large number of genes. Direct interaction between the p50 subunit of NF-κB and another leucine zipper-containing transcription factor, NF-IL-6, has also recently been demonstrated (36). In addition, while this article was under review, Stein et al. published work demonstrating physical interaction between NF-κB and the leucine zipper-containing C/EBP family of transcription factors (52). This interaction has been shown to be important in activation of the IL-8 gene (51a). It has been shown that c-Jun directly interacts with a member of the helix-loop-helix family of transcription factors, MyoD (5). The interaction between these proteins inhibited their ability to transactivate. The leucine zipper and helix-loop-helix domains of c-Jun and MyoD, respectively, were shown to be involved in this direct interaction. Furthermore, specificity of interaction was demonstrated by the observation that c-Fos, which heterodimerizes with c-Jun by means of the leucine zipper, was unable to interact with MyoD. Therefore, it would appear that the leucine zipper domain within a transcription factor is capable of determining the specificity of interaction with other leucine zipper-containing factors as well as interaction with unrelated domains in other transcription factors.

The results of our study suggest that associations between members of the Rel and basic/leucine zipper families of transcription factors may play a central role in the specific activation of a large number of target genes by NF-κB, in particular, the specific activation of the E-selectin gene in response to cytokines.

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