Activation of Transcription Factor NF-κB by the Adenovirus E3/19K Protein Requires its ER Retention

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Abstract. We have recently shown that the accumulation of diverse viral and cellular membrane proteins in the ER activates the higher eukaryotic transcription factor NF-κB. This defined a novel ER-nuclear signal transduction pathway, which is distinct from the previously described unfolded protein response (UPR). The well characterized UPR pathway is activated by the presence of un- or malfolded proteins in the ER. In contrast, the ER stress signal which activates the NF-κB pathway is not known. Here we used the adenovirus early region protein E3/19K as a model to investigate the nature of the NF-κB–activating signal emitted by the ER. E3/19K resides in the endoplasmic reticulum where it binds to MHC class I molecules, thereby preventing their transport to the cell surface. It is maintained in the ER by a retention signal sequence in its carboxy terminus, which causes the protein to be continuously retrieved to the ER from post-ER compartments. Mutation of this sequence allows E3/19K to reach the cell surface. We show here that expression of E3/19K potently activates a functional NF-κB transcription factor. The activated NF-κB complexes contained p50/p65 and p50/c-rel heterodimers. E3/19K interaction with MHC class I was not important for NF-κB activation since mutant proteins which no longer bind MHC molecules remained fully capable of inducing NF-κB. However, activation of both NF-κB DNA binding and κB-dependent transactivation relied on E3/19K ER retention: mutants, which were expressed on the cell surface, could no longer activate the transcription factor. This identifies the NF-κB–activating signal as the accumulation of proteins in the ER membrane, a condition we have termed "ER overload." We show that ER overload–mediated NF-κB activation but not TNF-stimulated NF-κB induction can be inhibited by the intracellular Ca^{2+} chelator TMB-8. Moreover, treatment of cells with two inhibitors of the ER-resident Ca^{2+}-dependent ATPase, thapsigargin and cyclopiazonic acid, which causes a rapid release of Ca^{2+} from the ER, strongly activated NF-κB. We therefore propose that ER overload activates NF-κB by causing Ca^{2+} release from the ER. Because NF-κB plays a key role in mounting an immune response, ER overload caused by viral proteins may constitute a simple antiviral response with broad specificity.

THE inducible transcription factor NF-κB is a central mediator of the human immune response (5). In most cell types, NF-κB is sequestered in an inactive, cytoplasmic complex by binding of IκB, an inhibitory subunit (4). Exposure of cells to a variety of pathological stimuli, such as bacterial or viral infection, inflammatory cytokines, and UV irradiation activates the transcription factor (5). Active NF-κB is rapidly released from the cytoplasmic complex by phosphorylation-controlled degradation of IκB (6, 25, 46, 49, 50). The transcription factor translocates to the nucleus, where it binds cognate DNA sequences, activating transcription of a large variety of genes including cytokine, hematopoietic growth factor, adhesion molecule, and other cell surface protein genes (for a complete list see reference 5).

We have recently shown that internal cellular stress, caused by the accumulation of proteins in the endoplasmic reticulum (ER) and by agents interfering with ER function, potently activates NF-κB (41). Treatment of cells with the glycosylation inhibitors tunicamycin and 2-deoxyglucose or with brefeldin A, which inhibits protein export out of the ER, strongly induces NF-κB. Likewise, overexpression of the influenza virus wild-type hemagglutinin or of immunoglobulin μ heavy chains in the absence of light chains activates the transcription factor. This represents a novel ER-nuclear signal transduction pathway, which is pharmacologically distinct from the unfolded-protein response (UPR)[1] described previously. Several agents such

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1. *Abbreviations used in this paper*: DTT, dithiothreitol; CAT, chloramphenicol acetyl transferase; CTL, cytotoxic T lymphocyte; EMSA, electrophoretic mobility shift assay; Luc, luciferase; OA, okadaic acid; UPR, unfolded-protein response.

as the glucosidase inhibitor castanospermine and the reducing agent dithiothreitol (DTT), which activate the UPR pathway, did not induce NF-κB activity (41). Similarly, overexpression of the influenza virus wild-type hemagglutinin activates NF-κB, but not the UPR pathway (40). The ER must therefore be able to emit distinct signals which selectively activate either the NF-κB pathway, the UPR, or both.

Human adenovirus causes respiratory, gastrointestinal, urinary, and ocular infections (17, 18). These may become persistent, causing the infectious virus to be shed from apparently healthy individuals several years postinfection (16). The adenovirus early region protein E3/19K is thought to contribute significantly to the establishment of persistent infections. E3/19K binds MHC class I proteins and prevents their expression on the cell surface by retaining them in the ER (1, 9). Thus, compared to uninfected cells, adenovirus-infected cells display fewer MHC class I molecules on their surface. MHC class I complexes serve to present foreign peptides to the immune system, allowing virus-infected cells to be recognized and destroyed by cytotoxic T lymphocytes (CTLs). Thus, because the expression of newly synthesized, peptide-loaded MHC class I molecules is prevented, adenovirus infected cells become protected from CTL lysis (10).

The ER retention of E3/19K is well investigated. It depends on sequences located in the carboxy terminus of the protein. A dilysine motif positioned three and four residues from the COOH terminus is both necessary and sufficient for ER retention (29). Introducing these residues into the COOH terminus of the cell surface protein CD8 confers ER residency on this protein. Moreover, CD4 and CD8 can be retained in the ER by addition of a polyserine tail with two lysines positioned three and four amino acids from the COOH terminus. In an independent study, Gabathuler and Kvist (19) showed indirectly that deletion of the two carboxy-terminal amino acids, which moves the critical lysine residues to the end of the protein, releases E3/19K from the ER. Likewise, deletion of the four carboxy-terminal residues or the two lysines and five additional amino acids, diminishes ER retention (19). However, one mutant, E3/19K-M125, which contains a deletion of the six COOH-terminal amino acids including the dilysine motif, is efficiently retained in the ER. More recently, it became clear that proteins with dilysine motifs obtain post-ER modifications and appear to be continuously retrieved to the ER from post-ER compartments (30). Binding of coatomer, a polypeptide complex located on the cytoplasmic side of ER and Golgi-derived membranes, might mediate their retrograde Golgi-to-ER transport (12, 33).

Assuming that E3/19K expression activates NF-κB, the underlying molecular signal could be further analyzed using mutants of the viral protein. One possibility is that the association of E3/19K with MHC class I molecules is necessary. In this case, it is the complexation between ER resident proteins that triggers NF-κB activation. The other possibility is that the signal relies on the retention and subsequent accumulation of E3/19K in the ER membrane. Here we report that the adenovirus E3/19K protein is a strong activator of NF-κB. Likewise, MHC class I overexpression in the absence of additional β_2-microglobulin expression induced NF-κB. The interaction between E3/19K and MHC class I was not necessary for NF-κB activation. However, there was a stringent requirement for ER retention. Mutant proteins, which escaped ER retention, no longer activated the transcription factor. NF-κB activation by wild-type E3/19K was dose dependent. This suggests that the copy number of ER resident membrane proteins rather than the complexation of proteins within the ER is the NF-κB–activating signal. E3/19K-mediated NF-κB induction was inhibited by pretreatment of cells with the intracellular Ca^{2+} chelator TMB-8. In addition, Ca^{2+} release from the ER induced by inhibition of the ER-resident Ca^{2+}-dependent ATPase with thapsigargin or cyclopiazonic acid (CPA), potently activated NF-κB. We therefore suggest that ER overload may activate NF-κB by releasing Ca^{2+} from the ER.

Materials and Methods

Cell Culture and Transfections

293 cells (Amer. Type Culture Collection, Rockville, MD; No. CRL 1573) and HeLa cells (Amer. Type Culture Collection; No. CCL 2) were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% FCS and 50 μg/ml penicillin-streptomycin (all from GIBCO-BRL, Gaithersburg, MD). Cells were plated 12–16 h before transfection at a density of 10^6 cells per 60-mm dish. Transfections were performed using calcium phosphate precipitation as previously described (23). The amounts of plasmids used are indicated in the figure legends. TMB-8, thapsigargin, and cyclopiazonic acid were purchased from Calbiochem Novabiochem Corp. (La Jolla, CA).

Plasmids

All E3/19K constructs contain the EcoRI D fragment of adenovirus 2 (26) in the pBluescript II KS vector. The mutants E3/19K-Ser11 and E3/19K-Ser83 have been previously described (45). The constructs E3/19K-K139/149S and E3/19K-K^d were generated by PCR-mediated oligonucleotide-directed mutagenesis (reference 27, details to be published elsewhere). The MHC class I K^k and K^d expression vectors have also been described (3, 32). The plasmid 6x-κB-tk-Luc contains three repeats of the HIV-1 tandem NF-κB sites in front of a minimal tk promoter and has been described previously (38). 6x-κB-tk-Luc and the parental tk-Luc vector were a generous gift of Dr. Markus Mayer (EMBL, Heidelberg, Germany). The IκB expression vector Rc/CMV-IκB has been described previously (52), it contains the entire IκB-α cDNA inserted as a HindIII fragment into Rc/CMV. The parental Rc/CMV vector was purchased from Invitrogen (San Diego, CA).

Electrophoretic Mobility Shift Assay and Antibody Supershifts

Total cell extracts were prepared using a high-salt detergent buffer (Totex) (20 mM Hepes, pH 7.9, 350 mM NaCl, 20% [wt/vol] glycerol, 1% [wt/vol] NP-40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 0.1% PMSF, 1% Aprotinin). Cells were harvested by centrifugation, washed once in ice cold PBS (Sigma Chem. Co., St. Louis, MO) and resuspended in four cell volumes of Totex buffer. After 30 min on ice, the lysates were centrifuged for 5 min at 13,000 *g* at 4°C. The protein content of the supernatant was determined and equal amounts of protein (10–20 μg) added to a reaction mixture containing 20 μg BSA (Sigma Chem. Co.), 2 μg poly (dI-dC) (Boehringer-Mannheim Corp., Indianapolis, IN), 2 μl buffer D+ (20 mM Hepes; pH 7.9; 20% glycerin, 100 mM KCl, 0.5 mM EDTA, 0.25% NP-40, 2 mM DTT, 0.1% PMSF), 4 μl buffer F (20% Ficoll 400, 100 mM Hepes, 300 mM KCl, 10 mM DTT, 0.1% PMSF) and 100,000 cpm (Cerenkov) of a ^{32}P-labeled oligonucleotide in a final volume of 20 μl. The AP-1–binding reaction contained 5 mM $MgCl_2$ in addition. Samples were incubated at room temperature for 25 min. For the supershift assays, 2.5 μl of antibody were added to the reaction simultaneously with the protein and incubated as described. Anti-p50, anti-p65, and anti-c-rel antibodies were purchased from Santa Cruz Biotechnology. NF-κB and

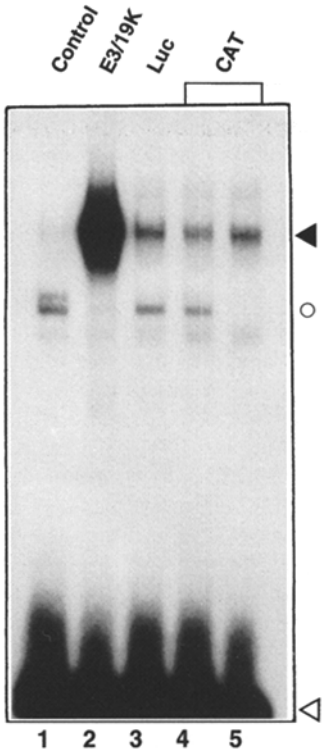


Figure 1. Expression of adenovirus E3/19K protein activates NF-κB. 293 cells were transiently transfected with the following plasmids: (Lane *1*) untransfected; (lane *2*) transfected with 6 μg E3/19K expression vector; (lane *3*) transfected with 6 μg luciferase expression vector; (lanes *4* and *5*) transfected with 6 μg of two CAT expression vectors driven by different promoters. 24 h after transfection total cell extracts were prepared and assayed in an EMSA using a high affinity κB-binding site as a probe. A filled arrowhead indicates specific NF-κB complexes. The open circle denotes nonspecific binding to the probe and the open arrowhead shows unbound oligonucleotide.

AP-1 oligonucleotides (Promega, Madison, WI) were labeled using γ-[^{32}P]ATP (3,000 Ci/mmol; Amersham Corp., Arlington Heights, IL) and T4 polynucleotide kinase (Promega).

Luciferase Assays

Cells were harvested 24 h posttransfection and luciferase (Luc) activity determined precisely as described (42). The cell pellet obtained from one 60-mm dish was resuspended in 150 μl of lysis buffer (25 mM glycylglycine, 1% Triton X-100, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT) and centrifuged at 13,000 *g* at 4°C for 5 min. 50 microliters of the supernatant were assayed in 150 μl assay buffer (25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, 15 mM KP_i, pH 7.5, 1 mM DTT, 1 mM ATP) using an LB 96 P luminometer (EG & G-Bertold, Bad Wildbad, Germany). Light emission was measured over a 30-s interval and the results are given in relative light units.

FACS Analysis

Cell surface staining of 293 cells was carried out as previously described (45). For intracellular staining, cells were incubated with antibodies in the presence of 0.075% Saponin (Sigma). The Tw1.3 monoclonal antibody recognizes a luminal epitope of E3/19K and was a generous gift of Dr. J.W. Yewdell, (NIH, Bethesda, MD) (13).

Immunoprecipitation

Cell labeling with [^{35}S]methionine, immunoprecipitation, and SDS-PAGE were carried out as previously described (9). The E3/19K antiserum, abbreviated C-tail in Fig. 7, is directed against the cytoplasmic tail of E3/19K (45). The anti-MHC class I rabbit antiserum (K-tail in Fig. 7) was raised against a peptide containing the 11 COOH-terminal amino acids of the K^d molecule (Burgert, H.-G., and M. Sester, unpublished data).

Results

Expression of Adenovirus E3/19K Protein Potently Activates NF-κB

To investigate whether expression of the ER-resident adenovirus E3/19K protein activates NF-κB, 293 cells were transiently transfected with a vector carrying the adenovirus 2 EcoRI D fragment. This sequence contains the entire E3/19K coding region as well as the E3 promoter (26). As controls, cells were transfected with expression plasmids for the cytoplasmic proteins luciferase (Luc) and chloramphenicol acetyl transferase (CAT). 24 h after transfection, total cell extracts were prepared and assayed for NF-κB DNA binding in an electrophoretic mobility shift assay (EMSA). Cells expressing E3/19K gave rise to a novel complex not found in mock-transfected cells and present only in small quantities in cells transfected with either Luc or CAT (Fig. 1). A faster migrating complex, which was already present in mock-transfected cells remained unchanged or was diminished in transfected cells.

NF-κB proteins constitute a large family of transcription factors, whose members can homo- and heterodimerize with each other (24). Hence, specific antibodies were added to DNA-binding reactions to identify the various NF-κB subunits in the E3/19K-induced complex. Addition of antibodies against the p50 subunit of NF-κB abrogated the entire complex. In return, more slowly migrating immune complexes were observed (Fig. 2, lane *2*). Antibodies to either p65 or c-rel caused only a partial reduction of protein-DNA complex formation (lanes *3* and *4*). Only the addition of both antibodies completely abolished complex formation (lane *5*). An antibody to E3/19K, used as a control, changed neither the amount nor the migration of the complex. Addition of a 50-fold excess of nonradioactive oligonucleotide containing a NF-κB–binding site effectively competed for complex binding while the same

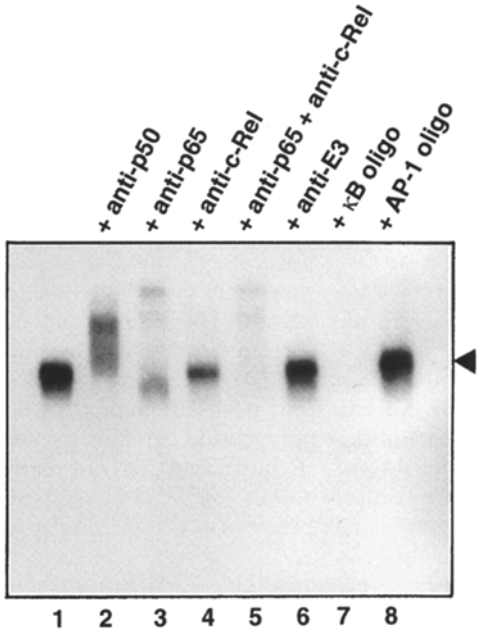


Figure 2. Identification of the NF-κB subunit composition. EMSA of total cell extracts from 293 cells transiently transfected with 6 μg E3/19K expression vector. (Lane *1*) Control; (lanes *2–6*) extracts were incubated with the antibodies indicated; (lane *7*) extracts were incubated with a 50-fold excess of unlabeled NF-κB oligonucleotide; (lane *8*) extracts were incubated with a 50-fold excess of unlabeled AP-1 oligonucleotide. A filled arrowhead points to the specific NF-κB complex.

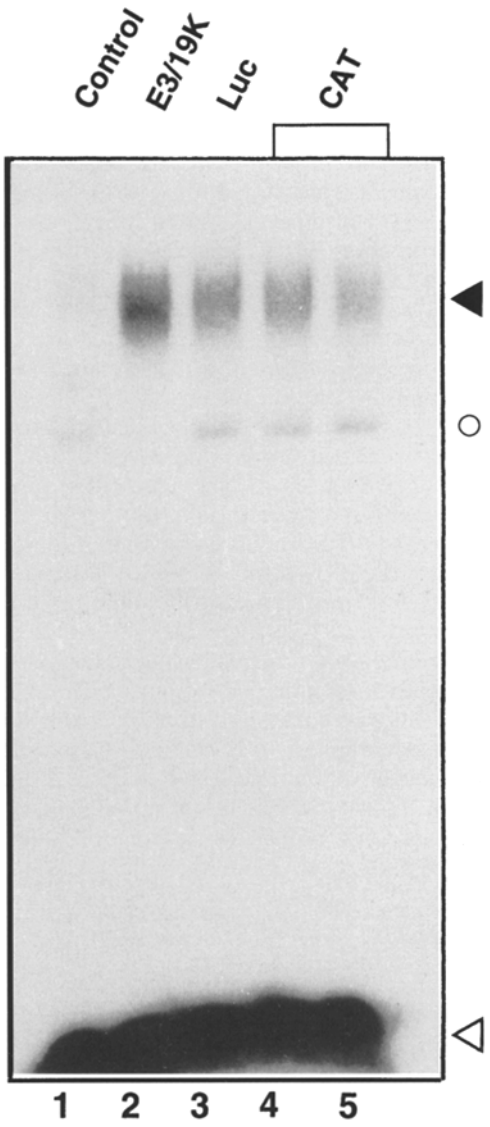


Figure 3. Effect of E3/19K expression on AP-1. The extracts shown in Fig. 1 were incubated in an EMSA reaction using a high affinity AP-1–binding site as a probe. A filled arrowhead indicates specific AP-1 complexes. The open circle denotes nonspecific binding to the probe and the open arrowhead shows unbound oligonucleotide.

amount of oligonucleotide carrying an AP-1–binding site had no effect (lanes *7* and *8*). In conclusion, in transiently transfected 293 cells, E3/19K potently induced DNA binding of a NF-κB complex composed of p50/p65 and p50/c-rel heterodimers.

Transfection and expression of E3/19K might represent a general stress situation for the cell, resulting in the activation of several stress-inducible transcription factors. We therefore tested whether E3/19K could activate AP-1, another stress-inducible transcription factor (2). The extracts used in Fig. 1 were incubated in an EMSA reaction with a DNA probe containing an AP-1–binding site. E3/19K expression also activated an AP-1 DNA-binding activity approximately fivefold. (Fig. 3, lane *2*). This complex was identified as AP-1 by competition and supershifting assays (data not shown). However, in contrast to NF-κB, AP-1 was almost equally activated by the transfection of plasmids encoding luciferase or CAT proteins (lanes *3–5*). AP-1 activation thus more likely results from manipulations of the $CaPO_4$ transfection rather than from the specific expression of E3/19K. Therefore, only NF-κB was selectively activated by E3/19K expression.

NF-κB Activation Is Independent of E3/19K Binding to MHC Class I Molecules

The ability of the E3/19K molecule to activate NF-κB may result from several of its properties. One possibility is that its binding to MHC class I molecules produces protein aggregates in the ER which impair the organelle and cause ER stress. We tested this hypothesis in two experiments. First, we compared wild-type E3/19K and two point mutants, which no longer bind MHC class I molecules, in their ability to activate NF-κB. In both point mutants, cysteine residues, either at positions 11 or 83, were mutated to serines which completely abolishes E3/19K binding to MHC class I molecules (45). 6 μg of either the wild-type or the mutant E3/19K expression plasmids were transfected into 293 cells and cell extracts prepared for NF-κB–binding assays 24 h later. Both point mutants remained fully capable of activating NF-κB to the same extent as the wild-type protein (Fig. 4 *A*, lanes *2–4*). This suggests that binding to MHC class I molecules is not required for E3/19K-mediated activation of NF-κB.

The E3/19K protein may be highly overexpressed in these assays thereby overriding the effect of MHC class I complexation. In a titration experiment, we found that transfection of 60 ng of E3/19K expression plasmid suffices to activate NF-κB, and that NF-κB activation increases linearly with the amount of plasmid transfected (data not shown; see Fig. 5). We also observed that transfection of an MHC class I expression plasmid activated NF-κB on its own. (Fig. 4 *B*, lanes *3* and *7*). MHC class I molecules require β_2-microglobulin binding for transport out of the ER (39). Cells overexpressing MHC class I may not contain sufficient amounts of endogenous β_2-microglobulin to transport all MHC molecules to the cell surface. MHC class I proteins thus accumulate in the ER, thereby activating NF-κB. We observed a similar effect when immunoglobulin μ heavy chains were expressed in the absence of light chains (41).

To further investigate the importance of E3/19K-MHC class I interactions for NF-κB activation, 293 cells were transfected with very low amounts of expression vectors. Cells were cotransfected with 60 ng of E3/19K vector and 60 ng of an expression vector encoding either the MHC class I allele K^d, which is bound by E3/19K, or the allele K^k, which is not bound. We also transfected cells with 60 ng or 120 ng of the K^k expression vector alone. 24 h post-transfection, total cell extracts were analyzed for NF-κB DNA binding. While transfection of 60 ng of E3/19K or MHC K^k expression vector alone activated NF-κB (Fig. 4 *B*, lanes *2* and *3*), cotransfection of either 60 ng of K^d or K^k with 60 ng of E3/19K had no larger effect than the transfection of 120 ng of either vector alone (compare lanes *4–7*). Thus, the interaction of E3/19K with MHC class I proteins shows no synergistic effect on NF-κB activation. These re-

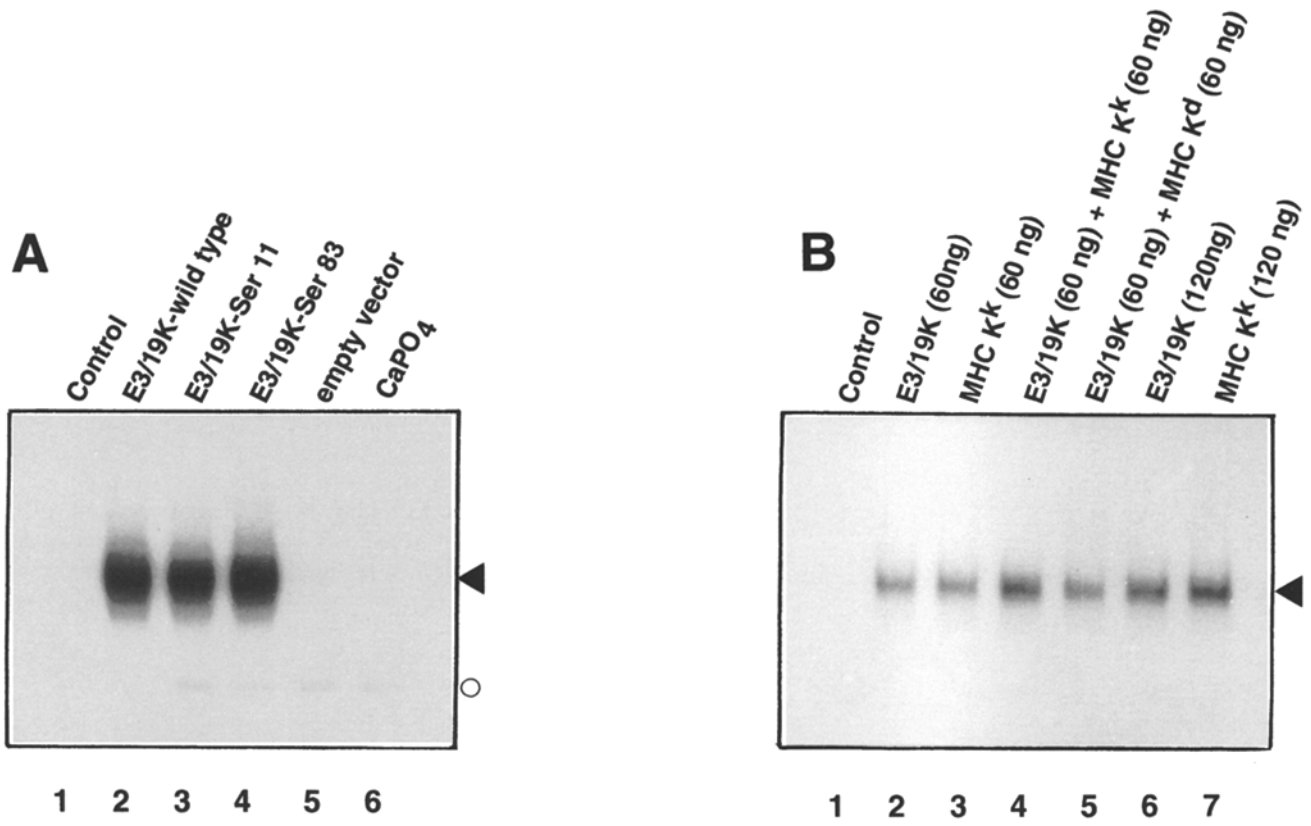


Figure 4. E3/19K-mediated NF-κB activation is independent of binding to MHC class I. (*A*) 293 cells were transiently transfected with 6 μg of either the wild-type E3/19K expression vector (lane *2*), or expression vectors for E3/19K point mutants (lanes *3* and *4*). (Lane *1*) Untransfected controls; (lane *5*) cells transfected with empty pBS vector; (lane *6*) cells treated with $CaPO_4$ precipitates containing no DNA. 24 h after transfection total cell extracts were prepared and assayed for NF-κB DNA binding in an EMSA. A section of a fluorogram is shown. A filled arrowhead indicates specific NF-κB complexes, the open circle denotes nonspecific binding to the probe. (*B*) 293 cells were transfected with the indicated amounts of either the wild-type E3/19K expression plasmid or expression plasmids for the murine MHC class I alleles K^d and K^k. 24 h after transfection total cell extracts were prepared and assayed for NF-κB DNA binding in an EMSA. A section of a fluorogram is shown. A filled arrowhead indicates specific NF-κB complexes.

sults corroborate our findings from the previous experiment that E3/19K activates NF-κB independent of its binding to MHC class I molecules.

Activation of NF-κB by E3/19K Requires ER Retention

Since NF-κB activation by E3/19K is independent of complex formation with MHC class I, we investigated whether its ER retention is necessary for induction of the transcription factor. Two mutant E3/19K proteins were constructed. The first, called E3/19K-K^d, contains amino acids 1-127 of E3/19K but the 15 COOH-terminal amino acids, in which the ER retention signal resides, were replaced by 40 amino acids from the COOH terminus of the MHC class I K^d molecule. This chimeric protein contains the luminal ER domain and the transmembrane segment of E3/19K fused to the cytoplasmic tail of the K^d molecule, and is slightly larger than wild-type E3/19K. A second mutant, called E3/19K-K139/140S, contains two point mutations. In this construct, the lysines at positions 139 and 140, which are situated four and three residues from the carboxy terminus of the protein and constitute the dilysine tag critical for ER retention (29), were replaced by serines.

We first tested whether the mutant E3/19K proteins are expressed on the cell surface, as was previously shown for similar mutants (19, 29). 293 cells were transfected with wild-type E3/19K, E3/19K-K^d, or E3/19K-K139/140S expression plasmids together with a plasmid encoding neomycin resistance. Stably transfected clones were selected and tested for protein expression by FACS analysis. Untransfected 293 cells and three clones expressing approximately equal amounts of E3/19K proteins were compared in FACS analysis for the subcellular distribution of the viral protein. Cells were stained using the anti-E3/19K antibody Tw1.3 by two procedures. To detect intracellular expression of the protein the membrane-permeabilizing detergent saponin was added to one set of samples (Fig. 5, *A–D*). A second set of samples was stained for surface expression of E3/19K (Fig. 5, *E–H*). All three cell lines expressed the E3/19K proteins to approximately the same levels. However, while wild-type E3/19K remained entirely intracellular, both the E3/19K-K^d and the E3/19K-K139/140S proteins appeared on the cell surface (compare panel *F* to panels *G* and *H*). Thus, replacement of the E3/19K COOH terminus or mutation of two critical lysine residues altered the intracellular distribution of the protein.

We subsequently investigated the effect of these mutations on the ability of E3/19K to activate NF-κB. Since

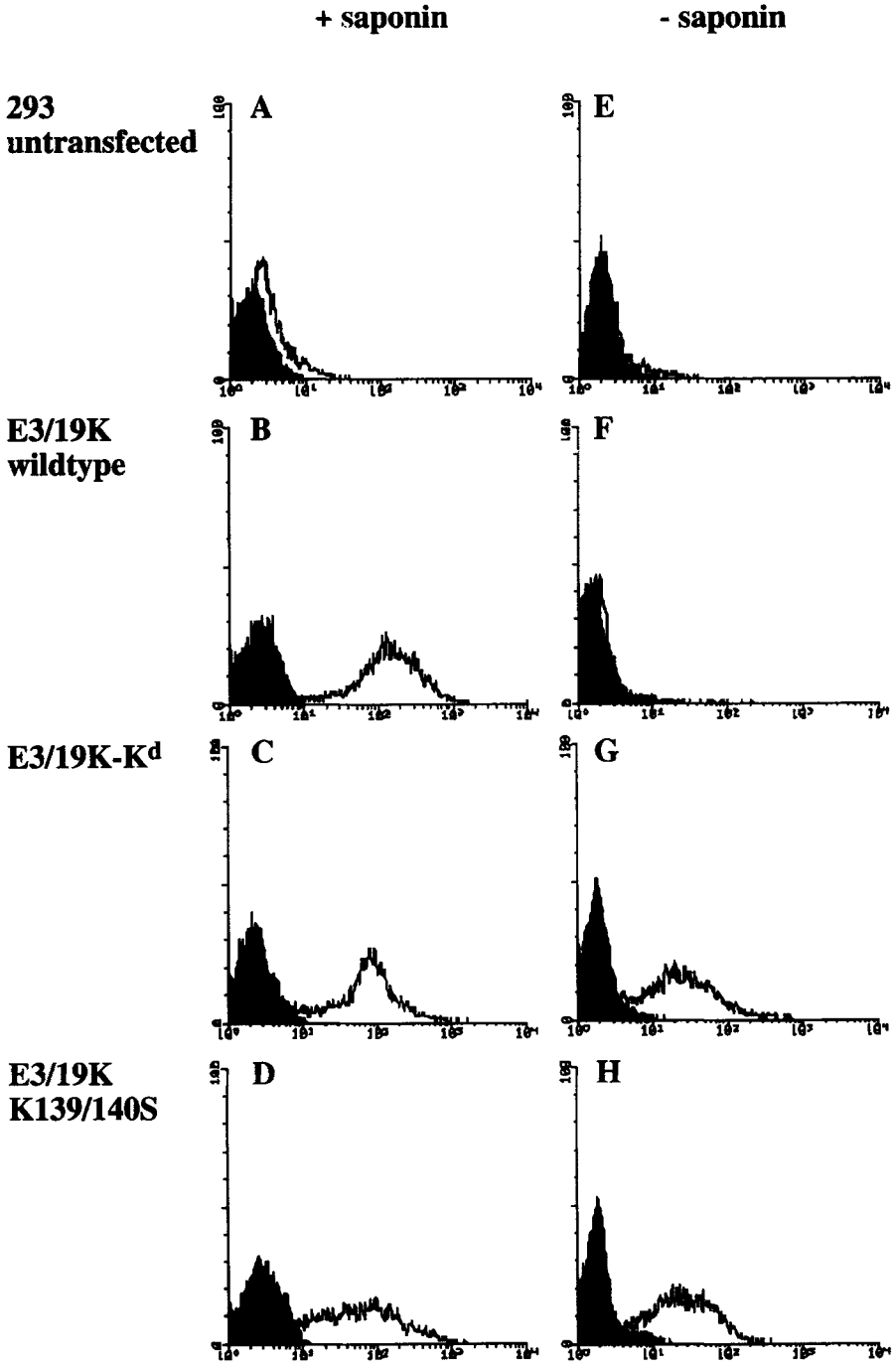


Figure 5. Expression of wild-type E3/19K, E3/19K-K139/140S, and E3/19K-K^d mutants in stably transfected 293 cells. 293 cells were stably transfected with expression vectors for wild-type E3/19K (*B* and *F*), or the mutants E3/19K-K^d (*C* and *G*) and E3/19K-K139/140S (*D* and *H*). Clones expressing approximately equal levels of proteins, as judged by immunoprecipitation (data not shown), were chosen for FACS analysis. Untransfected 293 cells were included as a control and are shown in *A* and *E*. Cells were stained with the anti-E3/19K antibody Tw1.3. In *A–D*, the detergent saponin was added in order to assess intracellular expression of the protein. *E–H* show staining for surface expression.

such an effect can be very subtle, the wild-type and the point mutant were compared in a titration assay. Between 60 ng and 6 μg of vectors encoding either the wild-type or the mutant protein were transfected into 293 cells. 24 h after transfection, total cell extracts were prepared and analyzed for NF-κB DNA binding. Transfection of as little as 60 ng of wild-type expression vector sufficed to activate NF-κB and the amount of complex increased as larger amounts of plasmid were used (Fig. 6, lanes *2–5*). In contrast, even the transfection of 6 μg of E3/19K-K139/140S plasmid, i.e., 100 times the amount required for detectable activation by the wild-type vector, failed to induce NF-κB DNA binding (Fig. 6, lanes *6–10*).

One possible explanation for these data is that in transiently transfected cells the mutant protein is expressed to a significantly lower level than the wild-type protein. We thus compared the transient expression levels of wild-type E3/19K, E3/19K-K139/140S, and E3/19K-K^d by in vivo labeling and immunoprecipitation. 293 cells were transfected with 6 μg of either the wild-type E3/19K expression plasmid, the E3/19K-K139/140S, or the E3/19K-K^d vector. 48 h after transfection half of the cells were harvested and analyzed for NF-κB DNA binding. The remaining cells were labeled with [^{35}S]methionine and cell lysates subjected to immunoprecipitation with two different antibodies. While expression of the wild-type E3/19K activates

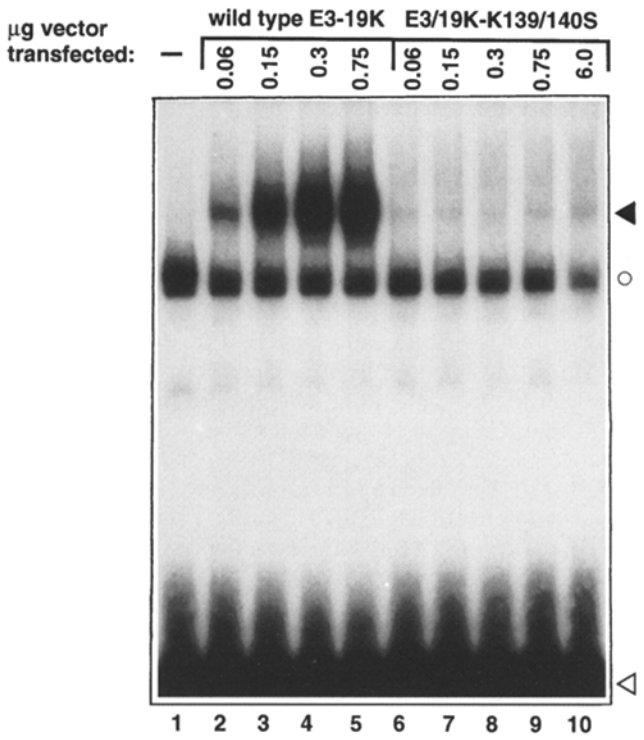


Figure 6. Dose response of NF-κB activation by wild-type E3/19K and E3/19K-K139/140S expression. 293 cells were transfected with the indicated amounts of wild-type or mutant E3/19K expression vector. 24 h after transfection total cell extracts were prepared and assayed for NF-κB DNA binding in an EMSA. A filled arrowhead indicates specific NF-κB complexes, the open circle denotes nonspecific binding to the probe and the open arrowhead shows unbound oligonucleotide.

NF-κB DNA binding under these conditions (Fig. 7 *A*, lane *2*), transfection of both the E3/19K-K139/140S point mutant and the E3/19K-K^d fusion protein failed to activate the transcription factor (Fig. 7 *A*, lanes *3* and *4*). Nonetheless, immunoprecipitation with two different antibodies showed that the proteins were expressed to equal levels in these cells (Fig. 7 *B*). Quantitative analysis by phosphoimaging determined that the E3/19K-K139/140S mutant was expressed at 104% and the E3/19K-K^d mutant at 114% of the wild-type protein in this experiment. Therefore, the difference in NF-κB activation does not reflect different transient expression levels of the mutant proteins, but rather their inability to elicit the NF-κB–inducing signal in the ER.

The Dilysine Motif Is Not Required for NF-κB Activation

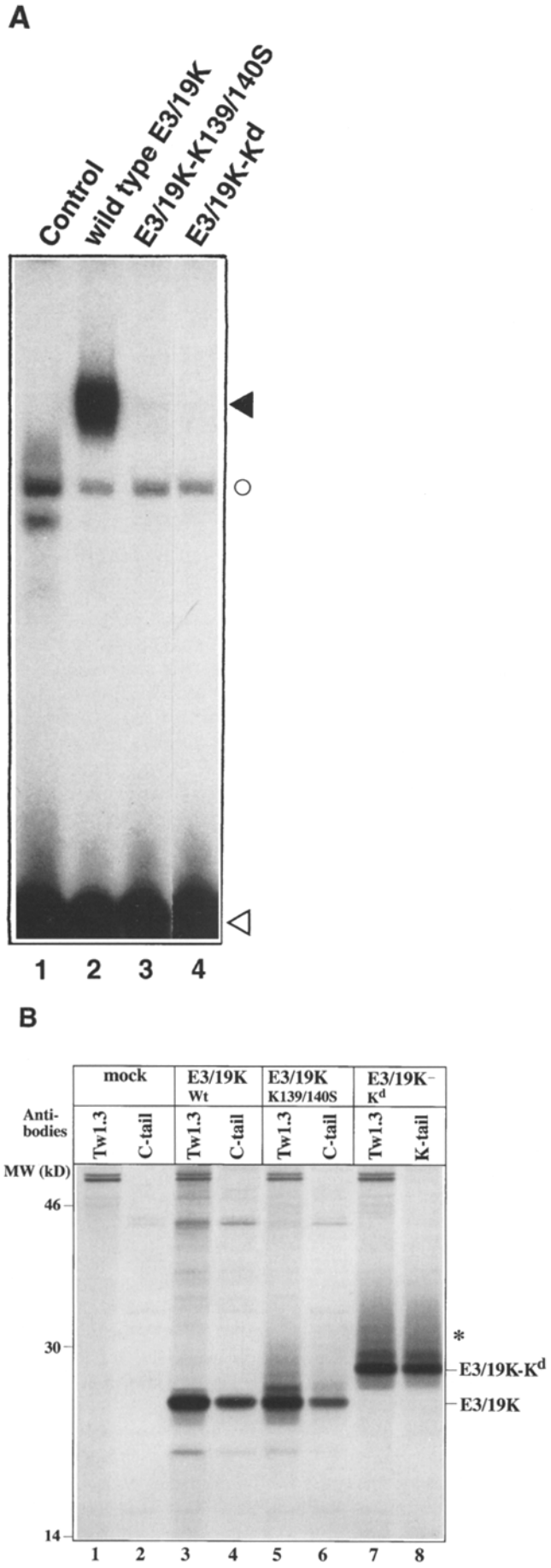
Mutation of the dilysine motif in the E3/19K-K139/140S construct has two simultaneous effects: for one, it relieves E3/19K ER retention, allowing the protein to escape to the cell surface. At the same time, however, the dilysine motif itself is destroyed. It has been postulated that this motif binds microtubules and coatomers, providing a link between the ER and the cytoplasm which might serve to transduce the NF-κB–activating signal (12, 14). Failure of the E3/19K-K139/140S mutant to activate NF-κB might thus result from either the loss of ER retention or from loss of dilysine motif-mediated signal transduction. We therefore investigated whether presence of the dilysine motif is required for NF-κB activation. In a previous study, Gabathuler and Kvist (19) described an E3/19K mutant, E3/19K-M125, which lacks the six carboxy-terminal amino acids, including the dilysine motif, but which is nontheless retained in the ER. In a pulse chase experiment, we confirmed that this mutant is retained in the ER as efficiently as wild-type E3/19K (Sester, M., and H.-G. Burgert, unpublished data). We compared the ability of wild-type E3/19K and E3/19K-M125 to activate NF-κB. 293 cells were transfected with 6 µg of expression vectors for either protein and cell lysates assayed for NF-κB DNA binding 24 h after transfection. Removal of the dilysine motif does not affect E3/19K-mediated NF-κB activation, since the E3/19K-M125 mutant induces the transcription factor to almost the same level as the wild-type protein (Fig. 8, compare lanes *2* and *3*). These data argue that the NF-κB–activating signal emitted from the ER is not mediated by the dilysine motif but is rather elicited by the retention and accumulation of E3/19K.

Wild-Type E3/19K but Not a Secreted Mutant Protein Induces κB-dependent Gene Expression

We tested whether the E3/19K-induced NF-κB is functional in that it can activate κB-dependent reporter gene activity. 293 cells were transfected with 5 ng of either a vector containing the luciferase gene driven by a minimal tk-promoter, or a vector containing the same promoter preceded by six copies of an NF-κB–binding site. In addition, the cells were cotransfected with 6 µg of the wild-type E3/19K or the E3/19K-K139/140S point mutant expression vectors. To ensure that increased reporter gene activity was specific for the activation of NF-κB, cells were also cotransfected with an expression vector for IκB-α, an inhibitory subunit, which prevents NF-κB activation when overexpressed (7). Expression of either the wild-type or the mutant E3/19K proteins did not affect basal tk-driven luciferase activity (Fig. 9, *left side*). κB-dependent reporter gene activity, however, was increased 17-fold by the expression of wild-type E3/19K, but not by the expression of the mutant protein (Fig. 9, *right side*). This activation was dependent on NF-κB, since it was entirely abolished by the overexpression of IkB. These data show that activation of transcription factor NF-κB by adenovirus E3/19K depends on ER retention of the protein and leads to nuclear gene expression.

E3/19K-induced NF-κB Activation Is Apparently Mediated by Release of Ca^{2+} from the ER

Retention of proteins in the ER must elicit a signal which reaches NF-κB in the cytoplasm. The ER is a reservoir for Ca^{2+}, which may be released into the cytoplasm upon stimulation. Since Ca^{2+} is a widely used second messenger, we investigated whether Ca^{2+} release from the ER might play a role in NF-κB activation by E3/19K. 293 cells were preincubated with increasing concentrations of the intracellular Ca^{2+} chelator TMB-8 (36). TMB-8 has been shown to inhibit Ca^{2+} release from the ER without affecting the influx of extracellular Ca^{2+} (11). 1 h after TMB-8 treatment, cells were either transfected with 6 µg of the



wild-type E3/19K expression vector or stimulated with 200 U/ml TNF. 4 h after transfection/stimulation, total cell extracts were prepared and analyzed for NF-κB DNA binding in an EMSA. Pretreatment with TMB-8 potently suppressed E3/19K-mediated NF-κB activation (Fig. 10 *A*). In contrast, it had virtually no effect on TNF-stimulated NF-κB induction (Fig. 10 *B*). Since NF-κB activation by E3/19K depends on efficient transfection and expression of the protein, we compared the amount of E3/19K protein in untreated and TMB-8–treated cells. Untreated 293 cells and cells pretreated with 1 mM TMB-8 for 1 h were transfected with 6 μg of the wild-type E3/19K expression vector. Mock-transfected cells were included as a control. 4 h after transfection, cells were labeled with [^{35}S]methionine for 75 min. Cell lysates were immunoprecipitated with a monoclonal antibody against E3/19K and analyzed by SDS-PAGE. Untreated and TMB-8–treated cells expressed identical amounts of E3/19K (Fig. 10 *C*, compare lanes *2* and *3*), indicating that the Ca^{2+} chelator affected neither transfection nor protein expression. The inhibitory effect of TMB-8 thus suggests a requirement for intracellularly released Ca^{2+} ions in E3/19K-mediated NF-κB activation.

Ca^{2+} release from the ER can be induced by inhibition of the ER-resident Ca^{2+}-dependent ATPase. Two structurally unrelated, selective inhibitors of this enzyme have been described: thapsigargin and cyclopiazonic acid (CPA) (22, 47). If ER overload activates NF-κB by causing Ca^{2+} efflux from the ER, treatment of cells with Ca^{2+}ATPase inhibitors should also induce the transcription factor. We investigated this hypothesis by treating HeLa cells with either 15 μM thapsigargin or 75 μM CPA for various times. Treatment with 50 ng/ml TPA, which was previously shown to induce NF-κB was included as a positive control. Treatment of HeLa cells with both thapsigargin and CPA strongly and rapidly activated NF-κB (Fig. 11). Activation was already seen 15 min after stimulation (lanes *2* and *7*) and reached maximal levels after 1–3 h (lanes *4*, *9*, and *10*) similar to TPA (lanes *12–16*). Induction by the agents was transient, decreasing to basal levels by 24 h (lanes *6*, *11*, and *16*). It has been shown that thapsigargin- and CPA-stimulated Ca^{2+} release is fast, occurring within seconds

Figure 7. Expression of wild-type and mutant E3/19K proteins in transiently transfected 293 cells and their effect on NF-κB activation. (*A*) 293 cells were transfected with 6 μg of expression vectors for the wild-type E3/19K (lane *2*), the E3/19K-K139/140S (lane *3*), or the E3/19K-K^d mutant (lane *4*). Lane *1* contains untransfected control cells. 48 h after transfection total cell extracts were prepared and assayed for NF-κB DNA binding in an EMSA. A filled arrowhead indicates specific NF-κB complexes. The open circle denotes nonspecific binding to the probe and the open arrowhead shows unbound oligonucleotide. (*B*) Immunoprecipitation of E3/19K proteins transiently transfected into 239 cells. 6 μg of expression vectors for the wild-type E3/19K (lanes *3* and *4*), the E3/19K-K139/140S (lanes *5* and *6*), or the E3/19K-K^d mutant (lanes *7* and *8*) were transfected into 293 cells. 48 h after transfection, cells were starved of methionine for 45 min and subsequently incubated with ^{35}S-labeled methionine for 75 min. Cell extracts were prepared and immunoprecipitated with two different antibodies for each protein as indicated. Lanes *1* and *2* contain lysates from mock-transfected cells. A star indicates slower migrating E3/19K proteins that have received post-ER modifications.

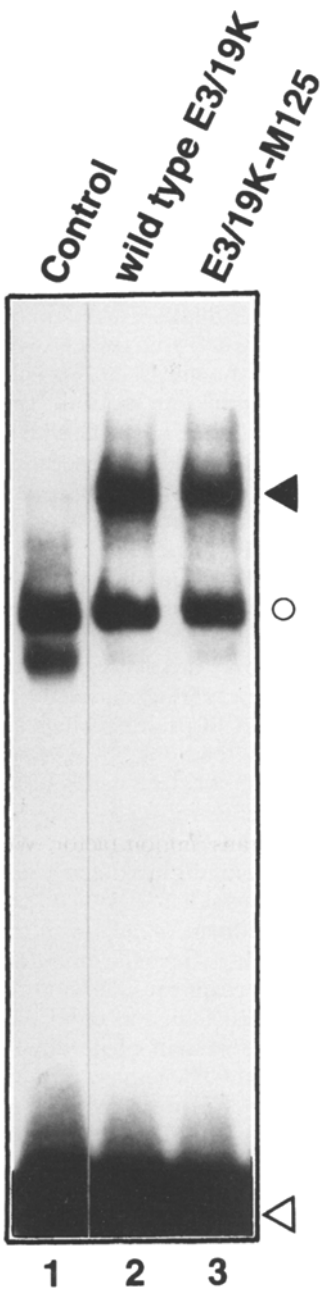


Figure 8. The dilysine motif is not required for E3/19K mediated NF-κB activation. 293 cells were transfected with 6 μg of expression vectors for the wild-type E3/19K (lane *2*) or the E3/19K-M125 mutant (lane *3*). Lane *1* contains untransfected control cells. 24 h after transfection total cell extracts were prepared and assayed for NF-κB DNA binding in an EMSA. A filled arrowhead indicates specific NF-κB complexes. The open circle denotes nonspecific binding to the probe and the open arrowhead shows unbound oligonucleotide.

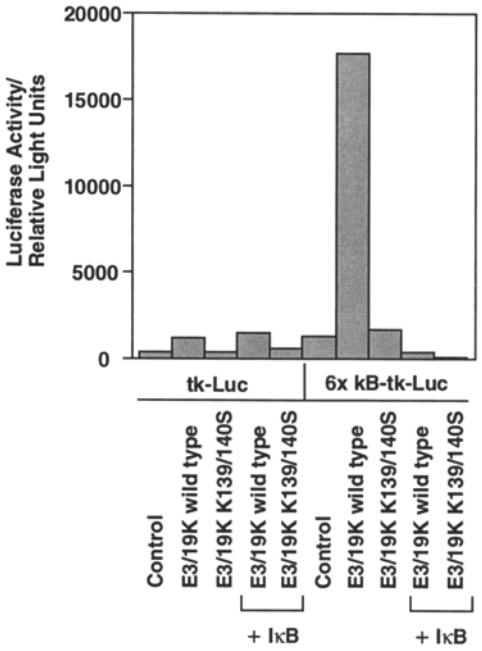


Figure 9. E3/19K-mediated κB-dependent gene expression requires an intact ER-retention signal. 293 cells were transfected with 5 ng of either the tk-Luc (*left side*) or the 6x-κB-tk-Luc (*right side*) plasmid. 6 μg of expression vectors for either the wild-type E3/19K or the E3/19K-K139/140S mutant were cotransfected together with 5 μg of either an IκB expression vector or empty Rc/CMV vector as indicated. Cells were harvested 48 h posttransfection and luciferase activity determined. Results represent averages of duplicate experiments.

after drug application (8). The rapid activation of NF-κB by Ca^{2+}ATPase inhibitors is consistent with the hypothesis that Ca^{2+} release from the ER can cause NF-κB activation. NF-κB activation by thapsigargin and CPA is dose dependent and can be inhibited by pretreatment of cells with the Ca^{2+} chelators TMB-8 and BAPTA-AM (data not shown). Taken together, our data suggest that Ca^{2+} efflux from the ER represents one cytoplasmic signal by which ER overload activates NF-κB.

Discussion

We have recently shown that the transcription factor NF-κB participates in a novel ER nuclear signal transduction pathway (41). The NF-κB–inducing pathway is distinguishable from the previously described UPR pathway, which is activated by the presence of un- or malfolded proteins in the ER (20). In contrast, the ER stress signal activating NF-κB is not well understood. To investigate the nature of this signal we used the adenovirus early region protein E3/19K. Two properties of this protein can potentially contribute to its capacity to activate NF-κB: its complexation with MHC class I molecules or its retention and subsequent accumulation in the ER. Since E3/19K has been studied in detail, several amino acid residues required for MHC class I binding and a dilysine motif required for ER retention have been identified (29, 45). This allows the introduction of minimal changes in the protein to abolish either property. In particular, the intracellular localization of the protein can be altered by point mutations without perturbing the ER luminal domain, an approach which is not possible with other NF-κB–activating proteins.

Point mutants of E3/19K which eliminate either MHC class I binding or ER retention were tested for their ability to activate NF-κB. Two mutants which no longer bind MHC class I molecules (45) remained fully capable of inducing NF-κB (Fig. 4 *A*). Moreover, accumulation of a certain number of E3/19K-MHC class I complexes appears to cause the same degree of ER stress as the accumulation of the same number of uncomplexed E3/19K molecules (Fig. 4 *B*). The sensor which signals ER stress must therefore recognize the number of proteins in the ER membrane but not their size or interaction in the ER lumen.

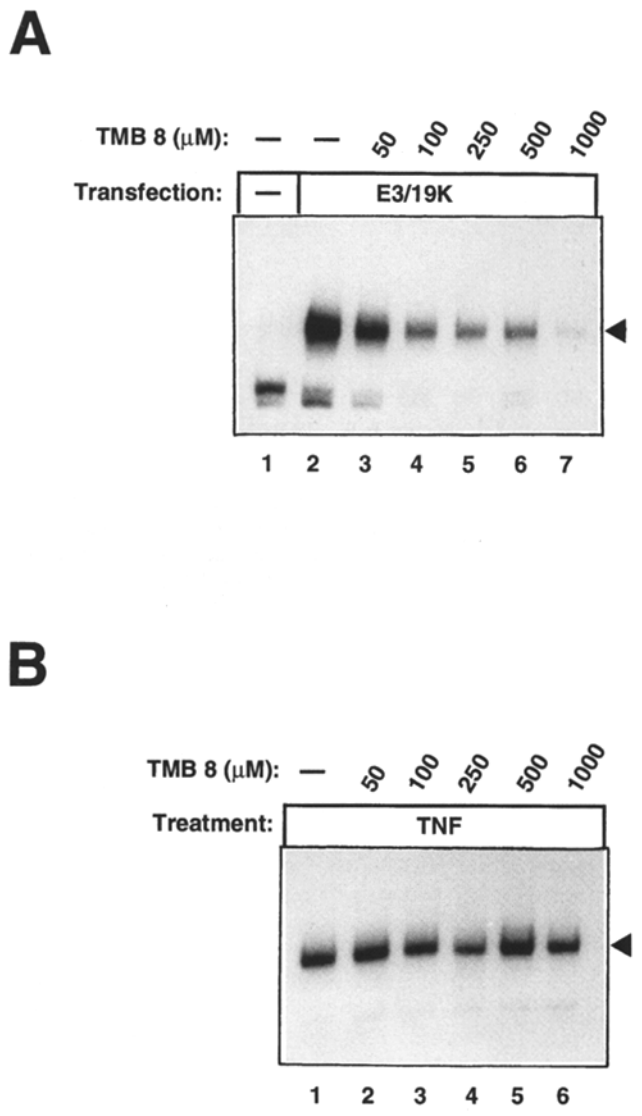


Figure 10. E3/19K-mediated but not TNF-stimulated NF-κB activation is inhibited by the intracellular Ca^{2+} chelator TMB-8. 293 cells were pretreated with increasing doses of TMB-8 as indicated for 1 h and subsequently either transfected with 6 μg of an E3/19K expression vector (*A*) or stimulated with 200 μ/ml TNF for 4 h (*B*). Cell extracts were prepared and analyzed in an EMSA using a high affinity κB-binding site as a probe. A filled arrowhead indicates specific NF-κB complexes. (*C*) Immunoprecipitation of E3/19K proteins transiently transfected into untreated 293 cells (lane *2*) and cells pretreated for 1 h with 1,000 mM TMB-8 (lane *3*). Cells were transfected with 6 μg of expression vectors for E3/19K (lanes *2* and *3*), lane *1* contains lysate from mock-transfected cells. 4 h after transfection, cells were starved of methionine for 45 min and subsequently incubated with ^{35}S-labeled methionine for 75 min. Cell extracts were prepared and immunoprecipitated with the anti-E3/19K monoclonal antibody Tw 1.3.

ER retention, however, is essential for the capacity of E3/19K to activate NF-κB. A point mutant in which two lysine residues were replaced by serines causing it to be expressed on the cell surface, no longer induced the transcription factor. Using this mutant, however, we cannot distinguish between the contribution of the dilysine motif itself and ER-retention: by mutating the two lysine residues to abolish ER retention, we concurrently destroy the dilysine tag. We therefore examined a second E3/19K mutant, in which the six COOH-terminal amino acids, including the dilysine motif at positions -3 and -4, are deleted. Several assays show that this construct is nonetheless retained in the ER (19 and Sester, M., and H.-G. Burgert, unpublished data). Deletion of the dilysine motif did not diminish the ability of E3/19K to induce NF-κB. This identifies the NF-κB activating ER-stress signal as the accumulation of proteins in the organelle. These data also explain why other proteins such as influenza hemagglutinin (40), immunoglobulin μ heavy chains (41) and MHC class I molecules (Fig. 4), which do not possess dilysine retention motifs but can nonetheless accumulate in the ER, and activate NF-κB. We have suggested that "ER overload", the congestion of the ER membrane with too many proteins, activates a signal transduction pathway which induces NF-κB. In this model, expression of E3/19K proteins which accumulate in the ER, causes ER overload. E3/19K mutants which are not retained in the ER do not significantly accumulate and therefore do not cause ER overload.

Two lines of evidence suggest that ER stress triggers the release of Ca^{2+}, which acts as a second messenger in the activation of NF-κB. First, E3/19K-mediated NF-κB activation can be inhibited by pretreatment of cells with the intracellular calcium chelator TMB-8. Second, inhibitors of the ER-resident Ca^{2+} ATPase, which cause a rapid release of Ca^{2+} from the ER, are potent activators of NF-κB. It has recently been shown that treatment of peritoneal macrophages with thapsigargin and CPA induces a rapid and dramatic increase in IL-6 mRNA expression and IL-6 secretion (8). In these cells, IL-6 transcription increases 10-fold after 15 min of treatment with thapsigargin. Since inducible IL-6 transcription is mediated by NF-κB (34), these data are explained by our demonstration of a rapid NF-κB activation in response to thapsigargin and CPA. Further studies will investigate whether the accumulation of proteins during ER overload elicits Ca^{2+} release by inhibiting the Ca^{2+}-ATPase. In this way, the enzyme might serve as the ER stress sensor.

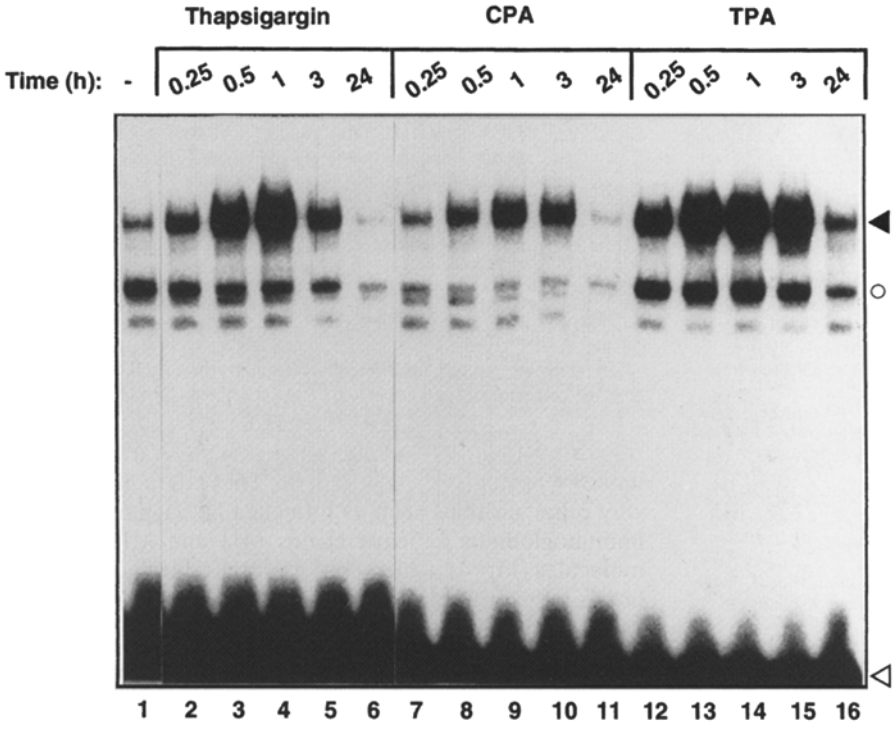


Figure 11. NF-κB is activated by two inhibitors of the ER-resident Ca^{2+} ATPase, thapsigargin, and cyclopiazonic acid. HeLa cells were treated with either 15 μM thapsigargin (lanes *2–6*), 75 μM cyclopiazonic acid (CPA, lanes 7–12), or 50 ng/ml TPA (lanes *13–16*) for various times as indicated. Treatment time is noted in hours. Equal amounts of protein from cell extracts were analyzed for NF-κB activity by EMSA. A filled arrowhead indicates the position of NF-κB DNA complexes. The open circle denotes a nonspecific activity binding to the probe and the open arrowhead shows unbound oligonucleotide.

TNF-mediated NF-κB activation is not inhibited by TMB-8. In contrast, we recently reported that NF-κB induction by the phosphatase inhibitor okadaic acid (OA) is also inhibited by preincubation with the Ca^{2+} chelator TMB-8 (44). OA has also been shown to disrupt ER function (35). Most likely, OA induces NF-κB by eliciting ER stress, rather than by inhibition of phosphatases, as was previously thought. NF-κB–activating agents can thus be divided into two classes: inducers such as TNF, which are not inhibited by intracellular Ca^{2+} chelators, and agents including phosphatase inhibitors and ER overload, which require intracellular Ca^{2+} release. With the exception of T cells, Ca^{2+} ions have not previously been implicated in NF-κB activation. In T cells, Ca^{2+} ionophores stimulate NF-κB very weakly on their own, but act synergistically with PMA to induce the transcription factor (48). By mobilizing intracellular Ca^{2+}, ER stress thus uses a novel messenger for NF-κB activation.

It has previously been shown that adenovirus infection induces TNF production in mice (21) and that TNF stimulates E3 protein expression (15, 31). What role may adenovirus E3/19K-mediated NF-κB activation play in the life cycle of the virus and the infected cell? NF-κB stimulates transcription of MHC class I genes (28, 43). This counteracts the effect of E3/19K, which binds MHC class I in the ER to prevent its surface expression. However, the adenovirus E3 promoter contains two functional NF-κB sites (reference 51, Deryckere, F., and H.-G. Burgert, manuscript in preparation). Activation of NF-κB thus increases transcription of both the E3/19K and MHC class I genes. This leads to the accumulation of more proteins in the ER, thereby eliciting more ER stress, leading to increased NF-κB activation and yet increased E3/19K and MHC class I expression. This is an example of a mutual adaptation of virus and host which may culminate in a chronically enhanced level of NF-κB activity and κB controlled gene expression. We have now shown that three structurally unrelated viral membrane proteins activate NF-κB: the truncated virion protein MHBS^t of HBV, wild-type hemagglutinin of influenza virus and wild-type E3/19K of adenovirus (37, 40). Since the transcription factor is known to induce genes for interferon and inflammatory cytokines in addition to MHC class I, ER overload by viral membrane proteins might represent a generalized antiviral response of the cell. Because the pathway is simply elicited by the accumulation of viral proteins in the ER membrane, it has very broad specificity. A cell would not need a specific mechanism to recognize a particular virus, but simply sense the ER stress caused by the novel production of secretory viral proteins. By activating NF-κB, a central mediator of the immune response, a fast and extremely efficient antiviral response is achieved. In the case of adenovirus and HIV-1, the virus has adapted to this response by selecting NF-κB motifs for regulation of its own protein transcription, thereby eventually counteracting the protective effects of NF-κB activation.

This work was supported by grants from the Deutsche Forschungsgemeinschaft (Ba-957/1-3) and the European Community Biotechnology Programme.

Received for publication 26 June 1995 and in revised form 2 October 1995.

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