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Cytokine-inducible expression in endothelial cells of an IκBα-like gene is regulated by NFκB

Rainer de Martin[1,4], Bernard Vanhove[1], Qi Cheng[1], Erhard Hofer[1], Vilmos Csizmadia[1,3], Hans Winkler[1,3] and Fritz H.Bach[1,2]

[1]Vienna International Research Cooperation Center (VIRCC), Brunnerstr. 59, A-1235 Vienna, Austria and [2]Sandoz Center for Immunobiology, Harvard Medical School, 185 Pilgrim Road, Boston, MA 02215, USA
[3]Present address: Sandoz Center for Immunobiology, Harvard Medical School, 185 Pilgrim Road, Boston, MA 02215, USA
[4]Corresponding author

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The transient expression of many different genes is mediated by the inducible transcription factor p50-p65 NFκB, which in turn is regulated by complex formation with its inhibitor IκBα. We describe here that in porcine aortic endothelial cells, either IL-1α, TNFα or LPS upregulates an inhibitor of NFκB which we refer to as ECI-6. ECI-6 is by structural and functional criteria an IκBα protein, the porcine homologue of MAD-3, pp40 and RL/IF-1. We have studied the promoter of the ECI-6/IκBα gene and provide three lines of evidence that its expression is directly regulated by NFκB. First, the 5′ regulatory region of ECI-6/IκBα contains two sites that bind NFκB in electrophoretic mobility shift assays. Second, expression following transfection of an ECI-6/IκBα promoter-luciferase reporter construct is dependent on a co-transfected NFκB-p65 subunit. Third, pretreatment of endothelial cells with antioxidants, agents that inhibit activation of NFκB, inhibit the expression of ECI-6/IκBα. We conclude that the regulated expression of ECI-6/IκBα could represent a novel feedback mechanism by which NFκB downregulates its own activity after transient activation of target genes has been achieved.

Key words: endothelial cells/inflammation/IκB/NFκB

Introduction

In response to inflammatory stimuli, endothelial cells (EC) upregulate a number of different genes, including interleukins (Locksley *et al.*, 1987; Sironi *et al.*, 1989), transcription factors (Colotta *et al.*, 1988; Opipari *et al.*, 1990), cell adhesion molecules (Simmons *et al.*, 1988; Bevilacqua *et al.*, 1989; Polte *et al.*, 1990), and factors of the coagulation system (Bussolino *et al.*, 1986; Nawroth *et al.*, 1986). These events, together with the downregulation of other genes and the loss of cell surface molecules, lead to an 'activated' phenotype and to functional changes of the cells, including the loss of barrier function that results in edema and hemorrhage. In addition, rather than maintaining anticoagulation, the activated EC promote coagulation.

Transcription of many of these genes involves NFκB, a transcription factor that is necessary, though not always alone sufficient, to induce transient expression of genes in a variety of cell types (for a review, see Baeuerle and Baltimore, 1988; Baeuerle *et al.*, 1991). NFκB is constitutively present in the cell as a p50-p65 heterodimer, and is retained in the cytoplasm by the physical association of the p65 subunit with IκBα. Nuclear transport of NFκB is accomplished by dissociation from IκBα, thereby unmasking its nuclear localization signal (Henkel *et al.*, 1992). This process very likely is controlled by phosphorylation-dephosphorylation of IκBα (Gosh and Baltimore, 1990). In addition, reactive oxygen intermediates seem to be common messengers in the signal transduction pathway leading to NFκB activation (Schreck *et al.*, 1992). Following dissociation from NFκB, IκBα is rapidly degraded (Beg *et al.*, 1993). However, besides its cytoplasmic function of controlling nuclear uptake of NFκB, IκBα has at least two other properties: it can also translocate to the nucleus (Zabel *et al.*, 1993) and it has the ability to displace NFκB from its DNA target sequence *in vitro* (Zabel and Baeuerle, 1990). Taken together, these studies indicate a possible function of IκBα in the nucleus.

The transient nature of NFκB-dependent gene transcription implies appropriate mechanisms for downregulation. In contrast to many reports describing the induction of NFκB, very little is known about its inactivation. Cycloheximide has been reported to block the inactivation and subcellular redistribution of NFκB (Baeuerle and Baltimore, 1988), and to potentiate the interleukin-1 (IL-1)-induced transcription initiation of endothelial leukocyte adhesion molecule-1 (ELAM-1) by augmenting NFκB binding activity to the ELAM-1 promoter (Gersha *et al.*, 1992). Given its ability to translocate to the nucleus and to displace NFκB from its binding site, IκBα could be a candidate for such a cycloheximide-sensitive protein.

In an attempt to better understand the molecular mechanisms underlying the inflammatory process and eventually to identify targets for therapeutic intervention, we isolated genes that are upregulated in cytokine-stimulated porcine EC by differential screening. One of these genes, ECI-6, shows structural and functional properties of IκBα. Since the expression of many genes that are induced during an inflammatory response involves NFκB, we have studied the regulation of the ECI-6/IκBα gene and demonstrate for the first time that NFκB can upregulate its own inhibitor by direct interaction with the IκBα promoter. This upregulation could represent a general mechanism by which NFκB restricts its own transcriptional activity to a period of time until functional IκBα is available, therefore rendering gene expression transient.

Results

Cloning and cDNA sequence of ECI-6

Differential screening of a cDNA library aimed at the

identification of genes expressed in activated, but not 'resting' endothelial cells resulted in the identification of cDNAs encoding the porcine homologues of IL-8 (Lindley *et al.*, 1988), MGSA (Richmond *et al.*, 1988), RANTES (Shall *et al.*, 1988), prostaglandin synthase (Kujubu *et al.*, 1991) and laminin B1 (Sasaki *et al.*, 1987), as well as two genes with no apparent homology to previously described sequences. In addition, one clone with strong homology to human MAD-3 (Haskill *et al.*, 1991), chicken pp40 (Davies *et al.*, 1991) and rat RL/IF-1 (Tewari *et al.*, 1992) was identified. We refer to this clone as ECI-6/IκBα.

The sequence of the ECI-6 cDNA of 1599 bp (excluding the poly(A) tail) is shown in Figure 1. It extends 125 bp 5′ of the first ATG that is within a Kozak consensus sequence (Kozak, 1989) for the predicted translation start site. The 532 bp 3′ untranslated region contains ATTTA motifs that are usually associated with RNA instability (Shaw and Kamen, 1986).

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GCGCTGCCGA GCCCACAACA GTCCGAGGCC ATCGTCCCGC CCGCCCGAGC CACCGCGAGC   60
AGCCACGCGC CGCGCAGCCT GTGGCCCGCG CACCTAGGGA GCAGCGCCCA AGCCCTCATC  120
GCGCC ATG TTC CAG CCC GCA GAG CCC GGC CAG GAG TGG GCC ATG GAG GGG  170
      M  F  Q  P  A  E  P  G  Q  E  W  A  M  E  G                   15
CCC CGG GAC GCG CTC AAG AAG GAG CGG CTA CTG GAT GAC CGC CAC GAC    118
 P  R  D  A  L  K  K  E  R  L  L  D  D  R  H  D                    31
AGC GGC CTG GAC TCC ATG AAG GAC GAG GAG TAC GAG CAG ATG GTG AAG    266
 S  G  L  D  S  M  K  D  E  E  Y  E  Q  M  V  K                    47
GAG CTG CGC GAG ATC CGC CTC GAG CCG CAG GAG GCG CCC CGC GGC GCC    314
 E  L  R  E  I  R  L  E  P  Q  E  A  P  R  G  A                    63
GAG CCC TGG AAG CAG CAG CTC ACC GAG GAC GGA GAC TCG TTC CTG CAC    362
 E  P  W  K  Q  Q  L  T  E  D  G  D  S  F  L  H                    79
TTG GCC ATC ATC CAT GAA GAG AAG GCA CTG ACC ATG GAA GTG GTC CGC    410
 L  A  I  I  H  E  E  K  A  L  T  M  E  V  V  R                    95
CAA GTG AAG GGA GAT CTG GCT TTT CTT AAC TTC CAG AAC AAC CTG CAG    458
 Q  V  K  G  D  L  A  F  L  N  F  Q  N  N  L  Q                   111
CAG ACT CCA CTC CAC TTG GCG GTG ATC ACC AAC CAG CCA GAA ATC GCT    506
 Q  T  P  L  H  L  A  V  I  T  N  Q  P  E  I  A                   127
GAG GCA CTT CTG GAA GCT GGC TGT GAT CCT GAG CTC CGA GAC TTT CGA    554
 E  A  L  L  E  A  G  C  D  P  E  L  R  D  F  R                   143
GGA AAT ACC CCT CTA CAC CTT GCC TGT GAG CAG GGC TGC CTG GCC AGT    602
 G  N  T  P  L  H  L  A  C  E  Q  G  C  L  A  S                   159
GTG GGA GTC CTG ACT CAG CCC CGC GGG ACC CAG CAC CTC CAC TCC ATT    650
 V  G  V  L  T  Q  P  R  G  T  Q  H  L  H  S  I                   175
CTG CAG GCC ACC AAC TAC AAT GGC CAC ACA TGT CTG CAC TTA GCC TCG    698
 L  Q  A  T  N  Y  N  G  H  T  C  L  H  L  A  S                   191
ATC CAT GGC TAC CTG GGC ATT GTG GAG CTG TTG GTG TCT TTG GGT GCT    746
 I  H  G  Y  L  G  I  V  E  L  L  V  S  L  G  A                   207
GAT GTC AAC GCT CAG GAG CCC TGC AAT GGC CGA ACC GCC CTG CAT CTT    794
 D  V  N  A  Q  E  P  C  N  G  R  T  A  L  H  L                   223
GCG GTG GAC CTG CAG AAT CCC GAC CTG GTG TCG CTC TTG TTG AAG TGT    842
 A  V  D  L  Q  N  P  D  L  V  S  L  L  L  K  C                   239
GGG GCT GAT GTC AAC AGA GTC ACC TAC CAG GGC TAC TCC CCG TAC CAG    890
 G  A  D  V  N  R  V  T  Y  Q  G  Y  S  P  Y  Q                   255
CTC ACC TGG GGC CGC CCA AGC ACT CGG ATA CAG CAG CAG CTG GGC CAG    938
 L  T  W  G  R  P  S  T  R  I  Q  Q  Q  L  G  Q                   271
CTG ACC CTA GAA AAC CTC CAG ATG CTT CCA GAG AGC GAG GAT GAG GAG    986
 L  T  L  E  N  L  Q  M  L  P  E  S  E  D  E  E                   287
AGC TAT GAC ACG GAG TCA GAG TTC ACA GAG GAT GAG CTG CCC TAT GAC   1034
 S  Y  D  T  E  S  E  F  T  E  D  E  L  P  Y  D                   303
GAC TGC GTG CTT GGA GGC CAG CGC CTG ACG TTA TGA GCTTTG GAAAGTGTCT 1086
 D  C  V  L  G  G  Q  R  L  T  L  *                               314
AAAAGACCAT GTACTTGTAC ATTTGTACAA AATCAAGAGT TTTATTTTTC TAAAAAAAAA 1146
GAAAAAAAGA AAAAAAAAGA AAAAAGGGTA TACTTATAAC CACACCGCAC ACTGCCTGGC 1206
CTGAAACATT TTGCTCTGGT GGATTAGCCC CGATTTTGTT ATTCTTGTGA ACTTTGGAAA 1266
GGCGCCAAGG AGGATCATCG GAATGCAGAG AGAACCTCTT TTAAACGGCA CCTTGGTGGG 1326
GCCTGGGGGA AAGGTTATCC CTAATTTGAT GGGACTCTTT TATTTATTGC GCTTCTTGGT 1386
TGAACCACCA TGGAGTCAGT GGTGGAGCCC AGGTGTATCT GGGAAATGTT AGAATCAGGT 1446
GTGTTGTTAA ACCTGTCAGT GGGGTGGGGT TAAAAGTCAC GACCTGTCAA GGTTTGTGTT 1506
ACCCTGCTGT AAATACTGTA CATAATGTAT TTTGTTGGTA ATTATTTTG GTACTTCTAAG 1566
ATGTATATTT ATTAAATGGA TTTTTACAAA CAG (A)n                          1599
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Fig. 1. Nucleotide and predicted amino acid sequence of ECI-6/IκBα. The five ankyrin repeats are underlined, the potential protein kinase C (RPSTR), casein kinase II (HDSGLDS) and tyrosine phosphorylation (DEEYEQMVK) sites are doubly underlined.

The predicted ECI-6 protein is structurally related to IκBα

Translation of the open reading frame of the ECI-6 cDNA predicts a protein of 314 amino acids (Figure 2) that shares strong homology with the three IκBα proteins MAD-3 (Haskill *et al.*, 1991), pp40 (Davies *et al.*, 1991) and RL/IF-1 (Tewari *et al.*, 1992). Features that are at least partially conserved between the four proteins include a consensus site for tyrosine phosphorylation (KDEEYEQM-VK), a potential casein kinase II phosphorylation site (DSGLDS) and five repeats of the ankyrin consensus sequence (Lux *et al.*, 1990). The carboxy-terminal region starting from position 281 (Figure 1) is rich in PEST residues found in proteins with rapid turnover (Rogers *et al.*, 1986). A protein kinase C (PKC) consensus phosphorylation site (RPSTR) is conserved except in pp40 (Nolan *et al.*, 1989).

IκBα activity of ECI-6

The potential IκBα activity of ECI-6 was demonstrated by inhibition of NFκB binding to its target sequence in electrophoretic mobility shift assay (EMSA). ECI-6 mRNA was *in vitro* translated in a wheat germ extract. As shown in Figure 3A, the protein product specifically inhibited the binding of proteins obtained from nuclear extracts of LPS-stimulated PAEC to a NFκB DNA target sequence in a dose-dependent manner. Binding of nuclear factors to the NFκB site was specific, as it could be inhibited by competition with unlabeled NFκB sites, but not with mutant NFκB (mκB) or unrelated (CRE) sites. In concert with these findings, when ECI-6 mRNA was translated using rabbit reticulocyte lysates (Figure 3B), the endogenous NFκB activity present

```
                1                                                  60
ECI-6       MFQPAEPGQE VAMEGPRDAL KKER...LL. .DDRHDSGLD SMKDEEYEQM VKELRERILE
HUMMAD3A    MFQAAERPQE VAMEGPRDGL KKER...LL. .DDRHDSGLD SMKDEEYEQM VKELQEIRLE
RATRLIF1    MFQPAGHGQD VAMEGPRDGL KKER...LV. .DDRHDSGLD SMKDEDYEQM VKELREIRLQ
CHKPP40     MLSAHRPAEP PAVEGC.EPP RKERQGGLLP PDDRHDSGLD SMKEEEYRQL VRELEDIRLQ
CONSENSUS   *--------- -*-**----- -***---*-- -********** ***-*-*-*- *-**--***-
                                                  CK-II       Tyr

               61                                                 120
ECI-6       PQEAPRGAEP VKQQLTEDGD SFLHLAIIHE EKALTMEVVR QVKGDLAFLN FQNNLQQTPL
HUMMAD3A    PQEVPRGSEP VKQQLTEDGD SFLHLAIIHE EKALTMEVIR QVKGDLAFLN FQNNLQQTPL
RATRLIF1    PQEAPLAAEP VKQQLTEDGD SFLHLAIIHE EKTLTMEVIG QVKGDLAFLN FQNNLQQTPL
CHKPP40     PREPPARPHA VAQQLTEDGD TFLHLAIIHE EKALSLEVIR QAAGDAAFLN FQNNLSQTPL
CONSENSUS   *-*-*----- *-******** -********* **-*-*--**-- *--**-**** *****-****
                                              ankyrin-1

               121                                                180
ECI-6       HLAVITNQPE IAEALLEAGC DPELRDFRGN TPLHLACEQG CLASVGVLTQ PRGTQHLHSI
HUMMAD3A    HLAVITNQPE IAEALLGAGC DPELRDFRGN TPLHLACEQG CLASVGVLTQ SCTTPHLHSI
RATRLIF1    HLAVITNQPG IAEALLKAGC DPELRDFRGN TPLHLACEQG CLASVAVLTQ TCTPQHLHSV
CHKPP40     HLAVITDQAE IAEHLLKAGC DLDVRDFRGN TPLHIACQQG SLRSVSVLTQ HCQPHHLLAV
CONSENSUS   ******-*-- ***-**-*** *---****** ****-**-** -*-**-**** -----**---
                ankyrin-2                          ankyrin-3

               181                                                240
ECI-6       LQATNYNGHT CLHLASIHGY LGIVELLVSL GADVNAQEPC NGRTALHLAV DLQNPDLVSL
HUMMAD3A    LKATNYNGHT CLHLASIHGY LGIVELLVSL GADVNAQEPC NGRTALHLAV DLQNPDLVSL
RATRLIF1    LQATNYNGHT CLHLASIHGY LGIVEHLVTL GADVNAQEPC NGRTALHLAV DLQNPDLVSL
CHKPP40     LQATNYNGHT CLHLASIQGY LAVVEYLLSL GADVNAQEPC NGRTALHLAV DLQNSDLVSL
CONSENSUS   *-******** ********-** *---**-*--* ********** ********** ****-*****
                              ankyrin-4                    ankyrin-5

               241                                                300
ECI-6       LLKCGADVNR VTYQGYSPYQ LTWGRPSTRI QQQLGQLTLE NLQMLPESED EESYDTES..
HUMMAD3A    LLKCGADVNR VTYQGYSPYQ LTWGRPSTRI QQQLGQLTLE NLQMLPESED EESYDTESEF
RATRLIF1    LLKCGADVNR VTYQGYSPYQ LTWGRPSTRI QQQLGQLTLE NLQTLPESED EESYDTES..
CHKPP40     LVKHGPDVNK VTYQGYSPYQ LTWGRDNASI QEQLKLLTTA DLQILPESED EESSESEP..
CONSENSUS   *-*-*-***- ********** *****-----* *-**--**-- -**-****** ***---*---
                                    PKC

               301                322
ECI-6       .EFTEDELPY DDCVLGGQRL TL
HUMMAD3A    TEFTEDELPY DDCVFGGQRL TL
RATRLIF1    .EFTEDELPY DDCVFGGQRL TL
CHKPP40     .EFTEDELMY DDCCIGGRQL TF
CONSENSUS   -********-* ***--**--* *-
```

Fig. 2. Comparison of the protein sequences of ECI-6/IκBα (ECI-6), MAD-3 (HUMMAD3A), RL/IF-1 (RATRLIF1) and pp40 (CHKPP40). Gaps (−) were introduced for optimal alignment, asterisks (*) indicate residues conserved in all four proteins. The five ankyrin repeats (ankyrin-1 to ankyrin-5), the PKC, casein kinase II (CK-II) and tyrosine (Tyr) consensus phosphorylation sites are underlined. The PILEUP program of the UWGCG software was used.

in these lysates (Davies *et al.*, 1991) is inhibited, whereas no inhibition is seen with control lysates.

ECI-6/IκBα is inducible by TNFα, IL-1 or LPS

ECI-6 specific mRNA was induced at least 10-fold within 2 h after stimulation of EC with either IL-1α, tumor necrosis factor α (TNFα) or lipopolysaccharide (LPS) (Figure 4). Low basal levels of expression were seen only after longer exposure (see also Figure 8). Expression was found in the presence of cycloheximide, suggesting that prior protein synthesis is not required for induction. Nuclear run-off experiments have demonstrated that *ECI-6* is at least partially regulated at the transcriptional level (R.de Martin *et al.*, in preparation).

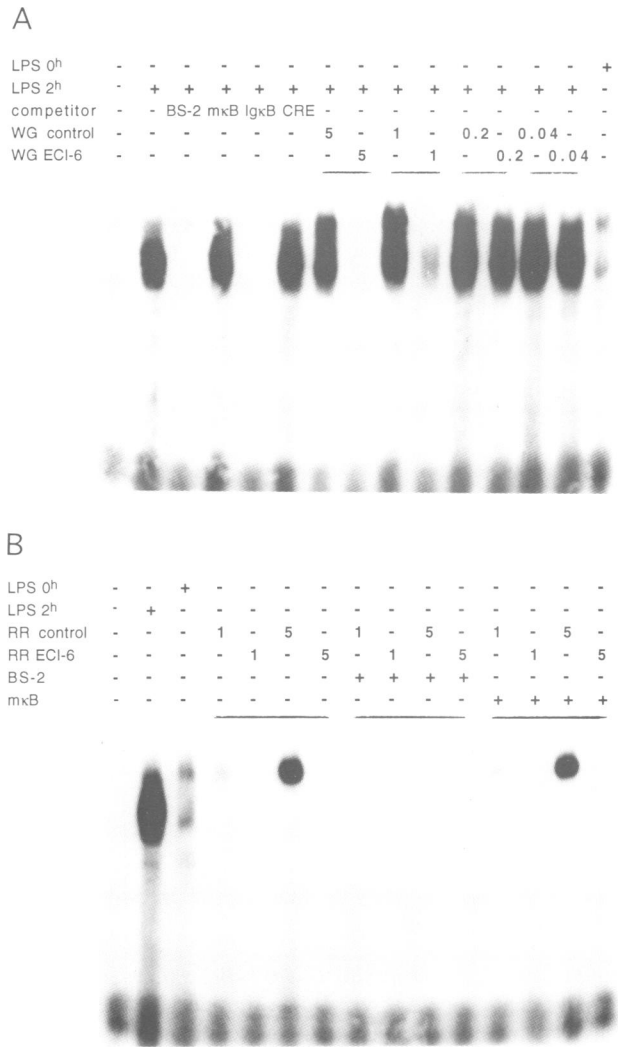

Fig. 3. Effect of *in vitro* translated ECI-6/IκBα on the binding of nuclear proteins to NFκB sites. ECI-6/IκBα translated *in vitro* in **(A)** wheat germ extracts was analyzed in combination with nuclear extracts of 2 h LPS stimulated (LPS 2h) or from unstimulated EC (LPS 0h). Amounts corresponding to 5−0.04 μl of the *in vitro* translation products, including ECI-6 (WG ECI-6) or control wheat germ extract (WG control), were added to the binding reaction as indicated above each lane. Specificity of binding of nuclear proteins to the labeled BS-2 oligonucleotide (a NFκB binding site from the ECI-6/IκBα promoter, see Figure 5) is demonstrated by competition with unlabeled oligonucleotides BS-2, Igκ B (a NFκB binding site from the Ig kappa light chain enhancer), mκB (a mutated NFκB site), or CRE (cyclic AMP responsive element). **(B)** ECI-6/IκBα was *in vitro* translated in rabbit reticulocyte lysates (RR ECI-6), that contain endogenous NFκB; RR control: reticulocyte lysates without ECI-6. Oligonucleotides used as cold competitors were as described in (A).

The promoter region of ECI-6/IκBα contains in vitro functional NFκB binding sites

Three positive clones were identified from a porcine genomic library by screening with an ECI-6/IκBα specific probe; phage DNA was analyzed by hybridization with specific oligonucleotides. The sequence of the 5′ upstream regulatory region was derived from a 0.9 kb *Hin*dIII−*Xho*I fragment (Figures 5A and 6A).

A single transcription start site was defined by primer extension (Figure 6B). RNase protection (Figure 6C) was carried out to rule out the possibility of introns in this region, thus confirming the results obtained with primer extension. Several longer, weaker bands in the RNase protection assay are most likely due to incomplete digestion of the probe.

In the upstream regulatory region (Figure 5A), sequences resembling a TATA box at positions −17/−22 and a potential SP-1 binding site at position −44/−49 are present. Two potential NFκB binding sites are located at positions −60/−70 and −221/−231 (designated BS-1 and BS-2). To investigate the binding of NFκB, we have used oligonucleotides corresponding to these sites (BS-1 and BS-2) in EMSA, either labeled or as competitors, for binding of NFκB proteins. As shown in Figure 5B, NFκB from nuclear extracts of EC stimulated with LPS for 2 h (LPS 2h) binds to a labeled oligonucleotide corresponding to the BS-2 site. The binding is specific in that it is inhibited only by oligomers with NFκB target sequences, but not by a mutated NFκB site. Binding is inhibited by unlabeled BS-1 oligonucleotide, confirming that this site represents a NFκB binding site as well.

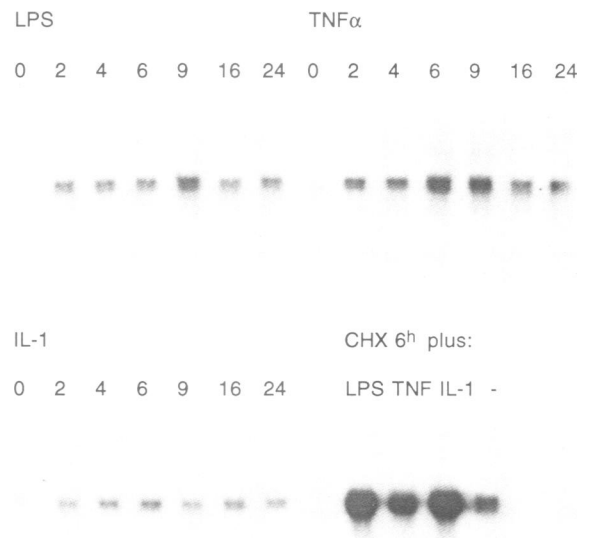

Fig. 4. Northern blot analysis of ECI-6/IκBα mRNA expression. Total cellular RNA from post-confluent porcine aortic endothelial cells stimulated with either IL-1α (100 U/ml), TNFα (100 U/ml), or LPS (10 ng/ml) alone for the indicated periods of time, or in combination with 10 μg/ml cycloheximide (CHX) for 6 h was probed for ECI-6/IκBα expression. Equal loading and transfer of mRNAs was confirmed by ethidium bromide staining.

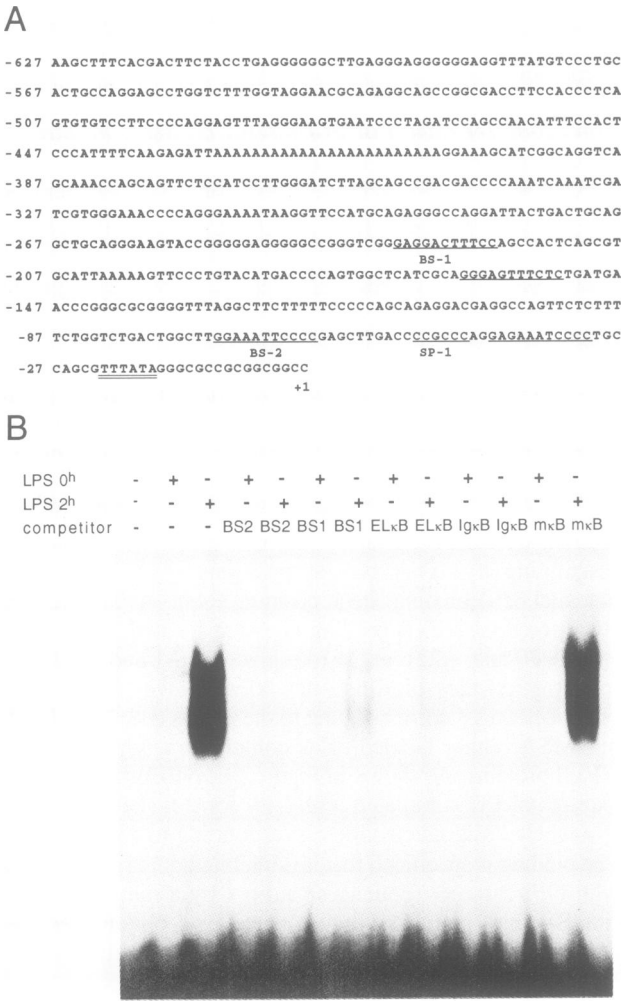

Fig. 5. (A) Nucleotide sequence of the 5′ regulatory region of the ECI-6/IκBα gene. An Sp-1 site and the NFκB binding sites (BS-1 and BS-2), as well as two other potential NFκB binding sites, are underlined. The TATA box is doubly underlined and the transcription start site marked by +1. **(B)** Binding of nuclear proteins to NFκB sites present in the ECI-6/IκBα promoter. Nuclear extracts were from EC treated with LPS for 2 h (LPS 2^h) or from unstimulated EC (LPS 0^h). A labeled oligonucleotide corresponding to the BS-2 site was used as a probe in all lanes. Oligonucleotides for competition (competitor) were as described in Figure 4 (ELκB: a NFκB binding site from the porcine ELAM-1 promoter; H.Winkler, in preparation) and are indicated above each lane.

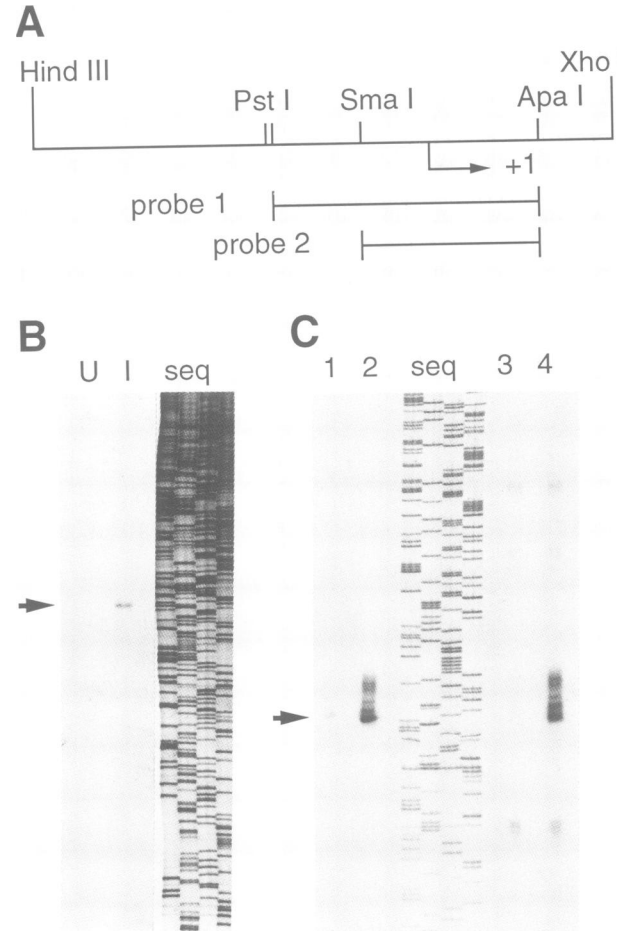

Fig. 6. Determination of the transcription start site. **(A)** Structure of a 0.9 kb *Hin*dIII−*Xho*I fragment spanning the promoter region of ECI-6/IκBα. Probes 1 and 2 were used for RNase protection. **(B)** Primer extension; RNA was from unstimulated (U) or from LPS-induced (I) EC; also shown is an unrelated DNA sequence as marker (seq). **(C)** RNase protection analysis: lanes 1 and 3: RNA from unstimulated EC; lanes 2 and 4: RNA from LPS-stimulated EC. *In vitro* transcribed probe 1 (lanes 1 and 2) or probe 2 (lanes 3 and 4) was used as indicated in (A); seq: DNA sequence. The position of the major protected fragment is indicated by an arrow.

The ECI-6/IκBα promoter is regulated by p65

Two fragments (Figure 7A) of the ECI-6/IκBα promoter of either 140 bp (containing the BS-2 site) or of 600 bp (containing both the BS-1 and the BS-2 site) were fused to a luciferase reporter gene in the vector UBT.Luc (de Martin *et al.*, 1993). These constructs were transfected into NIH3T3 cells, which have been reported to express IκBα in response to stimulation with serum (Tewari *et al.*, 1992). As shown in Figure 7B, expression of the reporter gene is dependent on the presence of a co-transfected vector encoding p65, whereas no expression is found after co-transfection of c-*rel*, another member of the NFκB family (Kieran *et al.*, 1990). The levels of luciferase are ~4-fold higher in the p600 as compared to the p140 construct, indicating that the BS-1 site contributes significantly to p65-dependent expression under these conditions. A more detailed study, involving mutagenizing the NFκB binding sites and co-transfecting combinations of various subunits of the NFκB family, will be necessary to elucidate the precise regulation of the IκBα promoter.

Inhibitors of NFκB inhibit ECI-6/IκBα expression

Antioxidants such as pyrrolidine dithiocarbamate (PDTC) have been demonstrated specifically to inhibit activation of NFκB, but not of several other transcription factors tested (Schreck *et al.*, 1992). Therefore we have used PDTC to assay whether or not expression of the ECI-6/IκBα gene is dependent on NFκB. LPS upregulated ECI-6/IκBα specific mRNA within 30 min. Levels increased further during the 4 h period studied (Figure 8). Pretreatment of EC with 30 μM PDTC for 1 h prior to stimulation with LPS abolished the induction of ECI-6/IκBα mRNA; no stimulation was seen with PDTC alone and levels of GAPDH mRNA were not affected.

Discussion

The ECI-6 clone described here was identified by differential screening of cytokine-stimulated EC. It shows strong

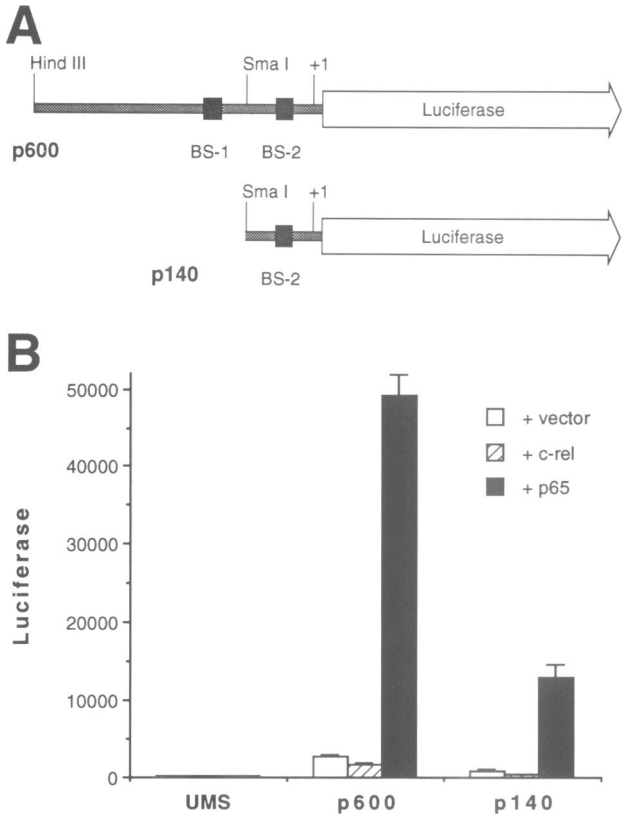

Fig. 7. Expression of ECI-6/IκBα–luciferase reporter genes in transiently transfected NIH3T3 cells. **(A)** Expression constructs containing either 600 bp (p600) or 140 bp (p140) fragments of the ECI-6/IκBα 5′ regulatory region fused to a luciferase reporter gene; BS-1, BS-2: binding sites for NFκB. **(B)** Luciferase expression levels after co-transfection with expression plasmids for p65 (CMV.p65), c-*rel* (CMV.c-rel) or vector without insert (vector); UMS: control luciferase vector. A vector expressing β-galactosidase from a RSV promoter was used as an internal control and values expressed as luciferase activity normalized for β-galactosidase expression.

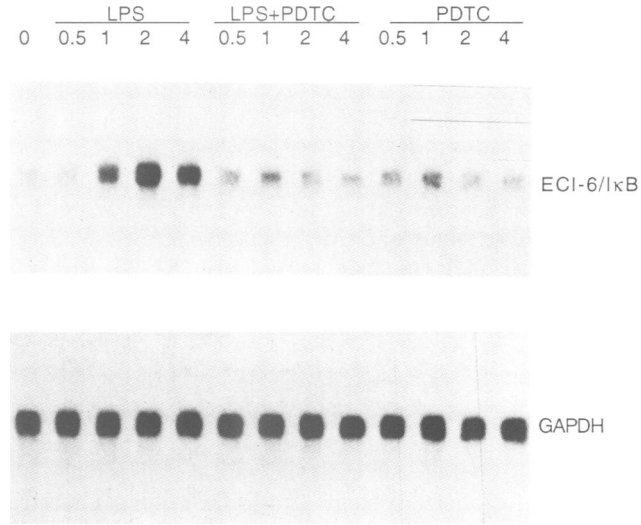

Fig. 8. Northern blot analysis of ECI-6/IκBα mRNA expression. EC were either stimulated with 5 ng/ml LPS, or preincubated with 30 μM PDTC 1 h prior to LPS stimulation, or incubated with PDTC alone as indicated. Total RNA was extracted at the times after LPS stimulation as indicated above each lane and analyzed with an ECI-6/IκBα specific probe. Filters were rehybridized with a GAPDH probe.

structural similarity to three IκBα genes: human MAD-3, rat RL/IF-1 and chicken pp40 (Davies *et al.*, 1991; Haskill *et al.*, 1991; Tewari *et al.*, 1992). IκBα inhibits the binding of heterodimeric p50–p65 NFκB to its DNA binding site by interacting with the p65 subunit of the transcription factor. Likewise, as demonstrated by EMSA, the ECI-6 protein inhibits the binding of NFκB to its binding site. Although we have not yet confirmed the involvement of the p65 subunit, ECI-6 very likely represents the porcine IκBα by structural and functional criteria.

Whereas regulated expression has not been described to date for pp40, both MAD-3 and RL/IF-1 are inducible genes that are upregulated upon adherence of monocytes to plastic surfaces and during liver regeneration, respectively. Nevertheless, our finding that the ECI-6/IκBα is inducible by TNFα, IL-1 and LPS in EC is unexpected, since the same agents have been reported to activate NFκB as well. However, the kinetics of expression of the two proteins are likely to be very different: biologically active NFκB has been demonstrated in the nucleus within minutes after stimulation due to post-translational activation, whereas the more time-consuming transcriptional upregulation of ECI-6/IκBα would lead to a delayed manifestation of its activity.

Since the expression of many inducible genes in endothelial as well as in other cell types involves NFκB, we have investigated the role of this transcription factor in the expression of ECI-6/IκBα. Cloning of the 5′ upstream regulatory region of ECI-6/IκBα revealed the presence of two potential binding sites for NFκB. Both of them represented NFκB binding sites in EMSA.

Evidence for a functional involvement of NFκB in the upregulation of ECI-6/IκBα was obtained by two experiments, the overexpression of and, vice versa, the inhibition of NFκB. Dithiocarbamates and iron chelators have been reported to act as potent and, in regard to several other transcription factors, specific inhibitors of NFκB, and have focused attention on reactive oxygen species as mediators of NFκB activation. In our experiments, pretreatment of EC with PDTC, a pyrrolidine derivative of dithiocarbamate, prior to stimulation with LPS, abrogated the inducibility of ECI-6/IκBα mRNA, corroborating the need for NFκB for its upregulation.

In a different type of experiment, we have fused sequences of the ECI-6/IκBα promoter to a luciferase reporter gene. Since the primary EC used in this study gave only weak transfection efficiencies in our hands, we have used NIH3T3 cells for transfection experiments. NIH3T3 cells have been shown to express endogenous IκBα (Tewari *et al.*, 1992). Expression of the reporter gene was entirely dependent on the expression of a co-transfected p65 subunit of NFκB. Since this subunit has strong transactivating but only low DNA-binding properties, it seems likely that p50, which mediates or increases the DNA binding of the p50–p65 heterodimer, is constitutively present in the cell in excess and that p65 is the limiting factor for ECI-6/IκBα expression. This would be in accordance with an inhibitory role for p50 in NFκB-dependent gene transcription. It also shows that EC specific factors are not necessary for ECI-6/IκBα promoter activity. Co-transfection with an expression vector for c-*rel* did not result in expression of the reporter gene.

The role of proteins regulating transcription factor activity has been demonstrated in several other systems where downregulation occurs by interaction with inhibitory

subunits. Examples include the following. (i) DNA binding of a member of the helix−loop−helix family, MyoD, mediates muscle cell specific differentiation. MyoD is inhibited by protein ID, a helix−loop−helix protein lacking the basic region necessary for DNA binding (Benezra *et al.*, 1991; Sun *et al.*, 1991). (ii) The *Drosophila* transcription factor Cf1-a is controlled by I-POU, a POU-domain protein expressed in neurons during development (Treacy *et al.*, 1991). (iii) The family of cAMP responsive element binding proteins and their modulators (CREB/CREM/CREMτ) represent a complex system of multiple interacting transcription factors, some of which antagonize each other (Foulkes *et al.*, 1992). (iv) IP-1 is a dominant inhibitor of a leucine zipper protein binding to the AP-1 site (Auwerx and Sussone-Corsi, 1991). Moreover, jun−fos complexes, which promote gene transcription in the early G_0-G_1 transition phase of the cell cycle, are replaced by complexes containing fos-related proteins (fra-1, fra-2, fos B) during late G_1 (Kovary and Bravo, 1992). During the nerve growth factor-induced transcription of the tyrosine hydroxylase gene, c-fos is found in complex with fosB at the time that transcription has terminated. Since ectopically expressed FosB can shut off tyrosine hydroxylase transcription, it could act as a repressor of c-fos-mediated transactivation (E.B.Ziff, personal communication).

Recent findings that IκBα can displace NFκB from its binding site *in vitro* and can translocate to the nucleus (Zabel and Baeuerle, 1990; Zabel *et al.*, 1993) would support a model, where IκBα could function as a regulatory subunit for NFκB also in the nucleus. Although our data do not directly demonstrate downregulation of NFκB activity by IκBα, they suggest that inducible expression of IκBα could restrict the action of NFκB to a limited period of time, thus rendering NFκB-dependent gene transcription transient.

Materials and methods

Preparation of cDNA library and differential screening

A cDNA library from porcine aortic EC stimulated with a combination of human recombinant IL-1α plus human recombinant TNFα (100 U/ml each, Genzyme) was prepared in the vector λ Zap (Stratagene). Two replicas were screened with ^{32}P-labeled first-strand cDNA probes reverse transcribed from uninduced and induced EC, respectively. Before hybridization, the 'induced' probe has been subtracted twice with a 10-fold excess of photobiotinylated (InVitrogen) 'uninduced' RNA. Phages that gave more strongly positive signals with the 'induced' as compared to the 'uninduced' probe were subcloned by *in vivo* excision and sequenced.

Screening of genomic library

A porcine genomic library (Clonetech) was screened with a labeled ECI-6/IκBα probe. DNAs from purified positive phages were characterized by Southern analysis after digestion with different restriction enzymes with a ^{32}P-labeled oligonucleotide derived from the 5′ region of the cDNA. A positive 0.9 kb *Hin*dIII−*Xho*I fragment was subcloned into pKSM13 (Stratagene) and sequenced.

Primer extension and RNase protection

A ^{32}P-labeled oligonucleotide complementary to bases 395−446 of the ECI-6/IκBα cDNA sequence (Figure 1) was used for primer extension as described previously (Sambrook *et al.*, 1989, section 7.81). For RNase protection, a 430 bp *Pst*I−*Apa*I fragment derived from the 0.9 kb *Hin*dIII−*Xho*I ECI-6/IκBα genomic clone was subcloned into pKSM13 and linearized with *Xba*I (probe 1) or with *Sma*I (probe 2). ^{32}P-labeled antisense RNA was transcribed *in vitro* and RNase protection carried out as described previously (Ausubel *et al.*, 1990, unit 4.7). For each assay, 1 μg of poly(A)$^+$ RNA from either unstimulated or stimulated (100 ng/ml LPS plus 10 μg/ml cycloheximide for 6 h) EC was used.

In vitro transcription and translation

By PCR, a *Bam*HI site was introduced immediately upstream of the initiator ATG of the ECI-6/IκBα cDNA, the fragment cloned into pKSM13 and sequenced. Ten micrograms of this plasmid were linearized and RNA transcribed using T7 polymerase (Stratagene) in a total volume of 100 μl, including 0.25 mM m^{7}G(5′)ppp(5′)G. After DNase I treatment, 1/20 of the purified RNA was translated in 50 μl wheat germ extract or in rabbit reticulocyte lysate (Promega). Parallel reactions including [^{35}S]methionine were carried out to confirm the correct size of the translation products by PAGE.

Nuclear extracts and electrophoretic mobility shift assays

Nuclear proteins were extracted from EC stimulated with 100 ng/ml LPS for 2 h or from non-stimulated cells (Dignam *et al.*, 1983). The double-stranded oligonucleotide BS-2 was labeled by filling in the overhangs with Klenow enzyme in the presence of radioactive nucleoside triphosphates, and 0.2 ng (100 000 c.p.m.) used per lane in EMSA. Oligonucleotides for EMSA had the following sequences (only the top strand is given): BS-1: AATTCGTCGGGAGGACTTTCCAGCCAG; BS-2: AATTCGGCTTG-GAAATTCCCCGAGCG; ELκB, a binding site from the porcine ELAM-1 promoter (H.Winkler, unpublished): AATTCATGCTGCTGGGAATTC-CTCTGTATG; IgκB, a NFκB binding site from the human immunoglobulin kappa light chain enhancer (Leonardo *et al.*, 1987): AATTCAGAGGGG-GATTTCCCAGAGG; mκB, a mutated NFκB site: AGCTTAGATTTT-ACTTTCCGAGAGGA; CRE, cAMP responsive element: GATCCGAT-TCTGACATCACCG. A 500-fold molar excess of unlabeled oligonucleotides was used for competition experiments. The resulting complexes were separated on 5% polyacrylamide gels.

EC culture and Northern blot analysis

Enzymatic isolation and culture of EC from porcine aorta was performed as described previously (Warren, 1990). RNA was extracted, fractionated on agarose/formaldehyde gels, transferred to Hybond N membranes (Amersham) and hybridized as described by Zipfel *et al.* (1989). Equivalent loading and transfer of RNA was confirmed by ethidium bromide staining of the gel and of the membrane after transfer.

Plasmids, transfections and luciferase assays

Expression plasmids for human p65 and c-*rel* were obtained from Dr W.C.Greene (Ruben *et al.*, 1991). A 600 bp fragment of the ECI-6/IκBα promoter with an additional *Hin*dIII site 20 bp downstream of the transcription start site was generated by polymerase chain reaction (PCR), ligated into a *Hin*dIII cut luciferase expression vector UBT.Luc (de Martin *et al.*, 1993) and sequenced, resulting in the plasmid p600. The p140 construct was generated by excision of a 460 bp *Sma*I fragment and religation of the vector.

NIH3T3 cells were transfected using Lipofectin (BRL) according to the manufacturer's protocol. As an internal control for transfection efficiency, a vector expressing β-galactosidase from the RSV promoter was included. Luciferase and β-galactosidase levels were determined 40 h later (de Wet *et al.*, 1987).

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