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Induction of the gene encoding mucosal vascular addressin cell adhesion molecule 1 by tumor necrosis factor α is mediated by NF-κB proteins

(transcription regulation)

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ABSTRACT **Mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1) is involved in trafficking of lymphocytes to mucosal endothelium. Expression of MAdCAM-1 is induced in the murine endothelial cell line bEnd.3 by tumor necrosis factor α (TNF-α), interleukin 1, and bacterial lipopolysaccharide. Here we show that TNF-α enhances expression of a firefly luciferase reporter directed by the MAdCAM-1 promoter, confirming transcriptional regulation of MAdCAM-1. Mutational analysis of the promoter indicates that a DNA fragment extending from nt −132 to nt +6 of the gene is sufficient for TNF-α inducibility. Two regulatory sites critical for TNF-α induction were identified in this region. DNA-binding experiments demonstrate that NF-κB proteins from nuclear extracts of TNF-α-stimulated bEnd.3 cells bind to these sites, and transfection assays with promoter mutants of the MAdCAM-1 gene indicate that occupancy of both sites is essential for promoter function. The predominant NF-κB binding activity detected with these nuclear extracts is a p65 homodimer. These findings establish that, as with other endothelial cell adhesion molecules, transcriptional induction of MAdCAM-1 by TNF-α requires activated NF-κB proteins.**

Mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1) is involved in homing of lymphocytes to mucosal vasculature through interaction with L-selectin and the α4β7 integrin molecules expressed on lymphocytes (1–4). In addition to its role in normal lymphocyte trafficking to mucosal lymphoid tissue, MAdCAM-1 may be involved in some conditions of chronic inflammation (2, 5, 6).

Inflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin 1 stimulate transcription of the genes encoding endothelial cell adhesion molecules including E-selectin, ICAM-1, and VCAM-1, and NF-κB binding sites located in the promoters of these genes have been implicated in cytokine induction (7–14). NF-κB activity is mediated by homo- or heterodimeric combinations of the NF-κB-family proteins p50/p105, p52/p100, p65 (RelA), c-Rel, and RelB. NF-κB DNA-binding activity is induced in most cell types, including endothelial cells, by a variety of agents such as TNF-α, interleukin 1, intra- and extracellular pathogens, or reactive oxygen intermediates (15–18). The specificity of the NF-κB-mediated response is controlled by the NF-κB proteins present in the cell and their binding preference for NF-κB sites in target promoters (19–23).

Cell surface expression of MAdCAM-1 is induced by TNF-α, interleukin 1, and bacterial lipopolysaccharide in the murine endothelial cell line bEnd.3 (24, 25). Sequence analysis of the MAdCAM-1 promoter region has revealed the presence of two NF-κB binding motifs (S. Sampaio, M.T., and E. C. Butcher, unpublished work). To investigate the mechanism of TNF-α-induced expression of MAdCAM-1 in bEnd.3 cells and the role of the NF-κB sites, we conducted mutational analysis of the promoter region and DNA-binding experiments with cell extracts from cells stimulated with TNF-α. The data show that TNF-α induction of MAdCAM-1 gene expression in bEnd.3 cells is mediated primarily by the p65 member of the NF-κB family.

MATERIALS AND METHODS

Cell Culture and Transfection. The mouse endothelial cell line bEnd.3 (a gift from W. Risau, Max-Planck-Institut, Bad Nauheim, Germany.) was cultured (26) and transfected (12) as described. Four and a half micrograms of MAdCAM-1-luciferase DNA and 0.5 μg of cytomeglovirus (CMV) promoter–β-galactosidase plasmid DNA, employed as an internal reference for transfection efficiency, were used for each transfection. Recombinant murine TNF-α (R & D Systems) was used at 10 ng/ml. Luciferase (Promega) and β-galactosidase (Tropix, Bedford, MA) assays were performed as suggested by the manufacturer. All transfections were carried out in duplicate and repeated at least three times.

RNase Protection Assay. RNase protection assay was performed as described (27). The RNA probe used extends from +1000 to +1219 of the MAdCAM-1 coding sequence and yielded 220- and 131-nt products following RNase digestion, which correspond to alternatively spliced forms of the mRNA. A probe producing a protected product of 156 nt from β-actin mRNA was used as an internal control.

Recombinant Plasmids and Oligonucleotides. The sequences of the oligonucleotides used are as follows, with nucleotides that differ from the wild-type sequence in lowercase type: TO-1122, 5′-GCGCGGAGCTCAAGCTTCCACCGGTGCCT-3′; TO-1154, 5′-GCGCGCTCGAGGCCTGCCTCTGTCTTGTG-3′; TO-1250, 5′-AGCCCGACCCCCACTTG-3′; TO-1257, 5′-CAAAGTGGGGGTCGGGCTcccAAAGCCCCCCTGGGAAAGTC-3′; MO-12, 5′-AAAGCCCCCTGGGAAAGTgggACAGAGCCGGCAGAAGGGG-3′; MO-13, 5′-ACTTTCCCAGGGGGCTTT-3′; MO-16 (wild-type site), 5′-GGTCGGGCTGGGAAAGCCCCCTGGGAAAGTCCCACAGAGCCGG-3′; TO-410 (E-selectin mutant NF-κB site), 5′-GTTTTTGGATGCCATTGGaagcttCCTCTTTACTGGATG-3′; HO-14 (AP-1 site), 5′-TCGAGTGACTCAGCGCG-3′; MO-18 (M1 site) has ccc at nt 10–12 instead of GGG in MO-16; MO-20 (M2 site) has ggg at nt 31–33 instead of CCC in MO-16; MO-22 (M3) has the combined changes of MO-18 and MO-20. The E-selectin wild-type NF-κB site (TO958) has been described (12).

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Abbreviations: MAdCAM-1, mucosal addressin cell adhesion molecule 1; TNF-α, tumor necrosis factor α.

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The MAdCAM-1–luciferase fusion plasmid p1727 (nt −1727 to +6) was constructed by PCR amplification of a genomic clone with TO-1122 and TO-1154 and subcloning of the PCR product between the *Sac* I and *Xho* I sites of the promoterless luciferase expression vector pGL2-basic (Promega). Exonuclease III was used to generate 5′ deletion mutants. The endpoints of the deletion were defined by sequencing. Site-directed changes in the NF-κB sites were introduced by two rounds of PCR followed by insertion of the fragment into pGL2. For pM1 two separate PCRs were carried out with p1727 as template and TO-1257 and TO-1122 or TO-1250 and TO-1154 as primers. The products of the first PCR were mixed and reamplified with TO-1122 and TO-1154. pM2 was constructed similarly with primers MO-12, MO-13, TO-1122, and TO-1154. pM3 was constructed in the same manner as pM2 except that pM1 was used as template.

Electrophoretic Mobility-Shift Assays. Nuclear extracts were prepared as described (12) except that the nuclear pellet was suspended in 0.6× volume of buffer B (10 mM Tris, pH 7.9/100 mM KCl/2 mM $MgCl_2$/0.1 mM EDTA/1 mM dithiothreitol with proteinase inhibitors), mixed with 0.06× volume of 4 M ammoinium sulfate in 10 mM Tris (pH 7.9), mixed gently for 20 min, and centrifuged for 20 min. The supernatant was used for DNA-binding reactions. Binding assays were performed as described (12). Antibodies directed against human p50 and p52 were kindly provided by Zhaodan Cao (Tularik Inc.). Antibody directed against a 14-aa peptide starting at position 348 of mouse p50 and those against human p65, c-Rel, RelB, Ets-1/Ets-2, and AP-2 were from Santa Cruz Biotechnology.

RESULTS

Time Course of MAdCAM-1 mRNA Induction by TNF-α. TNF-α induces the cell surface expression of MAdCAM-1 on the murine endothelial cell line bEnd.3, and this induction is caused by an increase in the level of MAdCAM-1 mRNA (24, 25). To confirm and extend this observation we determined the kinetics of MAdCAM-1 mRNA accumulation following stimulation of cells with TNF-α. Total RNA was isolated from bEnd.3 cells exposed to TNF-α for various times and the amount of MAdCAM-1 mRNA was determined by RNase protection analysis (Fig. 1). As expected, no MAdCAM-1-specific bands were observed in unstimulated cells. Cells stimulated with TNF-α revealed two bands that corresponded in size to the alternatively spliced MAdCAM-1 transcripts, which differ by the inclusion or lack of the mucin-like and third immunoglobulin-like domains (S. Sampaio, M.T., and E. C. Butcher, unpublished work). Both transcripts were induced rapidly following induction and their levels remained elevated for up to 36 hr. The kinetics observed for mRNA accumulation here are consistent with those observed previously for cell surface expression of the protein (24, 25).

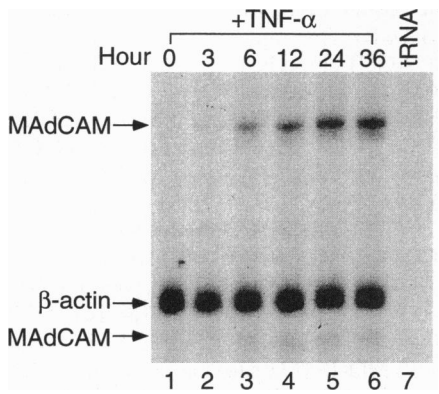

FIG. 1. Induction of MAdCAM-1 mRNA by TNF-α. Forty micrograms of total RNA isolated from unstimulated bEnd.3 cells (lane 1) or cells stimulated with TNF-α for 3, 6, 12, 24, or 36 hr (lanes 2–6) or yeast tRNA (lane 7) was hybridized with RNA probes derived from MAdCAM-1 or β-actin cDNA and then digested with RNase. The protected fragments corresponding to the long and short form of the MAdCAM-1 message and the β-actin message are indicated. Fifty times more MAdCAM-1 probe than β-actin probe was used.

Functional Analysis of the Mouse MAdCAM-1 Promoter. We next determined whether the induction of MAdCAM-1 gene expression by TNF-α resulted from an increased utilization of its promoter. For this purpose, the reporter plasmid p1727 was constructed by fusing nt −1727 to +6 of the mouse MAdCAM-1 gene to the firefly luciferase coding sequence. p1727 was transfected into bEnd.3 cells, and 36 hr later the amount of luciferase produced in the presence or absence of TNF-α was determined. The reporter plasmid directed a low but measurable amount of luciferase in the absence of TNF-α, confirming that the 1.7-kb DNA fragment included the promoter for the MAdCAM-1 gene. Cells transfected with p1727 and exposed to TNF-α consistently showed a 3- to 5-fold enhancement of luciferase activity compared with unstimulated cells, indicating that induction of MAdCAM-1 expression by TNF-α occurs at the transcriptional level and that 1.7 kb of upstream sequence of the MAdCAM-1 gene is sufficient to mediate TNF-α induction. The increase in luciferase activity was observed as soon as 3 hr after stimulation and was sustained for 24 hr (Fig. 2*A*).

Although MAdCAM-1 expression is restricted to endothelial cells (1), the 1.7-kb promoter fragment and one with an additional 6.3 kb of upstream sequence yielded similar luciferase activity and were induced to the same extent in mouse fibroblasts and human Hep G2 hepatoma cells as in bEnd.3 (data not shown), indicating that the sequences conferring cell type-specific expression must reside, at least in part, elsewhere in the MAdCAM-1 locus.

Definition of the TNF-α Response Element of MAdCAM-1. To delineate the TNF-α-inducible element(s) in the MAdCAM-1 promoter, deletion derivatives of the 1.7-kb fragment were constructed by truncation of the 5′ end and analyzed in transfection assays. Luciferase activity directed by the MAdCAM-1 promoter in the absence of TNF-α was detected with all promoter mutants tested except one with only 41 bp of upstream sequence (Fig. 2*B*). Strikingly, removal of sequence up to −132 had no effect on TNF-α inducibility. A promoter fusion that lacked an additional 20 bp (−112 to +6) was only slightly (<1.5-fold) responsive to TNF-α, and the one extending from −99 to +6 was totally nonresponsive. Thus the region −132 to −99 appeared to be critical for TNF-α-induced expression the MAdCAM-1 gene.

This 34-bp region harbors two putative NF-κB sites. The more distal of these, hereafter termed site 1, is at −121 to −112 and is identical (GGAAAGCCCC) to an NF-κB site found in the vimentin promoter (28). The more proximal site, termed site 2, is located at −108 to −99 and is identical (GGAAAGTCCC) to a site in the long terminal repeat of human immunodeficiency virus (29). To directly test the role of these putative NF-κB binding sites in TNF-α-induced expression of MAdCAM-1, and determine whether these were the only sites within the entire 1.7-kb fragment that were essential for induction, mutations were introduced that selectively inactivated one or both sites. Promoter mutants that had either site 1 (pM1), site 2 (pM2), or both sites (pM3) mutated showed no significant induction by TNF-α, confirming the importance of these regulatory elements (Fig. 2*C*). The results indicate that the two NF-κB like sites are the sole determinants of TNF-α inducibility of the MAdCAM-1 promoter and that neither site is dispensable.

Identification of NF-κB Binding Sites in the MAdCAM-1 Promoter. To demonstrate that the two regulatory sites defined in the aforementioned functional studies indeed bind NF-κB proteins, we performed electrophoretic mobility-shift assays with nuclear extracts prepared from untreated cells or

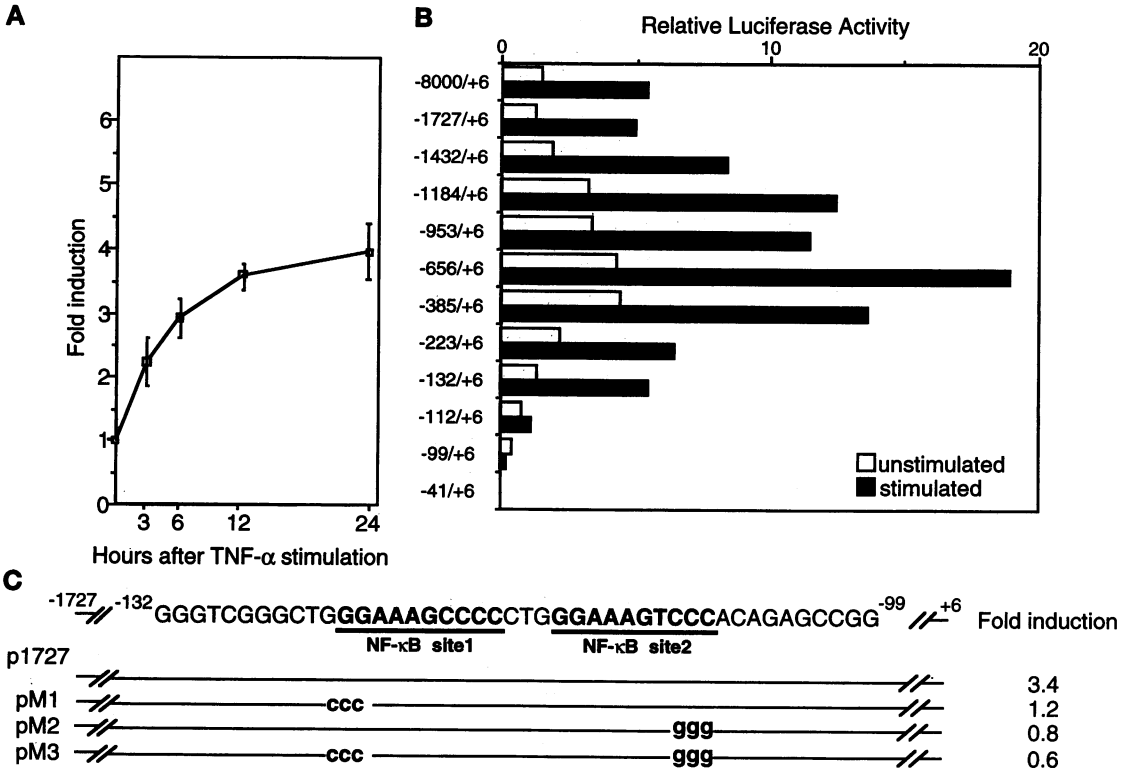

FIG. 2. Promoter activity of MAdCAM-1 promoter–luciferase fusion plasmids. (*A*) Time course of induction by TNF-α. Cells transfected with the MAdCAM-1–luciferase fusion plasmid p1727 were stimulated with TNF-α (10 ng/ml) for the indicated times prior to harvest. (*B*) Promoter activity of deletion mutants. Cells were transfected with the indicated plasmids, and luciferase activity was determined in cells that were left unstimulated or stimulated with TNF-α for 24 hr. Numbers at left indicate the MAdCAM-1 nucleotides present in the reporter plasmid. Data shown are from one experiment. Similar results were obtained in two additional transfection experiments. (*C*) TNF-α induction of site-specific mutants. Cells were transfected with plasmids having the wild-type sequence (p1727) or site-specific mutants (pM1, pM2, or pM3) and analyzed as in *B*. The fold increase in luciferase activity observed with TNF-α treatment is given. The top line represents the sequence of the relevant portion of the MAdCAM-1 promoter, and the two NF-κB sites are underlined. The three mutant derivatives pM1, pM2, and pM3 are given below. Nucleotide substitutions (lowercase type) were introduced in the −1727 promoter context.

cells exposed to TNF-α for various times. The DNA probe used extended from −131 to −89 and included both regulatory sites. Two new DNA–protein complexes, a major one termed complex I and a minor one termed complex II, were detected in extracts prepared from TNF-α-stimulated bEnd.3 cells as compared with unstimulated cells (Fig. 3*A*). The two complexes were detected in extracts prepared from cells treated with TNF-α from 6 to 36 hr and their relative abundance did not vary with length of induction.

Probes which had mutations in site 1 or site 2 (M1 and M2; Fig. 3*B*) showed similar binding activity as the wild-type probe. In contrast, no inducible DNA–protein complexes were detected with a probe having mutations in both sites (M3; Fig. 3*B*). The simplest interpretation of these data is that the two regulatory sites have equivalent capacity to bind the same nuclear factors and that the two complexes observed with the wild-type probe result from occupancy of a single site by different proteins from the extract, both of which are present in limiting amounts. To confirm this notion, binding reactions were performed with ≈3 times as much nuclear extract. Under these conditions two additional DNA–protein complexes (complex III and complex IV) were detected with the wild-type probe. Detection of these additional complexes was dependent on the integrity of both site 1 and site 2, consistent with the interpretation that the new complexes resulted from occupancy of both sites (Fig. 3*C* and data not shown).

To characterize the observed TNF-α-induced DNA-binding activity, competition experiments were performed with various oligonucleotides. The NF-κB binding site from the E-selectin promoter (12) was able to compete effectively for the formation of all four DNA–protein complexes (I–IV), whereas a mutated form of this site, which no longer binds NF-κB, showed no such activity (Figs. 3*C* and 4*A*). An oligonucleotide with a binding site for an unrelated transcription factor (AP-1) also failed to compete. The results of these competition experiments establish that the TNF-α-inducible DNA-binding activities detected in bEnd.3 extracts are likely to be composed of NF-κB proteins.

To further characterize the complexes observed with sites 1 and 2 of the MAdCAM-1 promoter, nuclear extracts from TNF-α-treated cells were incubated with the antibodies directed against individual members of the NF-κB family before addition of the oligonucleotide probe to the binding reaction mixture. Antibodies directed against p50, p65, and RelB affected binding, whereas those directed against p52 and c-Rel, as well as unrelated transcription factors (Ets-1/Ets-2 or AP-2), had no effect (Fig. 4*B*). These experiments indicate that both complexes are composed of NF-κB proteins, with complex I representing p65 homodimers and complex II representing p50/RelB heterodimers. A second antibody directed against a peptide derived from mouse p50 was used to confirm the absence of p50/p65 heterodimers (data not shown).

The prominent NF-κB binding activity detected in bEnd.3 extracts with the MAdCAM-1 probe appeared to consist of p65 homodimers. To determine whether this detection of p65 homodimers reflected a preference of the MAdCAM-1 NF-κB sites for p65 homodimers or whether it reflected the relative abundance of this dimer in bEnd.3 extracts, binding assays were performed with the E-selectin NF-κB site and also with extracts from a different cell line. Binding reactions with nuclear extract from the human endothelial cell line ECV304 yielded a single TNF-α-inducible DNA–protein complex on

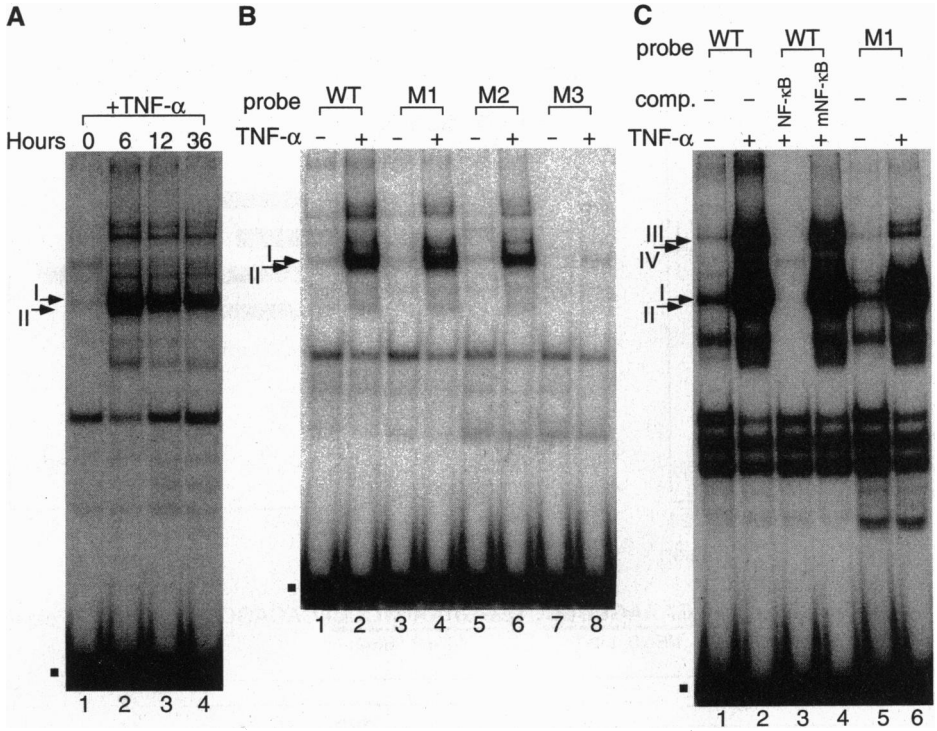

FIG. 3. Electrophoretic mobility-shift assays with the MAdCAM-1 regulatory element. (*A*) Three micrograms (protein) of nuclear extract from unstimulated (lane 1) or cells stimulated with TNF-α for 6 hr (lane 2), 12 hr (lane 3), or 36 hr (lane 4) was incubated with radioactivity labeled wild-type probe (WT) and the binding reaction mixtures were resolved in a polyacrylamide gel. The two DNA–protein complexes induced by TNF-α are indicated with arrows; the square marks the free probe. (*B*) Extracts from unstimulated cells (− lanes) or cells stimulated with TNF-α for 6 hr (+ lanes) were incubated with the indicated probes: wild-type sequence (WT), mutation in site 1 (M1), mutation in site 2 (M2), or mutations in both sites (M3). Arrows indicate the DNA–protein complexes detected with WT, M1, or M2. (*C*) Nuclear protein (10 μg) from unstimulated (−) or TNF-α-stimulated (+) cells was incubated with probe WT or M1. Two additional complexes detected with the WT probe but not M1 are indicated as III and IV.

both the MAdCAM-1 and E-selectin NF-κB sites (Fig. 5). Experiments with antibodies directed against individual NF-κB proteins indicated that the inducible complex observed in ECV304 extracts consisted of p50/p65 heterodimers. In contrast, the NF-κB complex formed with the E-selectin NF-κB site and bEnd.3 extracts appeared to consist of p65 homodimers (Fig. 5). These results indicate that the MAdCAM-1 NF-κB sites can bind p50/p65 heterodimers and that p65 homodimers constitute the major NF-κB binding activity in bEnd.3 extracts.

DISCUSSION

Our results demonstrate that regulation of MAdCAM-1 expression by TNF-α occurs at a transcriptional level and identify

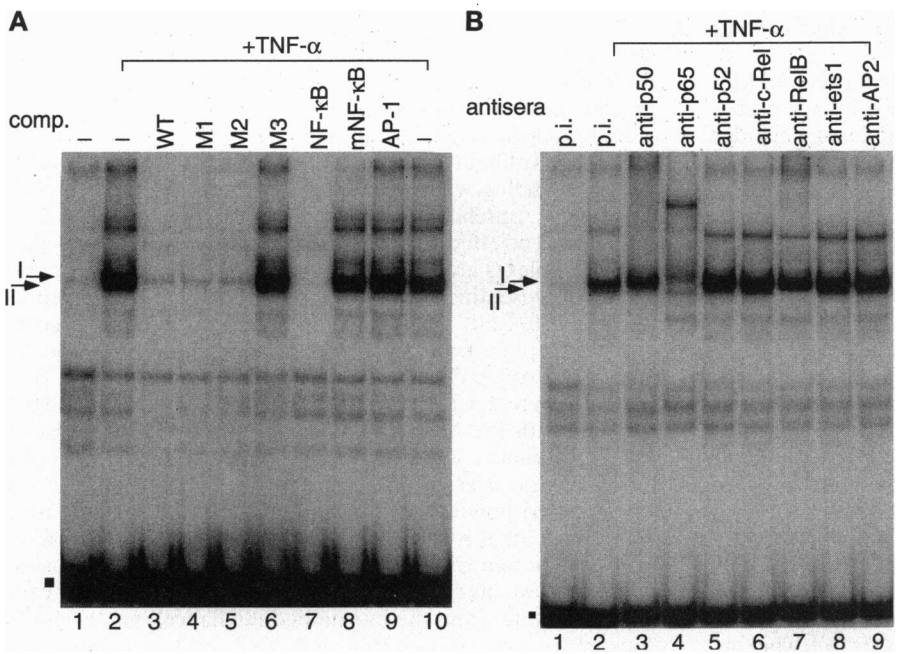

FIG. 4. Characterization of DNA–protein complexes detected in mobility-shift assays by competition and antibodies. (*A*) Extracts from cells incubated with TNF-α for 6 hr (lanes 2–10) were incubated with the wild-type probe WT. Binding reaction mixtures included the indicated unlabeled oligonucleotides as competitors in a 30-fold molar excess: −, none; WT, wild-type sequence; M1, mutation of site 1; M2, mutation of site 2; M3, mutations of sites 1 and 2; NF-κB, NF-κB site 3 from the E-selectin promoter; mNF-κB, mutant NF-κB site 3 from the E-selectin promoter; AP-1, consensus AP-1 binding site. (*B*) Antibodies directed against the indicated proteins were incubated with extracts from TNF-α-stimulated cells for 30 min at 4°C prior to addition of the wild-type probe (WT). All binding reaction mixtures included 1 μl of preimmune serum (p.i.) to normalize the amount of total protein added. Arrows indicate the two TNF-α-inducible complexes observed with the wild-type probe.

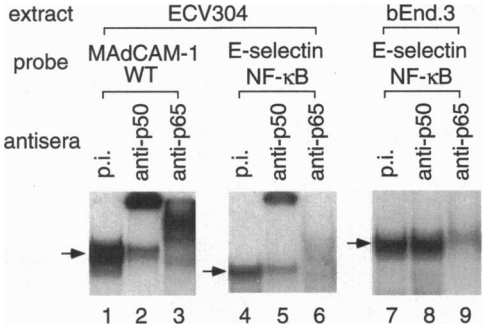

FIG. 5. Characterization of NF-κB binding activity in different cell lines. Extracts from ECV304 (lanes 1–6) or bEnd.3 cells (lanes 7–9) were incubated with TNF-α for 6 hr. The radiolabeled probe used was MAdCAM-1 wild-type oligonucleotide (WT, lanes 1–3) or E-selectin NF-κB site 3 (lanes 4–9). Incubation of extracts with preimmune serum (p.i., lanes 1, 4, and 7) or anti-p50 (lanes 2, 5, and 8) or anti-p65 (lanes 3, 6, and 9) was as in Fig. 4*B*. Arrow shows the DNA–protein complex observed.

regulatory NF-κB sites in the promoter that are essential for induction. Mutational analysis of the promoter demonstrated that as little as 132 bp of upstream sequence was sufficient for basal promoter function and TNF-α induction. Although sequence analysis had revealed the presence of several transcription factor binding sites upstream of −132 (S. Sampaio, M. Takeuchi, and E. C. Butcher, unpublished work), none of these seem to be essential for expression in transient transfection assays for promoter function. In contrast, integrity of both NF-κB sites (at −121 and −108) was found to be essential for induction, indicating that the two NF-κB sites may not be redundant.

TNF-α treatment of cells is known to activate NF-κB (18), and binding experiments with nuclear extracts confirmed that the regulatory sites involved in MAdCAM-1 expression bound NF-κB proteins. DNA-binding and competition experiments with wild-type and mutant oligonucleotide sequences indicated that the two NF-κB sites in MAdCAM-1 had similar binding properties. Further, the two sites were occupied sequentially with addition of increasing amounts of extract to the binding reaction mixture, and there was no evidence for any cooperative interaction. Thus, although the two sites appear to be noninteracting and equivalent in function, a single site is insufficient for TNF-α induction.

Antibodies directed against the various NF-κB proteins were used to identify the different family members present in the DNA–protein complexes detected with the MAdCAM-1 probe. These experiments revealed that the DNA-binding activities consisted of p65 homodimers and p50/RelB heterodimers. The most prominent complex was composed of p65 homodimers, which is consistent with the observation that the sequence of the two NF-κB sites on the noncoding strand in the MAdCAM-1 promoter, GGGGCTTTCC and GGGACTTTCC, match well with the consensus binding site for p65, GGGRNTTTCC (19). Alternatively, the detection of p65 homodimers may reflect the abundance of NF-κB proteins in the bEnd.3 cellular extracts used in the binding experiments, since the major NF-κB complex formed on the E-selectin NF-κB site with these extracts is also a p65 homodimer. We have not determined which of the two NF-κB dimers binding to the MAdCAM-1 promoter affects its expression. Both p50/RelB and p65/p65 can activate transcription (18, 30), and therefore it is likely that binding of any of these forms would result in activation of the MAdCAM-1 promoter.

In conclusion, the NF-κB family of transcription factors appears to play a central role in cytokine-induced expression of MAdCAM-1 gene as it does in regulating the expression of other leukocyte cell adhesion molecule genes expressed in endothelial cells (9–14, 31).

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