

# Characterization of an Immediate-Early Gene

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## Summary

We have cloned a group of cDNAs representing mRNAs that are rapidly induced following adherence of human monocytes. One of the induced transcripts (MAD-3) encodes a protein of 317 amino acids with one domain containing five tandem repeats of the *cdc10/ankyrin* motif, which is 60% similar (46% identical) to the ankyrin repeat region of the precursor of NF- $\kappa$ B/KBF1 p50. The C-terminus has a putative protein kinase C phosphorylation site. In vitro translated MAD-3 protein was found to specifically inhibit the DNA-binding activity of the p50/p65 NF- $\kappa$ B complex but not that of the p50/p50 KBF1 factor or of other DNA-binding proteins. The MAD-3 cDNA encodes an I $\kappa$ B-like protein that is likely to be involved in regulation of transcriptional responses to NF- $\kappa$ B, including adhesion-dependent pathways of monocyte activation.

## Introduction

NF- $\kappa$ B is an important transcription factor originally characterized as an I $\kappa$ B enhancer binding protein (Sen and Baltimore, 1986). In addition, binding sites for NF- $\kappa$ B are present in the regulatory regions of certain cytokine genes, the interleukin-2 (IL-2) receptor gene, class I and II histocompatibility antigen genes, an acute phase response gene, and several viral enhancers, including HIV-1 (Lenardo and Baltimore, 1989; Shimizu et al., 1990; Liebermann and Baltimore, 1990; Fan and Maniatis, 1989; Visvanathan and Goodbourn, 1989; Lenardo et al., 1989; Böhnelein et al., 1988; Baldwin and Sharp, 1988; Blanas et al., 1989; Ron et al., 1990; Nabel and Baltimore, 1987). These sites appear to be critical for appropriate regulated or cell type-specific gene expression. Stimulation by a number of agents including PMA, IL-1 $\beta$ , TNF $\alpha$ , and lipopolysaccharide results in the induction of nuclear NF- $\kappa$ B DNA-binding activity (Sen and Baltimore, 1986; Osborn et al., 1989; Israël et al., 1989; Lowenthal et al., 1989; Shirakawa et al., 1989; Lenardo and Baltimore, 1989). NF- $\kappa$ B is a complex of two proteins, p50 and p65 (Baeuerle

and Baltimore, 1989). The cDNA encoding the p50 DNA-binding subunit of NF- $\kappa$ B has been cloned, and the predicted protein was shown to be related to the *Drosophila dorsal* maternal effect gene and to the *c-rel* proto-oncogene product (Ghosh et al., 1990; Kieran et al., 1990; Bours et al., 1990; Meyer et al., 1991). Furthermore, it appears that the p50 DNA-binding subunit is processed from a 105 kd precursor protein that has six C-terminal repeats of the ankyrin consensus sequence (Kieran et al., 1990; Ghosh et al., 1990). Ankyrins are known to constitute a family of proteins that regulate interactions between a variety of membrane structures and the cytoskeleton (Lux et al., 1990). Proteins bearing multiple repeats of these structures are thus highly likely to be associated with receptors and the cytoskeleton. The DNA-binding activity KBF1 is the homodimer of the NF- $\kappa$ B p50 DNA-binding subunit (Kieran et al., 1990). The cloning of the cDNA encoding the p65 NF- $\kappa$ B subunit has recently been reported and the predicted protein sequence demonstrates homology to *c-rel* as well (Nolan et al., 1991; Ruben et al., 1991).

In many cells, NF- $\kappa$ B is localized in the cytoplasm where it is associated with a 36 kd inhibitor protein, I $\kappa$ B (Baeuerle and Baltimore, 1988a, 1988b; Ghosh and Baltimore, 1990; Zabel and Baeuerle, 1990). The p65 NF- $\kappa$ B subunit appears to be the target of interaction of I $\kappa$ B (Baeuerle and Baltimore, 1989; Ghosh and Baltimore, 1990; Urban and Baeuerle, 1990; Nolan et al., 1991). Evidence has been presented that phosphorylation of I $\kappa$ B blocks its ability to inhibit NF- $\kappa$ B DNA-binding activity, since protein kinase C (PKC) and several other kinases activate NF- $\kappa$ B DNA-binding activity in vitro (Ghosh and Baltimore, 1990; Shirakawa and Mizel, 1989).

Adherence of monocytes to plastic results in the rapid induction of many known cytokines, including IL-1, TNF $\alpha$ , colony stimulating factor-1, and the down-regulation of other constitutively expressed receptors and mediators including *c-fms* and lysozyme (Haskill et al., 1988; Eierman et al., 1989). We have described the cloning of a series of novel cDNA clones from a monocyte library constructed from monocytes adhered on plastic for 30 min (Sporn et al., 1990; Haskill et al., 1990, 1991). We demonstrate here that another unique cDNA clone, MAD-3, encodes a protein that appears by several criteria to be an I $\kappa$ B-like molecule.

## Results

We have studied gene expression in monocytes adhering to different substrates as models of mononuclear phagocyte maturation and of inflammation (Haskill et al., 1988; Eierman et al., 1989). One gene, MAD-3, is expressed early after monocyte adherence to plastic. By Northern analysis the transcripts are 1.6 kb in size and 0.04% abundant in 30 min plastic-adherent monocytes (Sporn et al., 1990). Adherence resulted in at least an 80-fold induction and expression was transient: levels returned to baseline within 8 hr (data not shown). Inclusion of cycloheximide

# A

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TGCCGCCGTCCCGCCCGCCAGCGCCCCAGCGAGGAAAGCAGCGCGCAGCCCGCGGCCAGC 60
GCACCCCGCAGCAGCGCCCGCAGCTCGTCCGCGCCATGTTCCAGGGCGCCGAGCGCCCA 120
          M F Q A A E R P Q 9
GGAGTGGGCCATGGAGGGCCCCCGCGAGCGGGTGAAGAAGGAGCGGCTACTGGACGACCG 180
E W A M E G P R D G L K K E R L L D D R 29
CCACGACAGCGGCCCTGGACTCCATGAAAGACGAGGAGTACGAGCAGATGGTCAAGGAGCT 240
H D S G L D S M K D E E Y E Q M V K E L 49
GCAGGAGATCCGCTCGAGCGCAGGAGGTGCGCGCGGCTCGGAGCCCTGGAAGGAGCA 300
Q E I R L E P Q E V P R G S E P W K Q Q 69
GCTCACCGAGGACGGGACTGTTCTCTGCACTGGCCATCCATGTAAGAAAAGCACT 360
          ankyrin I
  L T E D G D S F L H L A I I H E E K A L 89
GACCATGGAAGTATCCCGCAGGTGAAGGAGACCTGGCCCTTCTCAACTTCCAGAACA 420

T M E V I R O V K G D L A F L N F Q N N 109
CCTGCAGCAGACTCCACTCCACTTGGCTGTGATCACAACCGACCGAGAATGCTGAGGC 480
          ankyrin II
  L O Q T P L H L A V I T N Q P E I A E A 129
ACTTCTGGGAGCTGGCTGTGATCCTGAGCTCCGAGACTTTCGAGGAAAATACCCCTCACA 540

L L G A G C G D P E L R D F R G N T P L H 149
CCTTGCCTGTGAGCGGCTCGCTGGCCAGCGTGGGAGTCTGACTCAGCTCAGCCACC 600
          ankyrin III
  I A C E O G C L A S V G V L T O S C T T 169
CCCGCACCTCCACTCCATCCTGAAGGCTACCAACTACAATGGCCACAGTGTCTACACT 660

  P H L H S I L K A T N Y N G H T C L H L 189
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          ankyrin IV
  A S I H G Y L G I V E L L V S L G A D V 209
CAATGCTCAGGAGCCCTGTAATGGCCGACTGCCCTTCACTCGCAGTGGACCTGCAAAA 780
          ankyrin V
  N A Q E P C N G R T A L H L A V D L Q N 229
TCCTGACCTGGTGTACTCCTGTGAAGTGTGGGGCTGATGTCAACAGAGTTACCTACCA 840

F D L V S L L L K C G A D V N R V T Y Q 249

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G Q L T L E N L Q M L P E S E D E E S Y 289
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D T E S E F T E F T E D E L P Y D D C V 309
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F G G Q R L T L 317
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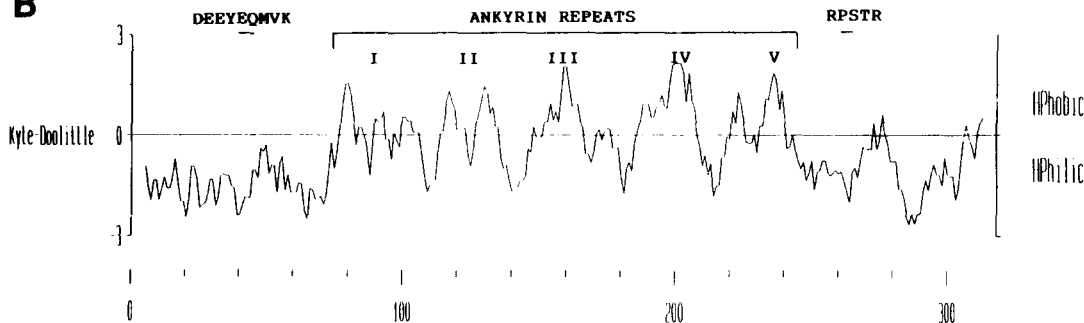
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Figure 1. Nucleotide and Amino Acid Sequence and Hydrophobicity Predictions for the MAD-3 cDNA

(A) Nucleic acid and predicted protein sequence of cDNA clone MAD-3. The DNA sequence and the amino acid translation predicted by the DNA sequence are also shown. The 1.6 kb size of the clone is close to that predicted from the transcript size on Northern analysis. The consensus tyrosine phosphorylation site and the possible phosphatidylinositol-3 kinase binding domain is underlined, the predicted PKC phosphorylation site is overlined, and the three ATTTA motifs are underlined and typed in bold. The ankyrin repeat domain (Lux et al., 1990) is typed in bold, and individual repeats are overlined and underlined.

(B) Hydrophilicity/hydrophobicity predictions for the proposed MAD-3 protein. The limits of the five ankyrin repeats are overlined and the location of individual ones are marked. The predicted phosphatidylinositol-3 kinase binding domain and the apparent PKC target sequences are also overlined. The plot was derived with the University of Wisconsin GCG program PEPLOT.

# B

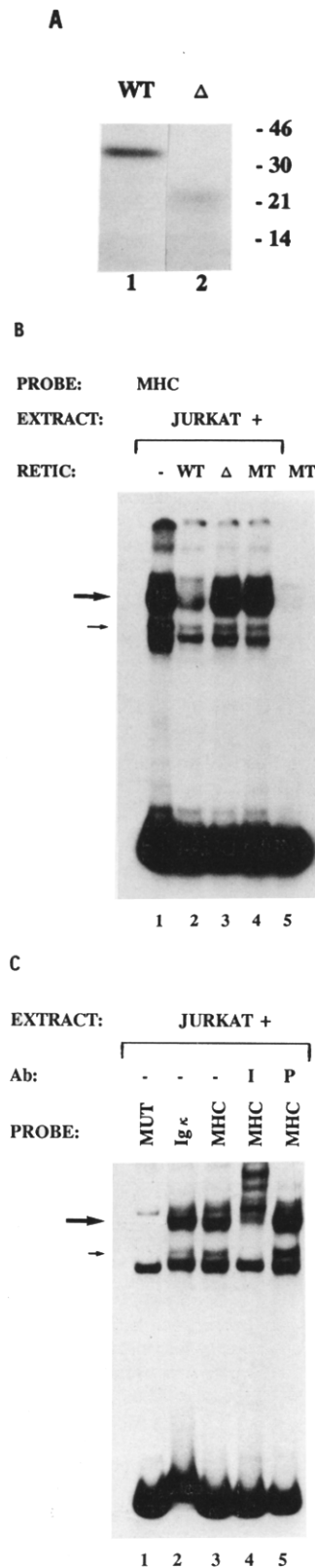


during adherence enhanced slightly the levels of mRNA, demonstrating that MAD-3 is an immediate-early gene induced by monocyte adherence (data not shown).

## Structure of MAD-3 cDNA

A full-length cDNA of MAD-3 was cloned from a library constructed from RNA pooled from fresh neutrophils and collagen-adherent monocytes. The probe was a 350 bp

cDNA isolated from the original  $\lambda$ gt10 library used to define adherence-induced cDNA clones (Sporn et al., 1990). The sequence of the MAD-3 cDNA of 1550 bp is shown in Figure 1A. It extends 94 bp 5' of a Kozak consensus sequence (Kozak, 1989) for the predicted start of translation. The 3' untranslated region contains three ATTTA motifs that usually signal rapid turnover of the mRNA (Caput et al., 1986; Shaw and Kamen, 1986). A poly(A) tail begins at the end of bp 1550.



**Figure 2. In Vitro Transcribed MAD-3 mRNA Translates a 36–38 kd Protein with Properties of IκB**

(A) 10% SDS–polyacrylamide gel analyzing reticulocyte lysates programmed with in vitro transcribed MAD-3 mRNA (lane 1, WT) or with MAD-3 mRNA transcribed from an *AccI*-digested plasmid (lane 2, Δ).

### Proposed Structure of the MAD-3 Protein

The predicted polypeptide specified by the open reading frame is composed of 317 amino acids and should encode a protein of approximately 34 kd. Secondary structure predictions suggest that the protein is arranged in three domains (Figure 1B). The N-terminal domain contains a hydrophilic stretch of 72 amino acids, which is marked by a consensus sequence (DEEYEQMVK) for tyrosine phosphorylation and is similar to the potential binding site of the phosphatidylinositol-3 kinase (amino acids 39–47) (Cantley et al., 1991). The C-terminal domain contains two regions of interest. One is a consensus sequence for PKC phosphorylation (RPSTR) (amino acids 260–264) (Noland et al., 1989). The other is a region rich in PEST residues (amino acids 264–314), which have been associated with rapid protein turnover (Rogers et al., 1986). The internal domain (amino acids 73–242) consists of five tandem repeats of the ankyrin consensus sequence (Lux et al., 1990). This region was found to be 60% similar (46% identity) to the homologous ankyrin repeat region of the NF-κB/KBF1 precursor (Ghosh et al., 1990; Kieran et al., 1990). The other domains of MAD-3 were dissimilar to NF-κB/KBF1.

### MAD-3 Encodes a 36–38 kd Protein with Properties of IκB

The presence of a consensus sequence for PKC phosphorylation, the predicted 34 kd size of the expressed protein, and the homology with the precursor of NF-κB/KBF1 suggested the possibility that MAD-3 encodes an IκB-like protein. To test this hypothesis the full-length MAD-3 cDNA sequence was cloned into the expression plasmid pcDNA 1 and RNA was transcribed using SP6 RNA polymerase. Since the reticulocyte lysate used for translation contained an endogenous NF-κB-like activity (data not shown), the lysates were depleted for this activity using a DNA affinity matrix specific for NF-κB. These NF-κB-depleted reticulocyte lysates demonstrated virtually no major histocompatibility complex (MHC) class I enhancer binding activity (Figure 2B, lane 5). The reticulocyte lysates were then used to

Protein was labeled with [<sup>35</sup>S]methionine. The mobilities of prestained molecular weight markers are shown.

(B) Gel mobility shift analyzing programmed reticulocyte lysates and nuclear extracts of PMA- and PHA-treated Jurkat T cells. For all lanes the class I MHC enhancer probe was used. The following protein sources were used: nuclear extracts of stimulated Jurkat T cells (lane 1), Jurkat extracts plus MAD-3 programmed lysates (lane 2, WT), Jurkat extracts plus lysates translated with mRNA from the *AccI*-deleted construct (lane 3, Δ), Jurkat extracts plus mock-translated reticulocyte lysates (lane 4, MT), and mock-translated reticulocyte lysates alone (lane 5, MT). The large arrow indicates the mobility of the NF-κB–DNA complex, and the small arrow indicates the mobility of the KBF1–DNA complex.

(C) Gel mobility shift assay characterizing the nuclear extracts of the stimulated Jurkat T cells. The following protein sources were used: extracts of stimulated Jurkat T cells (lanes 1–5) plus either antiserum to the p50 DNA-binding subunit of NF-κB (lane 4, I indicates immune antiserum) or preimmune (P) serum (lane 5). The DNA probes are as indicated above the figure: MUT (MHC double point mutant probe), Igκ, and MHC (class I MHC enhancer probe). The large arrow indicates the mobility of the NF-κB–DNA complex, and the small arrow indicates the mobility of the KBF1–DNA complex.

translate either full-length MAD-3 mRNA or mRNA derived from an *AccI* digest of the cDNA or were mock translated. *AccI* cuts the MAD-3 cDNA at the position corresponding to amino acid 186 in the fourth ankyrin repeat. The *in vitro* translated products, labeled with [<sup>35</sup>S]methionine, were electrophoresed on a 10% SDS-polyacrylamide gel. As predicted from the cDNA, the full-length MAD-3 mRNA and the mRNA from the *AccI*-digested plasmid revealed approximately 36 and 22 kd proteins (Figure 2A, lanes 1 and 2).

The MAD-3 translation products were then tested in a gel mobility shift assay for their ability to inhibit NF- $\kappa$ B activity. A class I MHC enhancer sequence (TGGGGATT-CCCCA), previously demonstrated to bind NF- $\kappa$ B (Baldwin and Sharp, 1988), was used as a DNA-binding probe. We chose to analyze whether NF- $\kappa$ B activity in Jurkat T cells stimulated with phytohemagglutinin (PHA) and phorbol 12-myristate 13-acetate (PMA) would be inhibited by the MAD-3 protein. The induction of NF- $\kappa$ B in these cells has been well documented (Nabel and Baltimore, 1987; Böhnlein et al., 1988), and, furthermore, there is an activity with properties of KBF1 in nuclear extracts of stimulated cells. As noted below, the DNA-protein complexes indicated by the arrows (Figure 2) appear by various criteria to be NF- $\kappa$ B and KBF1. Addition of the MAD-3 programmed lysates inhibited the DNA-binding activity associated with the slower NF- $\kappa$ B-DNA complex (indicated by the large arrow, Figure 2B, lane 2) in the stimulated Jurkat T nuclear extracts and only weakly affected the factor associated with the faster moving KBF1-DNA complex (indicated by the small arrow, Figure 2B, lane 2). Addition of either lysates programmed with the deleted mRNA or mock-translated lysates did not affect DNA-binding activity of either complex (Figure 2B, lanes 3 and 4).

To further characterize the DNA-binding activities in the nuclear extracts of the PMA- and PHA-stimulated Jurkat cells, several assays were performed. We first demonstrated that the two activities identified by the arrows are specific for the MHC enhancer probe, as they do not inter-

act with a double point mutated probe (Figure 2C, lane 1). We have previously shown that this mutant probe (TGC-GGATTCCCGA) is not bound by NF- $\kappa$ B (Baldwin and Sharp, 1988). The factors associated with the slower and faster complexes interact equally well with Ig $\kappa$  and class I MHC enhancer probes (Figure 2C, lanes 2 and 3), consistent with these activities being NF- $\kappa$ B and KBF1. Finally, the two DNA-protein complexes are recognized by antibodies against the p50 subunit of NF- $\kappa$ B (Figure 2C, lane 4) but not by preimmune serum (Figure 2C, lane 5). Thus, the MAD-3 protein strongly inhibits an authentic NF- $\kappa$ B activity from stimulated Jurkat T cells and may inhibit the Jurkat KBF1 activity very weakly.

To demonstrate that the observed inhibition is specific for the NF- $\kappa$ B activity, we analyzed the effect of the MAD-3 protein on other characterized DNA-binding proteins (Figure 3A). MAD-3 did not inhibit the DNA-binding activity of the major late transcription factor (Carthew et al., 1985; also known as USF), the Oct-1 factor (Singh et al., 1986), or H2TF1, a class I MHC enhancer binding factor (Baldwin and Sharp, 1987). We next analyzed whether NF- $\kappa$ B from another cell source would be inhibited by the translated MAD-3 protein. NF- $\kappa$ B from nuclear extracts of freshly isolated monocytes was inhibited by the MAD-3 protein, but the KBF1 activity found in these cells was unaffected (Figure 3B). Both the NF- $\kappa$ B and KBF1 activities in these extracts are recognized by antibodies to the p50 NF- $\kappa$ B subunit (data not shown). Thus, the MAD-3 protein is highly specific for NF- $\kappa$ B from several cell sources and has little or no effect on KBF1 DNA-binding activity (Figures 2B and 3B). These results are consistent with the observation that I $\kappa$ B interacts with the 65 kd subunit of NF- $\kappa$ B (Baeuerle and Baltimore, 1989; Ghosh and Baltimore, 1990; Urban and Baeuerle, 1990; Nolan et al., 1991), which is absent in KBF1 (Kieran et al., 1990). We therefore conclude that the MAD-3 translation product specifically inhibits NF- $\kappa$ B DNA-binding activity and does not inhibit the DNA-binding activity of KBF1, major late transcription factor, Oct-1, or H2TF1.

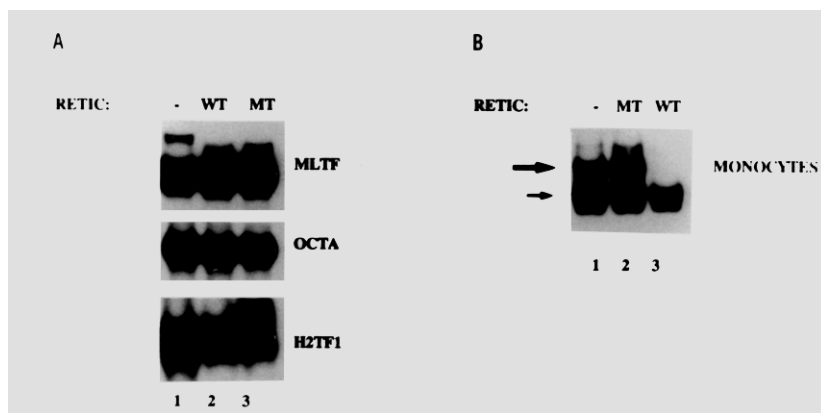


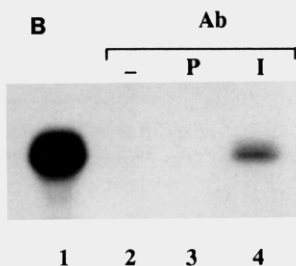
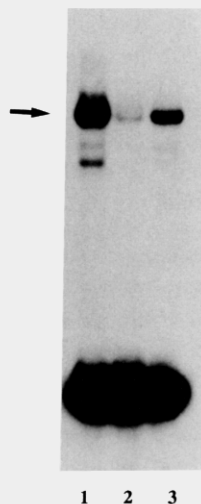
Figure 3. Specificity of Inhibition of DNA-Binding Activity by the MAD-3 Protein

(A) Gel mobility shift analyzing various DNA-binding activities. The adenovirus major late transcription factor (MLTF) and Oct-1 (OCTA) probes (as indicated) were incubated with nuclear extracts of stimulated Jurkat T cells (lanes 1-3) plus MAD-3 programmed lysates (lane 2, WT) or plus mock-translated lysates (lane 3, MT). The class I MHC enhancer probe was incubated with a phosphocellulose fraction from HeLa cells (lane 1) containing the DNA-binding activity H2TF1 (Baldwin and Sharp, 1987) plus MAD-3 programmed lysates (lane 2, WT) or plus mock-translated lysates (lane 3, MT).

(B) Gel mobility shift analyzing NF- $\kappa$ B in nuclear extracts of monocytes. The class I MHC

enhancer probe was incubated with nuclear extracts of freshly isolated monocytes (lane 1). Lane 2 included the addition of mock-translated lysates (MT) and lane 3 included the addition of MAD-3 translated lysates (WT). The large arrow indicates the mobility of the NF- $\kappa$ B-DNA complex, and the small arrow indicates the mobility of the KBF1-DNA complex.

**A** EXTRACT: AFF. NF- $\kappa$ B  
 PROBE: MHC  
 RETIC: - WT WT  
 DOC: - - +



**Figure 4. Direct interaction between MAD-3 and NF- $\kappa$ B**

(A) DOC releases NF- $\kappa$ B DNA-binding activity from the MAD-3 inhibition. Gel mobility shift using the class I MHC enhancer probe with the following binding conditions: DNA affinity-purified NF- $\kappa$ B (lanes 1–3) plus MAD-3 programmed lysates (lanes 2 and 3). Following incubation of the purified NF- $\kappa$ B with the MAD-3 programmed extract, DOC was added, followed by NP-40 incubation (lane 3). The arrow indicates the mobility of the NF- $\kappa$ B–DNA complex.

(B) Antibodies to NF- $\kappa$ B immunoprecipitate MAD-3. In vitro translated, [<sup>35</sup>S]methionine-labeled MAD-3 protein (lane 1) was incubated with NF- $\kappa$ B, followed by a no-serum control (lane 2), preimmune serum (P, lane 3), or antibodies to NF- $\kappa$ B (I, lane 4). Reactions for lanes 2–4 were then added to protein A–Sephacrose, and protein was recovered for SDS–polyacrylamide gel electrophoresis as described in the Experimental Procedures.

### Deoxycholate Treatment Dissociates Preformed MAD-3/NF- $\kappa$ B Complexes

NF- $\kappa$ B is found in the cytoplasm of many uninduced cells where it is bound to I $\kappa$ B, rendering it incapable of binding DNA (Baeyerle and Baltimore, 1988a, 1988b). This cytoplasmic form of NF- $\kappa$ B can, however, be liberated from I $\kappa$ B by treatment with sodium deoxycholate (DOC), thus making it competent for DNA binding (Baeyerle and Baltimore, 1988a). We first purified NF- $\kappa$ B by DNA affinity chromatography to remove any potential endogenous

NF- $\kappa$ B–I $\kappa$ B complexes. As with the crude nuclear extracts of stimulated Jurkat T cells (see Figure 2B), the MAD-3 translation product inhibited this partially purified NF- $\kappa$ B (Figure 4A, lane 2). Treatment of the NF- $\kappa$ B/MAD-3 reaction with sodium DOC followed by NP-40 incubation released NF- $\kappa$ B DNA-binding activity (Figure 4A, lane 3). Thus, the release of NF- $\kappa$ B DNA-binding activity from the reaction is derived from NF- $\kappa$ B–MAD-3 complexes and not from any endogenous NF- $\kappa$ B–I $\kappa$ B in the extract. Since NF- $\kappa$ B DNA-binding activity can be recovered from preformed NF- $\kappa$ B/MAD-3 by DOC treatment, we conclude that MAD-3 encodes a protein with properties of I $\kappa$ B.

### Antibodies to NF- $\kappa$ B Immunoprecipitate MAD-3

To determine if the translated MAD-3 protein directly interacts with NF- $\kappa$ B, an immunoprecipitation experiment was performed. The MAD-3–NF- $\kappa$ B complex was specifically immunoprecipitated by antibodies (Kieran et al., 1990) to the p50 DNA-binding subunit of NF- $\kappa$ B (Figure 4B, lane 4), but not by preimmune serum or by a no-serum control (Figure 4B, lanes 3 and 2, respectively). These results demonstrate that MAD-3 protein interacts directly with the NF- $\kappa$ B complex, presumably through the p65 subunit.

### Discussion

#### MAD-3 Encodes a Protein with I $\kappa$ B-like Activity

Several characteristics of the predicted amino acid sequence encoded by the MAD-3 cDNA led us to consider whether it had properties of I $\kappa$ B. The first was that the protein has homology with the ankyrin repeat domain of the NF- $\kappa$ B/KBF1 precursor, which is in the portion of the precursor that is removed from the DNA-binding subunit (Kieran et al., 1990; Ghosh et al., 1990). The second was that the predicted (34 kd) and expressed (36–38 kd) molecular sizes of the protein are quite similar to that (36 kd) of partially purified I $\kappa$ B (Ghosh and Baltimore, 1990; Zabel and Baeyerle, 1990). A third was that the MAD-3 protein has several putative protein kinase consensus target sequences, and it has been demonstrated that phosphorylated I $\kappa$ B will not inhibit the DNA-binding activity of NF- $\kappa$ B (Ghosh and Baltimore, 1990; Shirakawa et al., 1989).

MAD-3 protein that was translated in vitro (see Figure 2A) specifically inhibited the DNA-binding activity of NF- $\kappa$ B from different sources (Figures 2B and 3B). These included NF- $\kappa$ B activity induced by PMA and PHA treatment of Jurkat T cells and constitutive NF- $\kappa$ B activity found in human monocytes. Furthermore, the DNA-binding activity of NF- $\kappa$ B induced by lipopolysaccharide and cycloheximide treatment of a mouse pre-B cell line, NF- $\kappa$ B induced by PMA treatment of HeLa cells, and the endogenous NF- $\kappa$ B found in reticulocyte lysates were also inhibited by MAD-3 protein (data not shown). The specificity of the inhibition was demonstrated by the fact that other characterized DNA-binding activities are not inhibited by MAD-3 protein (Figure 3A). Furthermore, KBF1 DNA-binding activity from freshly isolated monocytes and from Jurkat cells is virtually unaffected by MAD-3 protein. Thus, the DNA-

binding activity of NF- $\kappa$ B, which is composed of 50 kd and 65 kd proteins (Baeuerle and Baltimore, 1989), is inhibited by the translated MAD-3 protein, whereas the DNA-binding activity of KBF1, which is apparently a homodimer of the 50 kd subunit (Kieran et al., 1990), is not or is only weakly affected (Figures 2B and 3B). These results are in agreement with previous data that demonstrate that I $\kappa$ B inhibits the DNA-binding activity of NF- $\kappa$ B through the 65 kd subunit (Baeuerle and Baltimore, 1989; Ghosh and Baltimore, 1990; Urban and Baeuerle, 1990; Nolan et al., 1991). Whether MAD-3 is identical to the I $\kappa$ B that has been described awaits purification of that activity and cloning of its cDNA. Furthermore, it is entirely possible that there are multiple I $\kappa$ B activities, possibly existing in a cell type-specific distribution. In fact, two forms of I $\kappa$ B have been identified (Zabel and Baeuerle, 1990). In addition, it will be important to determine if MAD-3 inhibits the DNA-binding activities of other members (Ballard et al., 1990) of the *rel* proto-oncogene family.

### **MAD-3 May Integrate Membrane Adhesive Interactions with the Cytoskeleton and Transcription**

Proteins bearing ankyrin repeats appear to play a prominent role in cell growth and differentiation (Lux et al., 1990). Erythrocyte ankyrins function as binding sites and anchors for integral membrane proteins, including the Na<sup>+</sup>+K<sup>+</sup>-ATPase and the voltage-dependent Na<sup>+</sup> channel, and brain ankyrin can bind tubulins and other proteins (discussed in Lux et al., 1990). The second domain of MAD-3 contains five tandem repeats of the ankyrin motif that have a high degree of homology to the precursor of the transcription factor KBF1/NF- $\kappa$ B (Kieran et al., 1990; Ghosh et al., 1990). This highly conserved motif is found in key differentiation-related genes of invertebrates, including the *notch* (Wharton et al., 1985), *lin-12* (Yochem et al., 1988), *fem-1* (Spence et al., 1990), and *cdc-10* proteins (Aves et al., 1985). It has been proposed that the structure of all these proteins and their manner of expression are consistent with their being receptors or adhesive proteins involved in intracellular signaling (Lux et al., 1990). The ankyrin motif is the postulated site of association between *notch* and an invertebrate version of a G protein  $\beta$  subunit (Hartley et al., 1988).

We propose that the ankyrin repeats of MAD-3 and the NF- $\kappa$ B/KBF1 precursor form the basis for a common address for subcellular localization, bringing the two proteins into close proximity. The precise location is still open to speculation, but it seems probable from the other known functions of human ankyrins that this is likely to be associated with cytoskeletal or receptor structures. The known induction of numerous cytokine genes by cytochalasin D and colchicine-sensitive adhesion events and the rapid translocation of PKC induced by adherence (Eierman and Haskill, 1991) suggest that transcription may occur through a pathway that integrates receptor signaling and induction of specific genes through cytoskeletal-bound transcription factors.

### **MAD-3 May Function as a Negative Regulator of Environmental Signals in Inflammatory Macrophages and a Feedback Inhibitor in Target Tissues**

The widespread usage of NF- $\kappa$ B in transcriptional responses to cytokines (Lenardo and Baltimore, 1989) raises the concern that cells that both secrete these cytokines and can respond to the same factors would require appropriate feedback inhibition. Monocytes and epithelial cells have recently been shown to synthesize an IL-1 receptor antagonist that may serve in part to limit IL-1 responsiveness (Eisenberg et al., 1990; Haskill et al., 1991). The I $\kappa$ B inhibitor offers the possibility of a more pleiotropic regulator of cytokine responsiveness. The low constitutive level of MAD-3 mRNA in monocytes but the high nuclear-associated activity of NF- $\kappa$ B in these same cells suggest that monocytes in the circulation are primed for rapid NF- $\kappa$ B-dependent responses. Adherence leads to translocation of PKC (Eierman and Haskill, 1991), which may lead to further NF- $\kappa$ B nuclear localization and signaling. However, we now show that this also results in the parallel induction of the MAD-3/I $\kappa$ B gene, which then has the ability to down-regulate further responsiveness to mediators that transcriptionally activate other genes via NF- $\kappa$ B. The rapid induction of the MAD-3 gene by adherence closely parallels that of IL-1 $\beta$  (Eierman et al., 1989) and thus provides an important potential mechanism for homeostatic regulation either through complex formation with NF- $\kappa$ B in the cytosol or through displacement of high affinity NF- $\kappa$ B bound to DNA (Zabel and Baeuerle, 1990).

### **Experimental Procedures**

#### **Isolation of a Full-Length Version of the MAD-3 cDNA**

We have previously reported isolation of a partial MAD-3 cDNA clone, which was derived from a  $\lambda$ gt10 cDNA library produced from 30 min adhered monocytes (Sporn et al., 1990). Differential screening of this library had identified a series of unique clones representing immediate-early genes induced by adherence. One of these, MAD-3, was a 350 bp fragment of a 1.6 kb mRNA. This partial clone was used to screen a second library constructed by Invitrogen in the pcDNA 1 vector. mRNA was derived from 30 min adhered monocytes and neutrophils. Screening of this library identified several apparently full-length clones. One of these was sequenced.

#### **DNA Sequence Analysis of cDNA Clones**

Chain termination sequencing was carried out with the USB sequence procedure employing dITP to eliminate compressions as necessary. Primers were synthesized to permit overlapping bidirectional sequencing of the entire cDNA clone.

#### **Computer Analysis**

All computer analysis and assembly of sequence information were carried out with the aid of the University of Wisconsin GCG programs. The FASTP program was used to identify the initial homology to ankyrin. Searches employing Wordsearch failed to find similar matches. GAP was used to determine the degree of homology between the NF- $\kappa$ B/KBF1 protein and the MAD-3 predicted protein sequence derived from TRANSLATE.

#### **In Vitro Transcription and Translation of MAD-3 mRNA**

Two micrograms of pcDNA 1 (Invitrogen) containing full-length MAD-3 cDNA was digested with BamHI, which cuts downstream of the cDNA insert, or with AclI. The digests were phenol-chloroform extracted, ethanol precipitated, and used to synthesize RNA in a 100  $\mu$ l reaction

for 1 hr at 37°C using SP6 RNA polymerase under conditions recommended by the manufacturer (Boehringer Mannheim). The RNA was extracted twice with phenol-chloroform, ethanol precipitated, and redissolved in 20 µl of water. Synthesis of RNA was confirmed by electrophoresis of the RNA on agarose gels. Rabbit reticulocyte lysates were first depleted of an endogenous NF-κB-like DNA-binding activity. This was performed by adding 100 µl of lysate to 20 µl of DNA affinity resin previously washed with deionized water (see description of preparation of the resin below). The binding reaction was performed, with frequent mixing, for 10 min at room temperature. The mixture was pelleted by brief centrifugation in a microfuge and the supernatant was removed for *in vitro* translation reactions. Four microliters of RNA was used for *in vitro* translation in rabbit reticulocyte lysates following the manufacturer's (Promega Biotech) recommendations. The [<sup>35</sup>S]methionine-labeled products were analyzed on a 10% SDS-polyacrylamide gel using the method of Laemmli (1970). The gel was dried and then exposed for autoradiography.

#### Gel Mobility Shift Assays

DNA-protein binding reactions were carried out in 10 mM Tris (pH 7.7), 50 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 2 µg of poly(dI-dC) (Pharmacia), and 10% glycerol in a final volume of 20 µl. The reactions contained 10 µg of nuclear extracts and/or 1 µl of rabbit reticulocyte lysates (either programmed with MAD-3 mRNA or mock translated) and 10,000 cpm of end-labeled MHC enhancer binding probe. p50 antiserum (0.5 µl) (gift of Alain Israël, Pasteur Institute) or 0.5 µl of preimmune serum was added directly to the binding reaction. The reactions were kept at room temperature for 15 min and then loaded on a nondenaturing 5% polyacrylamide gel using a Tris/glycine/EDTA buffer (Baldwin, 1990); they were then electrophoresed for approximately 2 hr at 20 mA. The gel was then dried and autoradiographed overnight at -70°C.

#### NF-κB-IκB Binding and Dissociation Reactions

Nuclear extracts (10 µg) containing NF-κB were reacted with 1 µl of MAD-3 programmed lysates or mock-translated lysates under the binding conditions described above. The reactions were kept at room temperature for 10 min followed by the addition of 2 µg of poly(dI-dC) and 10,000 cpm of radiolabeled DNA probe. The reactions were then loaded onto a 5% polyacrylamide Tris/glycine/EDTA gel and analyzed as described above. For the dissociation reactions, 0.8% sodium DOC was added to the binding reactions (minus poly(dI-dC) and probe) followed by 1.2% NP-40. Poly(dI-dC) and probe were added and incubated at room temperature for 15 min. These reactions were electrophoresed and analyzed as described above.

The DNA-binding probes are labeled HindIII-EcoRI digests of pUC plasmids containing oligonucleotides cloned into the polylinker with BamHI restriction ends. Sequences are as follows: the class I MHC enhancer probe, GGCTGGGGATCCCCATCT; the mutant MHC probe, GGCTGCGGATCCCCATCT (Baldwin and Sharp, 1987); the major late transcription factor probe, ACCCGTCACGTGGCCTACA; the Oct-1 probe, ATGCAAT; the Iκκ probe, CAGAGGGACTTCCGAGA.

#### Nuclear Extracts and DNA Affinity Chromatography

Jurkat T cells were grown in RPMI 1640 medium and 10% fetal calf serum. For stimulation of Jurkat T cells, PHA and PMA were added to final concentrations of 1 µg/ml and 50 ng/ml, respectively. Monocytes were isolated from donors as previously described (Haskill et al., 1988). Nuclear extracts were prepared by the method of Swick et al. (1989). The DNA affinity resin contained the MHC class I enhancer sequence covalently linked to cyanogen bromide-activated Sepharose 4B (Sigma). The resin was made and the purification of NF-κB was carried out essentially by the method of Kadonaga and Tjian (1986). Nuclear extracts of PMA- and PHA-stimulated Jurkat T cells were used for the NF-κB purification. Jurkat nuclear extracts were incubated with the resin for 20 min, and NF-κB was eluted with a salt gradient. Only one round of DNA affinity chromatography was performed. Different fractions were tested for the NF-κB DNA-binding activity by gel mobility shift assays, and positive fractions were used for the DOC experiment.

#### Immunoprecipitation of MAD-3

The binding reactions between NF-κB and the MAD-3 product were carried out as described above. After 10 min, 2.5 µl of antiserum against NF-κB (Kieran et al., 1990), preimmune serum, or 1 × binding buffer was added to the reactions and allowed to incubate for another 10 min at room temperature. The reactions were then added to 20 µl of protein A-Sepharose beads (Sigma) pre-equilibrated in 1 × binding buffer. After 15 min of frequent mixing, the reactions were briefly spun in a microfuge and the supernatants were removed. The beads were then washed as described (Springer, 1989), after which they were boiled for 5 min in 1 × SDS loading buffer, briefly spun, and the supernatants run on a 10% SDS-polyacrylamide gel as described above.

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#### **GenBank Accession Number**

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