A T-cell Enhancer Cooperates with NF-xB to Yield Cytokine Induction of E-selectin Gene Transcription in Endothelial Cells*

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Rob Hooft van Huijsduijnen†, James Whelan, Rosanna Pescini, Michael Becker-André, Anne-Marie Schenk, and John F. DeLamarter

From the Glaxo Institute for Molecular Biology, Chemin des Aulx, 1228 Plan-les-Ouates, Geneva, Switzerland

ELAM1 (endothelial leukocyte adhesion molecule 1, also known as E-selectin) is a highly tissue-specific adhesion molecule that is transiently and exclusively expressed on cytokine-induced endothelial cells. We have identified two proximal ELAM1 promoter elements and their DNA-binding factors that are, in addition to NF-xB, essential for ELAM1 transcription. Mutation of either element in promoter constructs carrying the first 383 nucleotides of the ELAM1 promoter markedly diminished the expression of a fused chloramphenicol acetyltransferase reporter gene. Although multimers of either element failed to display enhancer activity on its own, fusion of these to the NF-xB element had a strong stimulatory effect. This site, ACATCAT, is recognized by a factor we have called NF-ELAM1. The site corresponds to NF-ELAM1's preferential binding sequence (A/T)CA(G/T)CA(G/T) as determined in a target definition assay. This element is identical to the T-cell 5x enhancer found in the T-cell receptor-α, -β, and CD3δ genes. Our results suggest that the 5xNF-ELAM1 element can function as a modulator of NF-xB in endothelial cells both as well as a T-cell enhancer.

The human endothelial leukocyte adhesion molecule (ELAM1 also known as E-selectin) is a surface-expressed glycoprotein found exclusively on cytokine-induced endothelial cells (1). ELAM1 mediates adherence and extravasation of a subset of leukocytes (2–7). Expression of ELAM1 is correlated with several acute and chronic pathological conditions such as asthma and psoriasis (8). In this manner it plays an important role in the inflammatory response. Understanding the mechanisms of regulation involved in ELAM1 expression should shed light on the means by which the endothelium is activated to attract the appropriate immune cells to a site of injury.

Consistent with its role in leukocyte tissue infiltration, ELAM1 is induced transiently on endothelial cells where both its protein and mRNA levels peak about 4 h post-IL-1β or tumor necrosis factor 1α treatment, returning to near basal levels 20 h thereafter (3, 9). Studies on the mechanism(s) of this regulation have previously shown that both the ELAM1 promoter's transcription activity and its mRNA stability are under the control of newly synthesized proteins. Genomic sequences covering the ELAM1 promoter have recently been isolated (10–12). Within these sequences a binding site for the transcription factor NF-xB was found at position 117 relative to the transcription start site. In endothelial cells cytokine treatment activates NF-xB DNA binding activity. The importance of NF-xB for enhancing ELAM1 transcription was shown by mutation of the NF-xB site. The mutant chimeric ELAM1 promoter/CAT construct is unable to induce reporter activity in response to cytokine treatment of the transfected cells (10). In addition, cycloheximide plus IL-1 superinduces NF-xB DNA binding activity in endothelial cells. These results provide at least a partial explanation for the enhanced transcription activity of the ELAM1 promoter induced by protein synthesis inhibitors.

In spite of its central role in controlling the ELAM1 promoter, NF-xB by itself does not appear to be sufficient to mediate ELAM1 activation. The time course of NF-xB activation and ELAM1 induction do not correspond late after cytokine treatment. At 24-h post-IL-1 induction NF-xB DNA binding activity is still significant, whereas run-on transcription of ELAM1 mRNA is undetectable. Moreover, Whelan et al. (10) have demonstrated that sequences upstream of the NF-xB site between -233 and -117 are essential for the activity of ELAM1 promoter/CAT constructs, both in endothelial and non-endothelial cells.

We have undertaken to identify the factors which interact with NF-xB in causing cytokine induction of ELAM1 gene transcription. In this paper, we characterize the DNA-binding sites for a number of additional factors necessary for cytokine induction. We show that specific mutation of these sites correlates with loss of transcription activity. In addition we show here cooperation between factors binding one of these sequences and NF-xB. This cooperation appears to be a control feature of the mechanism by which cytokines induce ELAM1 transcription. The constitutive binding of this factor may also provide a means of repressing ELAM1 expression during the quiescent state.

EXPERIMENTAL PROCEDURES

Cell Lines—Human umbilical vein endothelial cells (HUVEC) 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), bovine brain extract containing heparin, 2% fetal bovine serum, gentamicin, and amphotericin (Clonetics, CA). IE-7 cells are simian virus 40 (SV40)-transformed primary HUVECs. This cell line was originally cloned under the name SGHEC-7 (32) and was grown in the endothelial growth medium described above containing geneticin (300 μg/ml).

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‡ To whom correspondence should be addressed. Tel.: 41-22-706-96-66; Fax: 41-22-794 69 65.

1 The abbreviations used are: ELAM1, endothelial leukocyte adhesion molecule 1/E-selectin; CAT, chloramphenicol acetyltransferase; IL-1, interleukin 1; HUVEC, human umbilical cord endothelial cell; TDA, target definition assay; PCR, polymerase chain reaction.

2 Fasani-Ghersa, P., Hooft van Huijsduijnen, R., Whelan, J. and DeLamarter, J. F. (1992) J. Biol. Chem. 267, 19226–19232.

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HeLa cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 50 μg each of penicillin and streptomycin/ml. IL-1 treatment (40 units/ml) was for 4 h in fresh medium prior to nuclear extract preparation or during 24 h following the day after DNA transfection of CAT constructs.

**Protein Preparations**—HUVEC (passage 5–6) and HeLa nuclear extracts were prepared as described (13) with modifications (14). Protein concentrations were adjusted to 3 μg/ml and aliquots stored at -70 °C.

**Probe Preparations**—The end-labeled probes corresponding to the ELAM1 promoter were prepared as described (15). Briefly, 10 pmol of one of the flanking oligonucleotides (18–34 nucleotides) were end-labeled with 10 μl (100 μCi) [γ-32P]ATP, in 20 mM Tris-Cl, pH 7.75, 10 mM MgCl₂, 50 mg/ml bovine serum albumin, 10 mM β-mercaptoethanol, and 10 units of polynucleotide kinase (Boehringer), in 15 μl of total volume. After 45 min at 37 °C, the enzyme was inactivated at 65 °C (10 min) and other components for the PCR (polymerase chain reaction) added; 10 μl of 10 × PCR buffer (0.5 M KCl, 0.1 M Tris-Cl, pH 8.4, 1.5 mM MgCl₂, and 1 mg/ml gelatin), 10 μl of dimethyl sulfoxide, 4 μl of dNTPs (5 mM each, pH 7.5, Pharmacia), 25 pmol of the other primer, ~0.5 μg of plasmid DNA containing the ELAM1 promoter insert as template, and H₂O to 100 μl. The sample was heated 5 min at 99 °C in a thermocycler (Ericomp, Twinblock, CA), cooled on ice, spun briefly to collect condensate, and supplemented with 0.5 μl (2.5 units) Taq polymerase (Cetus). Fifty μl mineral oil (Sigma) was overlaid and the sample was cycled 1.5 min at 94 °C, 1.5 min at 55 °C (25 cycles) and 0.5 min at 72 °C. The probe was concentrated by precipitation and either loaded directly on gel or methylated with dimethyl sulfate (5 μl/ml in cacodylate buffer, Ref. 20) and reprecipitated before gel purification.

Short probes for band shift experiments were generated by end-labeling as described above. After inactivation of the kinase, 25 pmol of the complementary oligonucleotide and 1 μl of 50 μl NaCl were added, the mixture was heated 2 min at 65 °C, and allowed to anneal for at least 30 min at room temperature.

The following oligonucleotides were used: NF-ELAM1 probe, 5'CAAGACATTTCTTAATTAAAGC and 5'GCTTTAAAAATCCAATTTCTTGTGGAC; NF-ELAM2 probe, 5'CAAGACATTCTTTACAGGTGCCT and 5'GCTTTAAAAATCCAATTTCTTGTGGAC.

The oligonucleotides were prepared on an Applied Biosystems synthesizer. All probes were purified on 0.5 × Tris-borate EDTA (TBE) (20) gels containing 15% acrylamide (1:19 acryl/bisacryl) for the annealed oligonucleotides and 10% for the PCR products. The bands were cut out of the gel, sliced, and soaked overnight in STE (0.1 M NaCl, 10 mM Tris-Cl, pH 7.5, and 1 mM EDTA). The eluate was used without further treatment.

The gel patterns were run on TBE gels and run in TBE gel.

**RESULTS**

Two DNA-binding Motifs in Addition to NF-κB Are Found in the ELAM1 Promoter Region Responsible for Cytokine Induction—We wished to determine what upstream sequences aside from the NF-κB-binding site might play a role in cytokine induction of ELAM expression. We have previously shown that the region from 233 to 117 nucleotides preceding the transcription start site is necessary for induction (10), yet contains no binding sequences for known factors (Refs. 10–12 and Fig. 1). To identify nuclear proteins which would recognize binding motifs on this DNA fragment, band shift analyses were carried out with a relatively long probe (~163 to ~75) covering this region of the ELAM1 promoter. To generate this fragment PCR synthesis was carried out with radiolabeled oligonucleotide primers (see Experimental Procedures). The resultant DNA fragment was specifically recognized by several factors from a nuclear extract of primary human umbilical cord vein endothelial cells (HUVECs) (Fig. 2). One of these bands is induced by IL-1 treatment. Competition with unlabeled NF-κB oligonucleotide (Fig. 2, lane 2) indicates that this band corresponds to NF-κB. We have named the other four complexes NF-ELAM1 and -1b and NF-ELAM2 and -2b. All complexes were stable in the presence of up to 5 μg of nonspecific competitor DNA (poly(dI-dC), suggesting tight and specific DNA-protein association.

![Fig. 1. ELAM1 promoter sequence. Numbering is relative to the transcription start site (+1, arrow) as determined previously (10). The TATA box, a CAAT sequence, the decameric NF-κB sequence, and NF-ELAM1 and -2 targets are boxed.](http://www.jbc.org/)

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All these bands could also be detected in HeLa nuclear extracts (data not shown).

**Determination of the NF-ELAM1-binding Site Suggests Identity to a T-cell Enhancer**—We next asked whether we could more precisely determine the sequences to which these factors bound. To accomplish this we undertook DNase I footprinting experiments using the same DNA region as in the band shift experiments for the footprinting substrate. A single footprint was observed between nucleotides −153 and −144, suggesting that these nucleotides interacted with DNA binding factor(s) (Fig. 3a). Both endothelial and HeLa cell extracts produced the same footprint (lanes 1–4 versus 6–8). A synthetic oligonucleotide spanning this footprint was tested in band shift assays using nuclear extracts from HUVEC cells (Fig. 3b). The pattern obtained suggests that the −153 to −144 site is recognized by NF-ELAM1a and −1b, the slower migrating complexes in Fig. 2. This was confirmed by the observation that this oligonucleotide, when used as unlabeled competitor, inhibited NF-ELAM1a and −1b binding to the labeled −163 to −75 fragment in band shift experiments (Fig. 2, EL1 lane). Although Fig. 2 also shows partial competition with NF-ELAM2b, results described below clearly show that this factor binds elsewhere. Since the NF-ELAM1a/b bands were competed away by an oligonucleotide carrying the wild type sequence (Fig. 3b, lane 3), whereas a mutant sequence did not (lane 4), NF-ELAM1a/b are specific for the −153 to −144 sequence revealed by the DNase I footprint. The “smear” corresponding to NF-ELAM1b possibly represents a partially degraded NF-ELAM1a complex, as it shows identical behavior with respect to the competitors. In addition these extra bands varied somewhat with different nuclear extracts, suggesting variable degradation of NF-ELAM1a. No difference was observed between extracts from noninduced (lane 1) or IL-1 induced (lane 2) cells. We conclude that the sequence between −153 and −144 is the binding site for a factor which is present in both endothelial cells and non-endothelial cells and which binds the element regardless of cytokine treatment of the cells.

To further define the precise binding sequence for NF-ELAM1, two approaches were undertaken. First, a panel of oligonucleotides was made carrying point mutations in the sequence protected in the DNase I footprint. The digest was performed in the absence (lane 5) or presence (other lanes) of nuclear extract. Lane 1, 10 μg of protein, uninduced HeLa extract; lane 2, 10 μg of protein IL-1 induced; lane 3, 18 μg of protein uninduced HeLa extract; and lane 4, IL-1 induced. Lane 6–8, 10, 20, and 30 μg of protein IL-1-induced HUVEC extract. The protected area is indicated by bars. Lane G, dimethyl sulfate-generated G ladder of the same DNA fragment, numbering of the G residues as in Fig. 1. Lane M, size markers. 

**FIG. 2.** Several nuclear factors bind the ELAM promoter between −163 and the NF-κB site. Band shift assay using a PCR-generated −163 to −75 ELAM1 promoter fragment and non-induced and IL-1-induced HUVEC nuclear extract. EL1, competition with unlabeled double-stranded ELAM1 oligonucleotide.  The competition with a double-stranded oligonucleotide carrying the NF-κB-site (−104 to −75 in the ELAM promoter).

**FIG. 3.** NF-ELAM1 binds at position −153 to −144 in the ELAM1 promoter. Panel a) DNase I footprint of the −247 to −75 promoter fragment. The digest was performed in the absence (lane 5) or presence (other lanes) of nuclear extract. Lane 1, 10 μg of protein, uninduced HeLa extract; lane 2, 10 μg of protein IL-1 induced; lane 3, 18 μg of protein uninduced HeLa extract; and lane 4, IL-1 induced. Lane 6–8, 10, 20, and 30 μg of protein IL-1-induced HUVEC extract. The protected area is indicated by bars. Lane G, dimethyl sulfate-generated G ladder of the same DNA fragment, numbering of the G residues as in Fig. 1. Lane M, size markers. Panel b, band shift assay using a double-stranded oligonucleotide spanning the −164 to −133 region (NF-ELAM1 site) and nuclear extracts from HUVEC induced or not with IL-1 (lanes 2–4 and lane 1, respectively). Lane 3, competition with 20 pmol of the unlabeled NF-ELAM1 recognition sequence (−163 to −133). Lane 4, competition with the block mutant (same sequence, but −154 to −144-mutated). The arrowhead points to the NF-ELAM1a band.
The NF-ELAM1- and -2-binding Motifs Are Required for ELAM Transcription—The functional importance of sequences around both the -150 and -100 nucleotides of the promoter in the cytokine induction of ELAM transcription was further tested in a reporter fusion assay. We have shown previously that both a 383- and 233-base pair ELAM1 promoter fragment fused to the CAT reporter gene display cytokine inducibility of the reporter (10). As we show here in Fig. 6a and b, a construct with only 181 base pairs upstream sequence is still IL-1-inducible. However, we also found that when the 10 nucleotides between -153 and -144 were altered in a “blocked mutation, these constructs no longer responded strongly to cytokine induction by reporter expression in the IE-7 endothelial cells (Fig. 6a, panel A and Fig. 6b) or in HeLa cells (Fig. 6a, panel B and Fig. 6c). Similarly, when the 3 Gs at position -104, -103, and -100 were replaced by Ts, CAT induction was markedly reduced (Fig 6). In contrast, mutation of a randomly chosen block of 10 nucleotides within this region (-161 to -170) had no effect on transcription. From these results we conclude that both the NF-ELAM1- and -2-binding sites are needed for full cytokine induction of the ELAM1 reporter. Thus, at least three DNA elements binding transcription factors (NFκB, NF-ELAM1, and NF-ELAM2)
NF-ELAM-1 and NF-κB Cooperation

NF-ELAM-1 Shows Cooperation with the NF-κB Enhancer Activity—We next wished to test whether the -144 to -153 sequence element (the NF-ELAM1-binding site) has enhancer activity of its own. Constructs carrying multimers of only this element linked to the enhancerless SV40 promoter fused to the CAT gene were tested for reporter expression in vector-transfected cells (Fig. 7A). As can be seen in Fig. 7b, constructs carrying one or three NF-ELAM1 elements do not display any constitutive enhancer activity. Moreover, enhancer activity is not found following IL-1 treatment of the cells. These results suggest that NF-ELAM1 does not act independently as a transcription enhancer either constitutively or when in an IL-1 stimulated milieu. We then constructed vectors that carry contiguous NF-ELAM1 and NF-κB elements to test the influence of the two elements together.

In these experiments the NF-ELAM1 motif combined with that of the NF-κB element acted to strongly augment the weak IL-1-inducible enhancer activity seen with that of an isolated NF-κB motif (Fig. 7b). As shown in a summary of the data in Fig. 7c, introduction of two NF-ELAM1 elements into a construct already carrying two NF-κB elements greatly increased cytokine-inducible reporter expression in both HeLa and HUVEC cells. We conclude that NF-ELAM1 is a DNA-binding factor which acts in concert with NF-κB to enhance cytokine-induced transcription from the ELAM1 promoter.

We next tested the isolated NF-ELAM2 element for enhancer activity. As can be seen in Fig. 7d, single or four consecutive copies of this element failed to augment constitutive or IL-1-induced transcription, even when linked to an NF-κB site. Even a construct carrying three copies of the NF-ELAM2-NF-κB tandem yielded only 13-fold induction (not shown), which compares poorly to a construct carrying just two NF-κB elements (Fig. 7b) that shows 9-fold acetylation induction.

FIG. 5. NF-ELAM-2a and -b bind around position -104 to -100 in the ELAM1 promoter. Panel a, methylation interference assay for NF-ELAM-2a. G ladders were generated from the unfraccionated probe (lanes 1 and 6) and probe retarded (lanes 2 and 4) or not (lanes 3 and 5) by NF-ELAM-2a from HUVEC (lanes 2 and 3) or HeLa cells (lanes 4 and 5). The G residues whose methylation interferes with NF-ELAM-2a binding are indicated by stars. Numbering of the G residues is as in Fig. 1. Panel b, band shift assay using nuclear extract from IL-1-induced HUVEC cells. The probe (WT) corresponded to the -132 to -65 ELAM promoter. In the mutated probe (MUT), the G residues at position -104, -103, and -100 had been mutated to Ts.

FIG. 6. Functional importance of the NF-ELAM1 and -2 elements. Panel a, CAT assay using -383 to +80 ELAM promoter-CAT constructs in endothelial (panel A) and HeLa (panel B) cells. Wild type (WT), non-mutated sequences. The position of the mutated nucleotides is indicated for the other constructs. Panels b and c, bar diagram of the CAT activity for various constructs in endothelial IE-7 (b) and HeLa (c) cells. The radioactivity in the spots was measured with an Ambis radioactivity scanner and the acetylated form calculated as percentage of total radioactivity per lane.
Band shift experiments with the oligonucleotides that were used to make this latter series of constructs showed good binding of NF-ELAM2a and -b (not shown), indicating that lack of NF-ELAM2 enhancer activity of the constructs cannot be ascribed to diminished factor binding.

Interestingly, all constructs carrying both NF-ELAM2 and \( \kappa B \) elements (including the one carrying three copies of each) showed reduced background acetylation (0.7% or less) in absence of IL-1 (compare with the last five constructs in Fig. 7b).

**DISCUSSION**

We have described two sequence elements and their binding factors, NF-ELAM1 and -2, that are both required for ELAM1 transcription. Interestingly, the -141 to -153 element recognized by NF-ELAM1a and -lb has no enhancer activity on its own, but is capable of up-regulating a neighboring NF-\( \kappa B \) element. Our mutation analysis clearly identifies both NF-ELAM1 and -2 elements as essential for ELAM1 promoter activity. Both elements have been very well conserved among human, mouse and rabbit ELAM1 promoters (10, 25, 26).

Moreover, DNA-binding factors comigrating with NF-ELAM1a and -b were detected in mouse heart and lung tissue (25).

Surprisingly, fine mapping of the NF-ELAM1 element revealed a sequence with a high degree of similarity to the \( \delta A \) element, reported to be a T-cell-specific enhancer (24).

Our data show that the \( \delta A \)-NF-ELAM1-binding site is unable to function as an independent enhancer in non-T-cells such as HUVEC, IE-7, and HeLa consistent with the published report. In contrast, we demonstrate a wider role for this element and its binding factor. We have shown that NF-ELAM1 acts as a modulator of NF-\( \kappa B \) activity in endothelial cells. The \( \delta A \)-NF-ELAM1 element thus appears to be involved in different activation pathways in T-cells compared to endothelial or other cells. Whereas in T-cells the element is active as an enhancer (presumably through a T-cell-specific factor) in other cells the element might require the cooperation of a second transcription factor (such as we have found with NF-\( \kappa B \)) to elicit its transcriptional activity.

We have also noted a similarity between the ACATCAT NF-ELAM1-binding site and the ACGTCT recognition sites for the CREB/ATF family. We initially dismissed the possible binding of these factors to the NF-ELAM1 element because 1) no CREB/ATF-binding sites had been described carrying the G to A transition as seen in the NF-ELAM1-binding sequence, and 2) our target definition assay clearly indicated a preference of NF-ELAM1 for the sequence containing an A. Nevertheless, Georgopoulos et al. (27) recently reported the cDNA cloning of a CRE-BP cDNA, whose encoded protein specifically recognizes the \( \delta A \) element. We are currently in the process of cloning cDNAs whose encoded proteins bind this element in the ELAM1 promoter.

Contrary to NF-ELAM1, we have been unable to detect cooperation between isolated NF-ELAM2 and \( \kappa B \) elements. Given our finding that three point mutations that abolish NF-ELAM2a and -2b binding also inhibit transcription, the
conclusion must be that the NF-ELAM2 element is highly dependent on its location and context in the ELAM promoter for activity. By contrast, basal level transcription from constructs carrying both NF-ELAM2 and αB elements is reduced as compared to constructs carrying one or more αB elements. One might therefore speculate that the NF-ELAM2 element and its binding factors are involved both in repressing leaky activity of the αB enhancer in the quiescent state and increased transcription upon IL-1 induction. Only the second function requires cooperation with other promoter elements. The hierarchy of cooperation that we have found is suggestive of a sequential order of events: DNA binding of NF-ELAM2 stabilizes NF-ELAM1, which in turn augments NF-αB binding and transactivation.

We have looked for cooperative DNA binding by NF-ELAM1, -2, and NF-αB in band shift experiments with DNA fragments carrying all sequence elements. All factors seem to bind independently (Figs. 2, 5b, Ref. 15 and data not shown). Moreover, we were unable to show cooperative binding to the isolated DNA fragment. However, due to the very high probe-to-complex ratio in band shift assays protein-protein cooperation may not be apparent in these experiments. Since two bands are obtained with the NF-ELAM1/-2 probe each the slower migrating complexes might consist of NF-ELAM1, -2, and/or NF-αB. However, as these bands are not affected by IL-1 induction, NF-αB is probably not involved. In addition, a complex between NF-ELAM1 and -2 would comigrate with the probe, which is something we do not see. The possibility remains that NF-ELAM1,-2, and NF-αB interact directly or indirectly in vivo or that their transcriptional activity is not a function of DNA binding cooperation but rather a synergistic interaction with the transcription complex.

NF-αB activation has been shown to be involved in cytokine induction of a number of genes (28, 29). Yet additional elements and factors would be expected to generate in each case a tissue-specific and temporally regulated response. In spite of high levels of activated NF-αB, ELAM1 transcription is essentially shut off 24-h post-IL-1 induction. This situation contrasts with the prolonged time course of intercellular adhesion molecule 1 induction in endothelial cells (30) that has also been suggested to depend on NF-αB activation (31). For ELAM1, the two elements and their corresponding factors reported here to be essential for transcription may be involved in down-modulation as well, a hypothesis we are currently pursuing.

The definition of the mechanisms involved in ELAM1 expression will allow a better understanding of the means by which the inflammatory response is regulated at the gene level. This understanding may in turn provide better means to control and influence this important element of the immune defense mechanism.

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