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A novel signal transduction pathway from the endoplasmic reticulum to the nucleus is mediated by transcription factor NF-κB

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The inducible, higher eukaryotic transcription factor NF-κB is activated by a variety of external stimuli including inflammatory cytokines, viral and bacterial infection and UV irradiation. Here we show that internal stress, caused by the accumulation of proteins in the endoplasmic reticulum (ER), also induces NF-κB DNA binding as well as κB-dependent gene expression. This was observed upon expression of immunoglobulin μ chains in the absence of light chains and by treatment of cells with several agents known to cause ER stress, such as tunicamycin, brefeldin A, 2-deoxyglucose and thapsigsargin. The transcription factor AP-1 was weakly induced under similar conditions. Overexpression of NF-κB subunits did not influence expression of the gene encoding grp78/BiP, a protein induced by various forms of ER stress. Likewise, the glucosidase inhibitor castanospermine, which induced grp78/BiP expression, failed to activate NF-κB, while the antioxidant dithiothreitol augmented grp78/BiP expression but prevented activation of NF-κB. Hence, NF-κB participates in a novel ER–nuclear signal transduction pathway distinct from the unfolded-protein-response described previously. We provide evidence that the ER can produce at least two distinct signals in response to a functional impairment. One is emitted by the presence of unfolded proteins, the other in response to overloading of the organelle, for example through the overexpression of secretory proteins.

Key words: ER/μ chain/NF-κB/PDTC/protein secretion

Introduction

Cells respond to a variety of external and internal stimuli by modifying their pattern of gene expression. This is mediated by inducible transactivating factors, which are activated in response to stimulation and subsequently induce or repress transcription of their target genes. While most inducible transcription factors described so far respond to external stimuli, such as a cytokines and hormones, little is known about factors which respond to internal stimuli, for example the stress caused by the accumulation of proteins in the endoplasmic reticulum (ER). Some newly synthesized proteins are processed through the ER, where they are folded and become glycosylated. These processes can be disturbed, for instance by the presence of mutant proteins, which are not folded correctly and accumulate within the ER (Kozutsumi *et al.*, 1988). Likewise, the expression of immunoglobulin μ chains in the absence of light chains leads to an accumulation of heavy chains in the ER (Thorens *et. al.*, 1985). Finally, a variety of pharmacological agents, such as tunicamycin (Duksin and Mahoney, 1982), brefeldin A (Misumi *et al.*, 1986) and 2-deoxyglucose (Resendez *et al.*, 1986) have been shown to lead to protein accumulation, either by inhibiting glycosylation (Elbein, 1987) or by blocking protein transport out of the ER (Fujiwara *et al.*, 1988). We will refer to these situations collectively as ER stress.

Cells respond to ER stress by inducing novel gene expression. Consequently, a signal must be transduced from the ER to the nucleus, which activates transcription in response to ER stress. Although fairly little is known about ER–nuclear signal transduction in mammalian cells, this pathway has been studied extensively in yeast. The presence of malfolded proteins in the ER activates a pathway termed the 'unfolded-protein-response' (UPR) (Shamu *et al.*, 1994). Several genes are activated by this pathway, for example, a family of ER-resident proteins called the glucose-responsive proteins (grps) (Kozutsumi *et al.*, 1988). These genes carry a conserved 28 bp element called the 'unfolded-protein-response element' (UPRE) in their promoters (Chang *et al.*, 1989). In yeast, a protein called the unfolded-protein-response factor binds this sequence in gel shift assays (Mori *et al.*, 1992). However, it has not been identified or characterized in detail. A constituitive human protein, p70CORE, has been shown to bind the grp78 UPRE, however, its function has not been investigated (Li *et al.*, 1994). Recently, two groups used a genetic screen to identify yeast mutants incapable of activating the UPR. An ER-resident protein kinase called IRE-1/ERN-1 was cloned, whose kinase activity is essential for ER nuclear signal transduction (Cox *et al.*, 1993; Mori *et al.*, 1993). However, neither the signal which activates the kinase nor its targets have been identified.

The inducible transcription factor NF-κB is present in an inactive, cytoplasmic form in most cell types (Baeuerle and Baltimore, 1988). Upon stimulation of cells with a large variety of pathological agents, such as inflammatory cytokines, UV irradiation as well as bacterial and viral infection, NF-κB activity is induced (for a review see Baeuerle and Henkel, 1994). Activation ocurrs via phosphorylation and proteolytic degradation of IκB, the inhibitory subunit (Beg *et al.*, 1993; Brown *et al.*, 1993; Henkel *et al.*, 1993; Sun *et al.*, 1994; Traenckner *et al.*, 1994). The released NF-κB dimer rapidly translocates to the nucleus, where it activates transcription of target genes such as interferons, cytokines, cell adhesion molecules and hematopoetic growth factors. Activation of NF-κB by all inducers tested to date is inhibited by a variety of

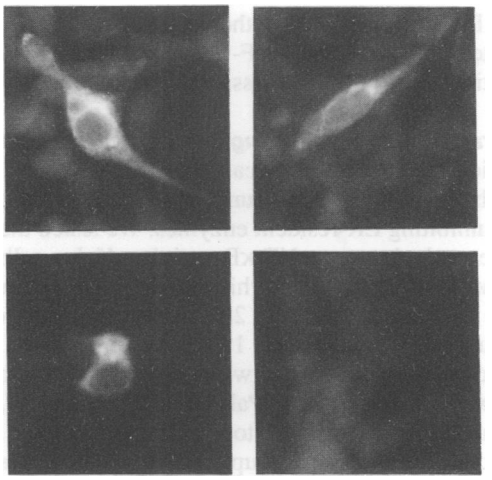

Fig. 1. Expression and subcellular distribution of Ig μ chains in 293 cells. 293 cells were transfected with 6 μg of a μ chain expression plasmid (top right and left and bottom left) or with 6 μg of an empty control vector (bottom right). Fourty eight hours after transfection, cells were fixed and stained with a FITC-conjugated goat anti-mouse IgM antibody. Photographs of fluorescence microscopy are shown enlarged 400 times.

antioxidants (reviewed in Schreck *et al.*, 1992). This finding, together with the observation that μM-concentrations of H_2O_2 activate NF-κB in some cell lines, suggests that reactive oxygen intermediates (ROIs) act as second messengers during NF-κB activation (Schreck and Baeuerle, 1991; Schreck *et al.*, 1991; Schmidt *et al.*, 1995).

We show here that a variety of agents which induce ER stress activate the transcription factor NF-κB. Since the activated NF-κB was capable of translocating to the nucleus and stimulating target gene transcription, this establishes a direct ER–nuclear signal transduction pathway. We provide evidence that NF-κB is unlikely to mediate the previously described UPR pathway. Hence, NF-κB mediates a novel signal transduction pathway connecting the ER and the nucleus in mammalian cells.

Results

Expression of the immunoglobulin μ chain activates NF-κB

In order to investigate whether the transcription factor NF-κB is activated by ER stress, we transfected 293 cells with an expression vector for the immunoglobulin μ chain. Lenny and Green (1991) have shown that in the absence of light chains, immunoglobulin μ chains are unable to exit from the ER; they therefore accumulate and cause ER stress. Since COS cells were used in these experiments, we first tested whether transfection of a μ chain expression vector also causes the protein to accumulate in the ER of 293 cells. Cells were plated onto coverslips 24 h prior to transfection with either a μ chain expression vector or an empty vector. Fourty eight hours after transfection, the cells were fixed and stained with a FITC-conjugated goat anti-mouse μ chain antibody. Figure 1 shows photographs of immunofluourescence microscopy. Control cells show only background staining, whereas cells transfected with the μ chain expression vector reveal a predominantly perinuclear and cytoplasmic localization of μ chain, consistent with the retention of this protein in the ER. We then tested whether NF-κB is activated in this system. 293 cells were transiently transfected with three different preparations of a μ chain expression plasmid. One plasmid preparation was purified using a $CsCl_2$ density gradient (Figure 2A, lanes 2, 5 and 6) and the other two were prepared on Quiagen columns (Figure 2A, lanes 3 and 4). This was done since it has been reported that plasmids

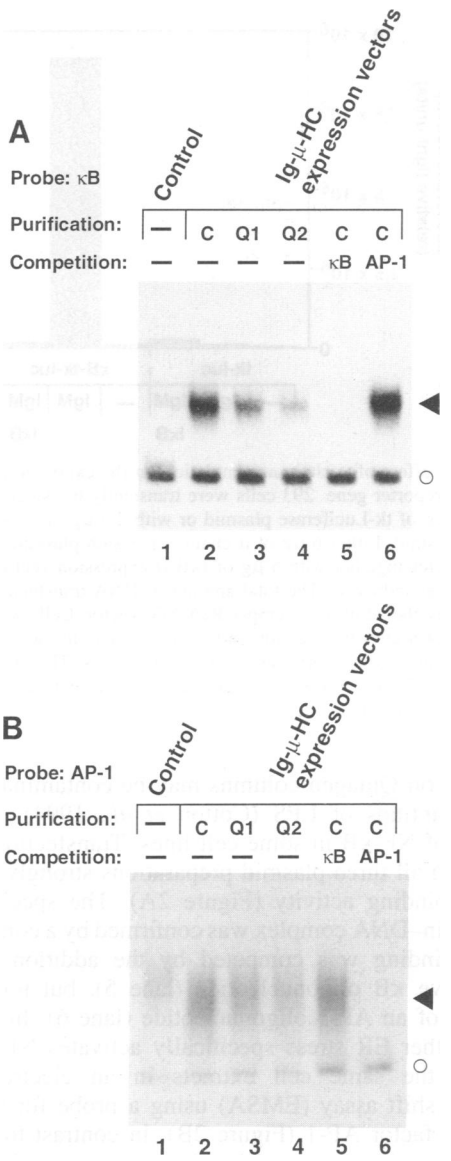

Fig. 2. The effect of μ chain expression on the activation of NF-κB and AP-1. 293 cells were transiently transfected with three preparations of μ chain expression vector. Twenty four hours after transfection, total cell extracts were prepared and analysed by EMSA using ^{32}P-labelled oligonulceotide probes containing either an NF-κB (**A**) or an AP-1 binding site (**B**). Lane 1, control cells transfected with empty vector; lanes 2, 5 and 6, cells transfected with 6 μg $CsCl_2$-purified μ chain expression vector; lanes 3 and 4, cells transfected with 6 μg of two different μ chain expression vector preparations purified on Quiagen columns; lane 5, extract incubated with a 50-fold molar excess of unlabelled NF-κB oligonucleotide; lane 6, extract incubated with a 50-fold molar excess of unlabelled AP-1 oligonucleotide. A section of a fluorogram is shown. The arrowheads point to the NF-κB and AP-1 complexes in (A) and (B), respectively. The open circle denotes a non-specific protein binding to the DNA probe.

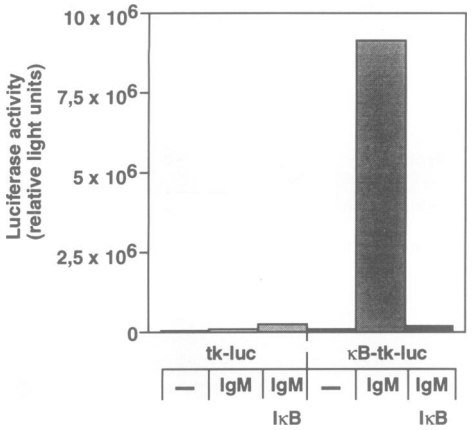

Fig. 3. The effect of μ chain accumulation on the expression of a κB-dependent reporter gene. 293 cells were transiently transfected with either 2.5 μg of tk-Luciferase plasmid or with 2.5 μg of 6x-κB-tk-Luc reporter plasmid. Either 6 μg of μ chain expression plasmid or 6 μg of μ chain vector together with 5 μg of IκB-α expression vector were co-transfected as indicated. The total amount of DNA transfected was equalized by the addition of empty Rc/CMV vector. Cells were harvested 24 h after transfection and luciferase activity determined. The results are given in arbitrary relative light units. The experiment was performed six times with similar results and a representative experiment is shown.

prepared on Quiagen columns may be contaminated with large quantities of LPS (Cotton *et al.*, 1994), a potent inducer of NF-κB in some cell lines. Transfection of 293 cells with all three plasmid preparations strongly induced NF-κB binding activity (Figure 2A). The specificity of the protein–DNA complex was confirmed by a competition assay. Binding was competed by the addition of non-radioactive κB oligonucleotide (lane 5), but not by the addition of an AP-1 oligonucleotide (lane 6). In order to test whether ER stress specifically activates NF-κB, we assayed the same cell extracts in an electrophoretic mobility shift assay (EMSA) using a probe for the transcription factor AP-1 (Figure 2B). In contrast to NF-κB, AP-1 is weakly activated by the expression of μ chain. The specific AP-1–DNA complex was identified by oligonucleotide competition (Figure 2B, lanes 5 and 6). Thus, ER stress induced by abberrant protein expression predominantly activates NF-κB.

We subsequently tested whether the NF-κB activated by μ chain expression is transcriptionally active. 293 cells were transfected with either a vector containing only the minimal tk promoter driving luciferase expression or with the same vector containing six κB sites upstream of the promoter. Two additional plasmids were co-transfected: the μ chain expression vector and an expression vector encoding IκB-α, the inhibitory subunit, which upon overexpression prevents NF-κB activation (Beg *et al.*, 1992). The total amount of DNA transfected was equalized by the addition of empty vector DNA, which does not affect NF-κB activity (data not shown). Cells were harvested 24 h after transfection and luciferase activity was determined. Transfection of the μ chain expression vector did not affect basal tk promoter activity (Figure 3). However, NF-κB-driven luciferase activity was increased 172-fold following transfection of μ chain. This induction was virtually abolished by the cotransfection of IκB-α, indicating that it was entirely dependent on NF-κB activation. Thus, ER stress caused by the accumulation of μ chains activates a functional NF-κB complex, capable of activating target gene expression in the nucleus.

Several ER stress-inducing agents activate NF-κB

A variety of pharmacological agents produce ER stress either by causing protein accumulation within the organelle or by inhibiting ER-resident enzymes. We tested the effect of five such drugs on NF-κB activity. HeLa cells were incubated with three drugs which lead to protein accumulation in the ER: tunicamycin, 2-deoxyglucose and brefeldin A (Duskin and Mahoney, 1982; Misumi *et al.*, 1986; Resendez *et al.*, 1986) as well as with the glucosidase inhibitor castanospermine (Palamarczyk and Elbein, 1985) and thapsigargin, an inhibitor of the ER-resident Ca^{2+}-dependent ATPase (Thastrup *et al.*, 1990). Cells were exposed to the compounds for 24 h after which cell extracts were prepared. Equal amounts of protein were analysed in an EMSA, using a ^{32}P-labelled high-affinity NF-κB binding site as a probe. Figure 4A (lanes 2–5) and C (lanes 2 and 3) show that incubation with tunicamycin, 2-deoxyglucose, brefeldin A and thapsigargin strongly induced the formation of a novel protein–DNA complex, while faster and slower migrating, constitutive activities either remained unchanged or were diminished. In contrast, no novel complex was induced in HeLa cells incubated with various concentrations of castanospermine (Figure 4B). Even concentrations of up to 1 mM castanospermine had no effect (data not shown).

Complexes binding the NF-κB sequence can consist of a variety of dimerized subunits, all members of the NRD (NF-κB-rel-dorsal) family of transcription factors (Grilli *et al.*, 1993; reviewed in Baeuerle and Henkel, 1994). The dimer composition can be analysed immunologically by adding antibodies to EMSA reactions. We analysed the subunit composition of 2-deoxyglucose-induced NF-κB (Figure 5). While anti-p50 antibody bound the entire NF-κB activity, causing it to migrate more slowly in the gel (lane 2), anti-p65 and anti-c-rel antibodies each only partially reduced the NF-κB binding activity (lanes 3 and 4). However, the binding activity was abolished entirely when both anti-p65 and anti-c-rel antibodies were added to the DNA binding reaction (lane 5). An antibody to IκB, the inhibitory subunit, was used as a control and affected neither the mobility nor the amount of NF-κB complex. An excess of non-radioactive κB oligonucleotide competed effectively for complex binding (Figure 5, lane 7), while an AP-1 oligonucleotide had no effect (lane 8), demonstrating that the complex binds specifically to the κB site. Taken together these data identify the ER stress-induced NF-κB as a mixture of p50/p65 and p50/c-rel heterodimers.

ER stress-mediated NF-κB induction has slower kinetics when compared with activation by external stimuli

Activation of NF-κB by agents such as PMA, TNF or IL-1 is very rapid, often occurring within minutes following stimulation (see, for instance, Henkel *et al.*, 1993). ER stress, however, is a slow, cumulative process which increases as more and more proteins accumulate in the ER over time. We therefore asked whether the kinetics of ER stress-induced NF-κB activation differ from those

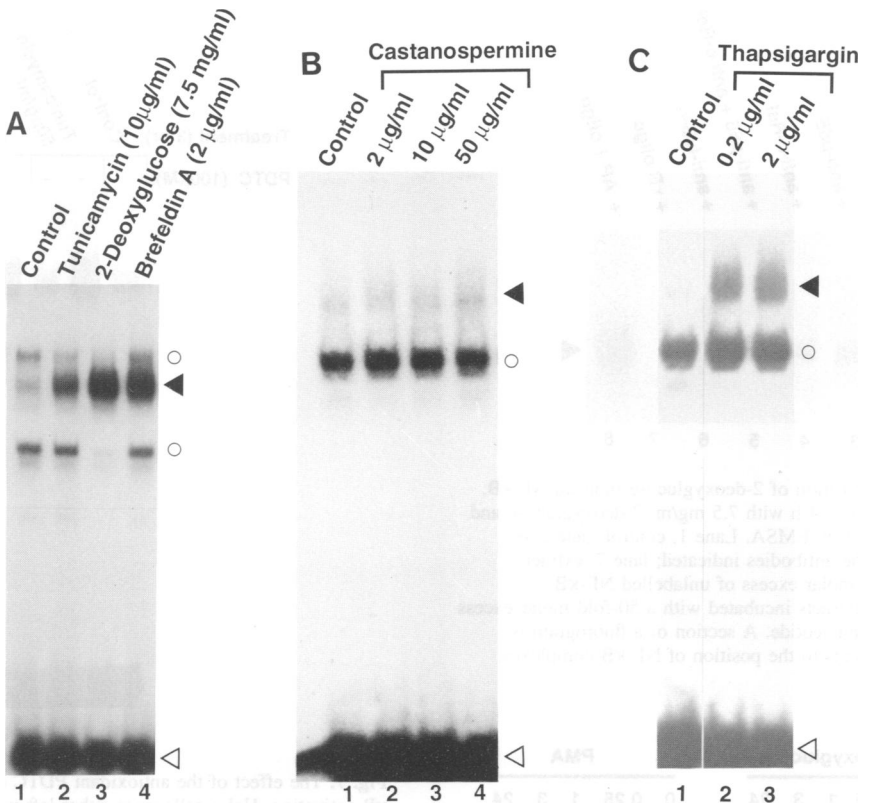

Fig. 4. The effect of ER stress-inducing agents on NF-κB activation. (**A**) HeLa cells were treated for 24 h with various agents at the concentrations indicated. Equal amounts of protein from cell extracts were analysed for NF-κB activity by EMSA. (**B**) The effect of castanospermine on NF-κB activation. Cells were treated for 24 h with the indicated concentrations of castanospermine. (**C**) The effect of thapsigargin on NF-κB activation. Cells were treated for 24 h with 0.2 μg/ml or 2 μg/ml thapsigargin. Sections of fluorograms are shown. A filled arrowhead indicates the position of NF-κB DNA complexes. The open circle denotes a non-specific activity binding to the probe and the open arrowhead shows unbound oligonucleotide.

observed following PMA stimulation. HeLa cells were treated either with 7.5 mg/ml of the glycosylation inhibitor, 2-deoxyglucose or with 50 ng/ml PMA, and harvested at various time points. Equal amounts of protein were analyzed in an EMSA. NF-κB was rapidly induced by PMA (Figure 6). An increase in NF-κB binding activity was detected as early as 15 min after treatment (lane 7). Maximal activation occurred 1–3 h after stimulation (lanes 8 and 9); after 24 h NF-κB binding activity was markedly reduced (lane 10). In contrast, 2-deoxyglucose-induced NF-κB activation first became detectable 3 h after treatment (lanes 1–4). It increased further with time and only reached levels comparable with 1 h of PMA stimulation after 24 h (lane 5). The slow and steadily increasing kinetics of 2-deoxyglucose-induced NF-κB activation are consistent with the hypothesis that the transcription factor was activated by the gradual accumulation of proteins in the ER.

ER stress-mediated NF-κB induction is inhibited by the antioxidant PDTC

Given the distinct kinetics of ER stress mediated NF-κB activation, we asked whether the activation occurs via a molecular mechanism related to that described for other NF-κB inducers. The effect of all previously characterized NF-κB-activating stimuli is inhibited by a variety of structurally unrelated antioxidants, suggesting the involvement of reactive oxygen intermediates (ROIs) as second messengers in the activation of the transcription factor (Schreck *et al.*, 1992; reviewed in Meyer *et al.*, 1994). We pre-incubated HeLa cells with 100 μM of the antioxidant pyrrolidinedithiocarbamate (PDTC) for 1 h prior to stimulation with ER stress inducers. Since PDTC does not retain its activity over 24 h, cells were incubated for 3 h with slightly higher doses of inducers than those used previously (compare Figure 4). Total cell extracts were prepared and equal amounts of protein analysed by EMSA. Pre-incubation with the antioxidant inhibited the NF-κB activation induced by all three ER stress-inducing agents tested (Figure 7). This suggests that protein accumulation in the ER may activate NF-κB via oxidative stress, a mechanism similar to that employed by other stimuli.

NF-κB does not influence expression of grp78/BiP

Expression of the grps, a family of ER-resident proteins, is induced by treatment of cells with tunicamycin, brefeldin A, 2-deoxyglucose, castanospermine and thapsigargin (Watowich and Morimoto, 1988; Price *et al.*, 1992) as well as by expression of μ chain (Lenny and Green, 1991). We tested whether the well-characterized grp protein, grp78, also known as the immunoglobulin chain binding protein, BiP, is transcriptionally activated by NF-κB. 293 cells were transfected with expression plasmids for the NF-κB subunits p50 and p65 as well as with the empty vector. RNA was harvested 24 h after transfection and 10 μg of total RNA analysed in a Northern blot. The blot

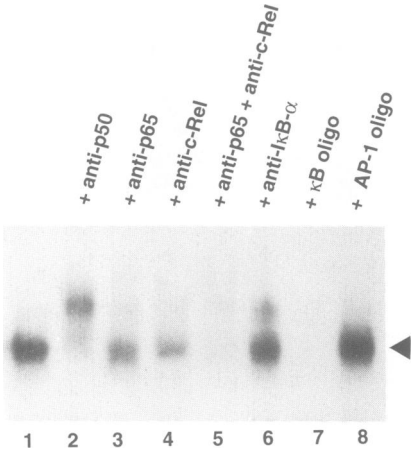

Fig. 5. The subunit composition of 2-deoxyglucose-induced NF-κB. HeLa cells were treated for 24 h with 7.5 mg/ml 2-deoxyglucose and total cell extracts analysed by EMSA. Lane 1, control; lane 2–6, extracts incubated with the antibodies indicated; lane 7, extract incubated with a 50-fold molar excess of unlabelled NF-κB oligonucleotide; lane 8, extracts incubated with a 50-fold molar excess of unlabelled AP-1 oligonucleotide. A section of a fluorogram is shown. An arrowhead points to the position of NF-κB complexes.

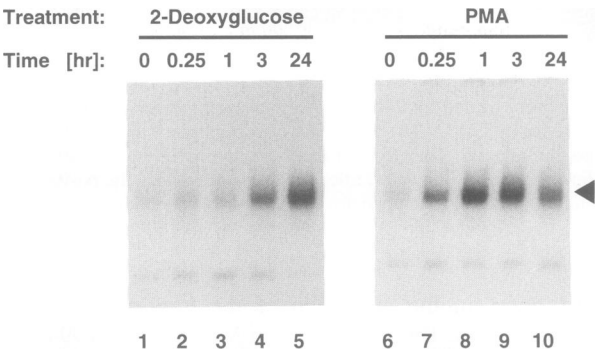

Fig. 6. Kinetics of NF-κB activation by 2-deoxyglucose and PMA. HeLa cells were treated for various times with either 7.5 mg/ml 2-deoxyglucose (lanes 1–5) or 50 ng/ml PMA (lanes 6–10) and total cell extracts analysed by EMSA. A section of a fluorogram is shown. A arrowhead points to the position of the NF-κB complex.

was probed either with the cDNA for IκB-α (Figure 8, lanes 4–6), a known NF-κB target gene (de Martin *et al.*, 1993; Sun *et al.*, 1993), or with a 950 bp fragment of the murine grp78/BiP cDNA (Figure 8, lanes 1–3). Since the NF-κB p65 subunit contains the transactivating domain of the transcription factor and can bind DNA as a homodimer, its expression is sufficient to induce the transcription of NF-κB target genes (Schmitz and Baeuerle, 1991). In contrast, a p50 homodimer has no transactivating potential and represses transcription. Accordingly, IκB-α mRNA levels were increased by transfection of the p65 expression vector (Figure 8, lane 5), but not by transfection of either the p50 expression vector or empty vector (lanes 4 and 6). In contrast, the level of grp78/BiP mRNA was not increased by p65 overexpression (lane 2), neither was it reduced by transfection of the p50 expression plasmid (lane 3). Thus, NF-κB appears not to regulate transcription of the grp78/BiP gene.

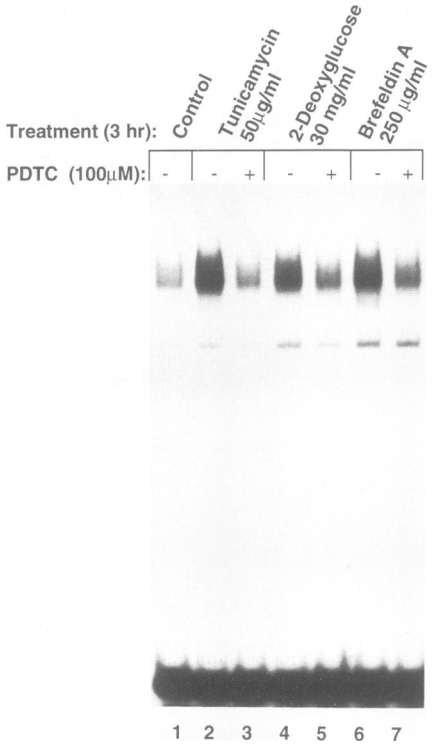

Fig. 7. The effect of the antioxidant PDTC on ER stress-mediated NF-κB activation. HeLa cells were either left untreated (lanes 1, 2, 4 and 6) or pre-treated for 1 h with 100 μM PDTC (lanes 3, 5 and 7). Subsequently, cells were stimulated with ER stress-eliciting agents at the indicated concentrations for 3 h. Equal amounts of protein obtained from total cell extracts were used in an EMSA. A fluorogram of a native gel is shown.

Several stimuli discriminate between NF-κB and BiP activation: evidence for a novel signal transduction pathway

Besides the observation that NF-κB fails to activate grp78/BiP transcription, a second result already suggested that NF-κB mediated ER–nuclear signalling is distinct from the previously described UPR: the glucosidase inhibitor castanospermine has been shown to induce grp78/BiP transcription (Watowich and Morimoto, 1988), but failed to activate NF-κB (Figure 4B). We thus directly compared the effect of castanospermine on the induction of grp78/BiP and IκB mRNA. In addition, we investigated the effect of TNF, a potent inducer of NF-κB, on the transcription of these genes. HeLa cells were treated for 4 h with either 50 μg/ml castanospermine or with 200 U/ml TNF. Since NF-κB activation is inhibited by the antioxidant and reducing agent dithiothreitol (DTT) (Pahl and Baeuerle, 1995), but grp genes are induced by reducing agents (Kim *et al.*, 1987), we pre-incubated two samples with 5 mM DTT for 1 h prior to stimulation. Total cell extracts were prepared and assayed for NF-κB activity by EMSA (Figure 9, top panel). In addition, RNA was harvested and analysed in a Northern Blot (Figure 9, lower panel). The blot was probed either with the IκB cDNA or with the cDNA for grp78/BiP. Equal quality and loading of the RNA was verified by hybridization to the cDNA for the glycolytic enzyme GAPDH. As shown above (see Figure 4B), castanospermine failed to activate NF-κB (Figure 9, lane 2). In contrast, TNF strongly induces NF-κB (Figure 9, lane 4). Pre-incubation with DTT prevents NF-κB induction by

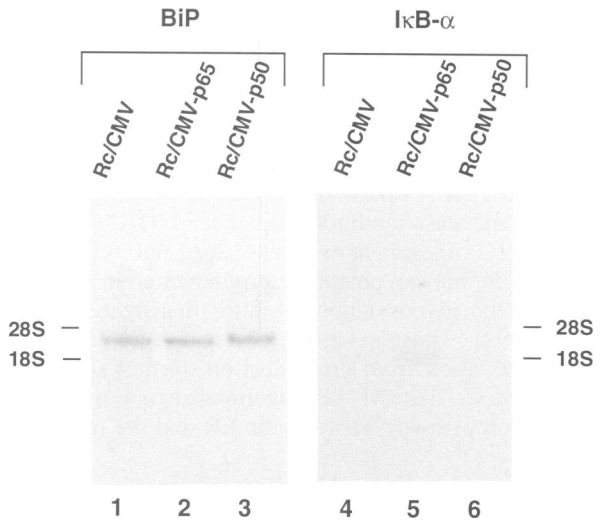

Fig. 8. The effect of overexpressing NF-κB subunits on the mRNA levels of grp78/BiP and IκBα. Northern blots were prepared using 10 μg total cellular RNA isolated from 293 cells transfected with 6 μg of either empty Rc/CMV vector or expression vectors for the NF-κB subunits p50 and p65 as indicated. Lanes 1–3 were probed with a 950 bp *Eco*RI fragment of the murine grp78/BiP cDNA; lanes 4–6 were probed with the complete human IκB-α cDNA. A fluorogram of the filter is shown. The positions of the 28S and 18S ribosomal RNAs are indicated.

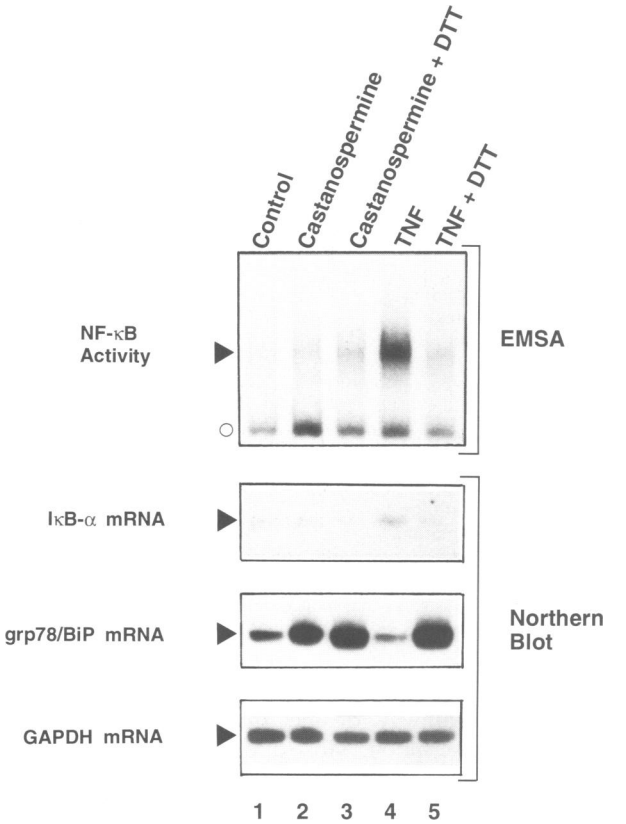

Fig. 9. The effect of castanospermine, TNF and DTT on the activation of NF-κB and the mRNA levels of grp78/BiP and IκBα. (Top panel) total cell extracts were prepared from untreated HeLa cells (lane 1) or cells treated for 4 h with either 50 μg/ml castanospermine (lanes 2 and 3) or 200 U/ml TNF (lanes 4 and 5). Cells in lanes 3 and 5 were pre-treated with 5 mM DTT for 1 h prior to stimulation. Cell extracts were analysed for NF-κB activity by EMSA. A section of a flourogram is shown. An arrowhead points to the position of NF-κB complexes and the open circles denote a non-specific activity binding to the DNA probe. (Bottom panels) a Northern blot was prepared using 10 μg of total cellular RNA isolated from HeLa cells treated as described above. As indicated, the blot was probed with the complete human IκB-α cDNA, a 950 bp *Eco*RI fragment of the murine grp78/BiP cDNA and with the complete cDNA for human GAPDH.

TNF (Figure 9, lane 5). Consistent with these observations, the mRNA for IκB is only induced by treatment with TNF (Figure 9, lane 4); induction does not occur in the presence of castanospermine or after pretreatment with DTT. In contrast, grp78/BiP mRNA is potently induced by castanospermine (Figure 9, lane 2), and this induction is augmented by pre-treatment with DTT (Figure 9, lane 3). TNF stimulation fails to induce grp78/BiP mRNA, but pretreatment with DTT activates the gene (Figure 9, lanes 4 and 5). Thus, the UPR gene grp78/BiP and the NF-κB target gene IκB are regulated inversely by castanospermine, TNF and DTT, confirming that NF-κB mediated ER–nuclear signalling is not triggered by the same stimuli as the previously described UPR but represents a novel pathway.

Discussion

The accumulation of unfolded or incorrectly processed proteins in the ER impairs the function of the organelle and thereby represents a threat to the cell. One response to this challenge is the induction of a family of genes encoding the grps. This family includes three ER-resident proteins, grp94, grp78/BiP and ERp72, which assist in protein folding (reviewed in Gething and Sambrook, 1992). Expression of grp proteins is induced by various ER stress-eliciting agents such as tunicamycin, brefeldin A, 2-deoxyglucose, castanospermine and thapsigargin (Watowich and Morimoto, 1988; Price *et al.*, 1992) as well as by the accumulation of mutant proteins in the ER (Kozutsumi *et al.*, 1988) and the expression of immunoglobulin μ chains (Lenny and Green, 1991). The promoters for grp78 and grp94 have been cloned from various species and show striking homology in a 28 bp region, which partially mediates ER stress inducibility (Chang *et al.*, 1989). Recently, a constituitive factor, p70CORE, was partially purified and shown to bind to this region in the human grp78 promoter (Li *et al.*, 1994). However, binding is unaltered by ER stress and its functional significance remains to be investigated. With the exception of a CCAAT motif, which binds members of the constitutive CTF/NF-1 family, no transcription factors mediating grp78 expression have been described (Wooden *et al.*, 1991). Moreover, deletion analysis and linker scanning of the rat grp78 promoter did not reveal any single element reponsible for ER stress-mediated induction of transcription (Wooden *et al.*, 1991). Rather, the promoter must contain multiple, functionally redundant elements.

Several stimuli distinguish between NF-κB and UPR mediated ER–nuclear signalling

In this study we have shown that the transcription factor NF-κB is induced under various conditions which promote the accumulation of proteins in the ER. It is thus tempting to speculate that NF-κB transduces the signal between the ER and the nucleus, which activates transcription of grp genes. However, we will discuss evidence that NF-κB-

Table I. Various stimuli discriminate between the activation of the UPR and the NF-κB pathways

Stimulus	Activation of:	
	UPR pathway	NF-κB pathway
2-Deoxyglucose	+	+
Brefeldin A	+	+
Tunicamycin	+	+
Thapsigargin	+	+
Heavy chain expression	+	+
Calcium ionophores	+	-/+
2-Mercaptoethanol	+	-
Dithiothreitol	+	-
Castanospermine	+	-
Okadaic acid	-	+
Cycloheximide	-	+
Tumor Necrosis Factor α	-	+
p65 overexpression	-	+
Expression of wild-type Influenza haemagglutinin	-	+

Three groups of inducers can be distinguished: (i) agents that activate both pathways, (ii) agents that induce only the UPR or (iii) the NF-κB pathway. For references see text.

mediated ER–nuclear signalling represents a novel signal transduction pathway, distinct from that which activates grp gene transcription. Most direct evidence for a novel signal transduction pathway comes from comparing the effects of castanospermine, TNF and DTT on the UPR and NF-κB pathways. While the UPR, measured by grp78/BiP transcription, is activated by castanospermine and DTT, these agents neither activate NF-κB DNA binding nor induce transcription of the NF-κB target gene IκB. In fact, DTT inhibits NF-κB activation. Conversely, TNF activates NF-κB but fails to induce grp78/BiP transcription. Several other conditions discriminate between NF-κB activation and induction of the UPR (see Table I). For example, overexpression of the NF-κB subunit p65, which activates transcription of the NF-κB target gene IκB-α, does not induce an increase in grp78/BiP mRNA levels. Likewise, expression of p50 does not decrease mRNA levels. This is consistent with the observation that the published promoter sequences of grp94 and grp78/BiP (Ting and Lee, 1988; Chang *et al.*, 1989) do not contain any sequences with homology to known NF-κB binding sites.

The ER stress-eliciting agents used in this study can be divided into three groups according to their effect on NF-κB activation and induction of the UPR. We have summarized these data in Table I. While several factors such as overexpression of the μ chain, tunicamycin, 2-deoxyglucose, brefeldin A and thapsigargin activate both pathways, castanospermine and DTT activate the UPR but not the NF-κB pathway, and TNF and p65 overexpression activate NF-κB but do not trigger the UPR. Several additional stimuli which discriminate between the UPR and the NF-κB pathways have been described. For instance, there are inducers of grp expression that do not activate NF-κB. Grps are induced by calcium ionophores (Wu *et al.*, 1981; Lee *et al.*, 1984) and by 2-mercaptoethanol (Kim *et al.*, 1987). Calcium ionophores only have a co-stimulatory effect on NF-κB in T cells (Tong-Starksen *et al.*, 1989) while 2-mercaptoethanol inhibits NF-κB activation (Schreck *et al.*, 1992). Conversely, there are inducers of NF-κB which do not induce grp expression and, therefore, act independently of ER stress. For example, grp induction depends on novel protein synthesis as it is inhibited in the presence of cycloheximide (Resendez *et al.*, 1986; Kim *et al.*, 1987). Since NF-κB induction is independent of protein synthesis, its activation is not blocked but rather enhanced by cycloheximide (Sen and Baltimore, 1986). Likewise, NF-κB is potently induced by the phosphatase inhibitor okadaic acid (OA) (Thévenin *et al.*, 1991). Treatment with OA does not activate grp transcription, but can potentiate grp induction in combination with the glycosylation inhibitor thapsigargin (Price *et al.*, 1992). Thus, a variety of conditions distinguish NF-κB activation from grp induction, further supporting the hypothesis that NF-κB functions in a novel signal transduction pathway between the ER and the nucleus.

ER overload mediates NF-κB activation

We have recently shown that expression of the influenza virion protein hemagglutinin (HA) induces NF-κB (Pahl and Baeuerle, 1995). The expression of wild-type HA does not activate grp transcription (Kozutsumi *et al.*, 1988). Only when mutant HA proteins, which cannot fold correctly, accumulate in the ER is grp transcription induced. The expression of glycosylation mutants in this system did not activate grp transcription, leading Gething and Sambrook (1992) to propose that the common primary signal for grp induction is the presence of malfolded proteins. By blocking glycosylation, agents such as tunicamycin and 2-deoxyglucose also elicit protein malfolding which may mediate the effect (Kozutsumi *et al.*, 1988). Hence, the pathway which activates grp transcription in reponse to ER stress has been named the unfolded-protein-response. NF-κB mediated ER–nuclear signalling is activated by a different, but partially overlapping, set of inducers suggesting that the ER stress signal activating NF-κB is different from that evoking the UPR. Overexpression of immunoglobulin μ chains and the influenza HA protein, that both glycoproteins are processed through the ER, but not overexpression of the unglycosylated, bacterial chloramphenicol acetyl transferase, induces NF-κB (Pahl and Baeuerle, 1995). As a result we propose that 'ER overload', the congestion of this organelle with protein, triggers NF-κB activation. By blocking transport out of the ER, the ER stress-inducing agents used in this study would also cause ER overload. The mechanism by which this ER stress triggers NF-κB activation is not clear. However, the signal most likely involves the production of ROIs, as these are required for NF-κB induction. The ER lumen, where the overload signal must originate, is an oxidizing environment and ER membranes contain cytochrome p450 enzymes, which are capable of generating ROIs. Likewise, the leakage of glutathione disulfide, a mild oxidant, or calcium from the overloaded organelle could cause oxidative stress.

Which physiological role does NF-κB activation by ER stress play? During viral infection, the cellular protein synthesis machinery is reprogrammed to produce large quantities of viral proteins, among them virion proteins such as HA, which are processed through the ER. Virus infection thus causes a sudden, drastic increase in the amount of proteins entering the ER, a situation which most likely leads to ER overload. By inducing NF-κB activity and consequently κB-dependent target gene

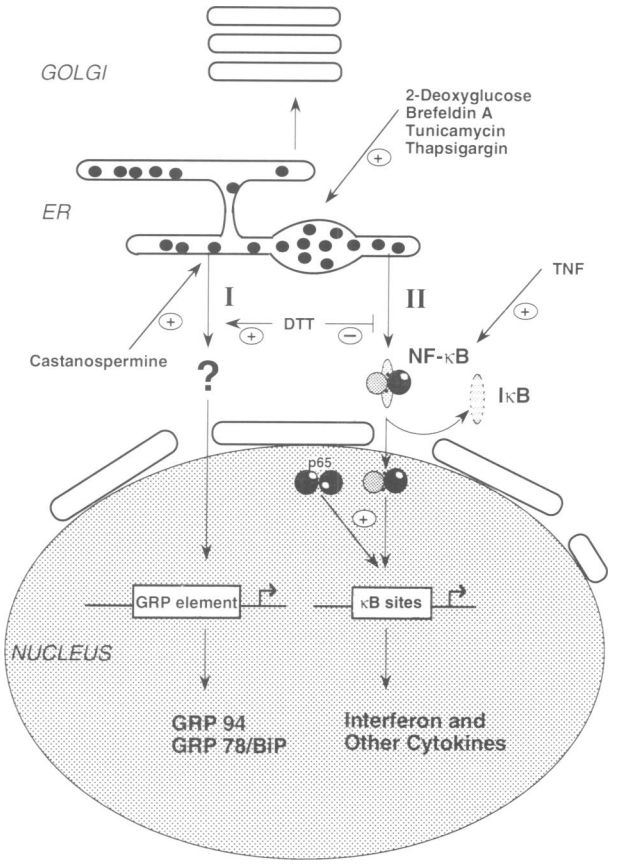

Fig. 10. The role of NF-κB in ER–nuclear signal transduction. The presence of malfolded proteins in the ER or treatment with castanospermine triggers pathway I, the 'UPR'. This leads to the transcription of the grp genes by an as yet unknown transcription factor. ER overload, a congestion of the organelle with excess proteins and TNF treatment activate NF-κB (pathway II) and result in the upregulation of NF-κB target genes, but not of grp78/BiP.

expression, ER overload may trigger the production of interferons and cytokines, which can act as antiviral agents. Therefore, ER stress-induced NF-κB activation may represent a simple, early, fast and effective antiviral response. We are currently investigating whether ER stress induces transcription of antiviral response genes.

Recent studies in the yeast *Sacchromyces cerevisiae* suggest a second possible role for ER stress induced NF-κB activity: the regulation of ER biosynthesis. Genetic screens in yeast have identified an ER-resident serine/threonine kinase called IRE-1/ERN-1, which is related to the cdc28/cdc2 family of protein kinases (Cox *et al.*, 1993; Mori *et al.*, 1993). Yeast cells carrying mutations in *ire-1/ern-1* fail to induce the UPR. Interestingly, *ire-1/ern-1* mutants are inositol auxotrophs. In yeast, the concentration of free inositol regulates membrane biosynthesis (Plautauf *et al.*, 1992). It has been proposed that the IRE-1/ERN-1 kinase controls membrane biosynthesis and by sensing the amount of unfolded proteins in the ER adjusts ER size to the current need (Shamu *et al.*, 1994). Higher eukaryotes are also capable of expanding their ER content, when the amount of protein requiring ER processing exceeds the capacity of this organelle (Schands *et al.*, 1973). In contrast to yeast, which appearently contain no NF-κB/IκB homologues (Moore *et al.*, 1993), higher eukaryotes have evolved two distinct ER–nuclear signalling pathways (see Figure 10). One responds specifically to the presence of unfolded proteins and activates grp transcription. A second pathway, mediated by NF-κB activation, may sense the amount of protein processed through the ER and signals ER overload. Such a signal could trigger increased ER biosynthesis to relieve the congestion in this organelle. It is interesting to note that mature B cells, which produce and secrete large amounts of immunoglobulins, increase their ER content and that these cells are among the few cell types which contain constitutively active NF-κB complexes (Sen and Baltimore, 1986). Whether NF-κB can control ER biosynthesis remains to be investigated.

Materials and methods

Cell culture and transfections

HeLa cells (ATCC # CCL 2) and 293 cells (ATCC # CRL 1573) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 50 μg/ml penicillin and streptomycin (all from Gibco-BRL). HeLa and 293 cells were plated 12–16 h prior to transfection at a density of 10^6 cells per 60 mm dish. Transfections were performed using calcium phosphate precipitation as previously described (Graham and van der Eb, 1973). The amounts of plasmids used are indicated in the figure legends. Tunicamycin, brefeldin A, 2-deoxyglucose, thapsigargin, castanospermine, DTT and PDTC were purchased from Sigma Biochemicals.

Plasmids

The μ chain expression plasmid was a kind gift of Dr M.Reth (Max-Planck-Institute, Freiburg). The murine BiP cDNA was generously given by Dr I.Haas (University of Heidelberg). 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 (Meyer *et al.*, 1993). 6x-κB-tk-Luc and the parental tk-Luc vector as well as the GAPDH cDNA were generous gifts of Dr Markus Mayer (EMBL, Heidelberg). The IκB expression vector Rc/CMV-IκB has been described previously (Zabel *et al.*, 1993). It contains the entire IκB-α cDNA inserted as a *Hin*dIII fragment into Rc/CMV. The parental Rc/CMV vector was purchased from Invitrogen.

Electrophoretic mobility shift assays

Total cell extracts were prepared using a high-salt detergent buffer (Totex) [20 mM HEPES, pH 7.9, 350 mM NaCl, 20% (w/v) glycerol, 1% (w/v) NP-40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 0.1% PMSF and 1% aprotinin]. Cells were harvested by centrifugation, washed once in ice-cold PBS (Sigma) and resuspended in four cell volumes of Totex buffer. The cell lysate was incubated on ice for 30 min, then 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), 2 μg poly(dI-dC) (Boehringer), 2 μl buffer D+ (20 mM HEPES; pH 7.9; 20% glycerine, 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 c.p.m. (Cerenkov) of a ^{32}P-labelled oligonucleotide in a final volume of 20 μl. The AP-1 binding reaction contained 5 mM $MgCl_2$ in addition to the above reaction mixture. 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 (Santa Cruz, CA). Anti-IκB antibody was prepared as described (Zabel *et al.*, 1993). NF-κB and AP-1 oligonucleotides (Promega) were labelled using [γ-^{32}P]ATP (3000 Ci/mmol; Amersham) and T4 polynucleotide kinase (Promega).

Luciferase assays

Cells were harvested 24 h post-transfection and luciferase activity determined precisely as described (Pahl *et al.*, 1991). The cell pellet obtained from one 60 mm dish was resuspended in 150 μl of lysis buffer [25 mM glycylglycine, 1% (v/v) Triton X-100, 15 mM $MgSO_4$, 4 mM EGTA, 1mM DTT] and centrifuged at 13 000 *g* at 4°C for 5 min. Fifty microlitres 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 a LB 96 P luminometer (EG & G-Bertold, Bad Wildbach, Germany). Light emission was measured over a 30 s interval and the results are given in relative light units.

Immunofluorescence staining

Cells were plated onto coverslips 24 h prior to transfection by the Ca-phosphate method (Graham and van der Eb, 1973). 48 h after transfection, cells were fixed for 5 min in 3.7% formaldehyde in PBS followed by a 1 min treatment in 0.2% (v/v) Triton X-100 in PBS. Non-specific binding was blocked by incubation in 10% fetal calf serum/PBS for 30 min. Cells were stained with a FITC-conjugated goat anti-mouse μ-chain specific antibody (Sigma), diluted 1:10 in PBS for 1 h. The stained coverslips were mounted onto slides and photographed using a Zeiss Axiovert 100 microscope.

Northern blots

Total cellular RNA was harvested using an acidic phenol extraction as previously described (Chomczynski and Sacci, 1987). 10 μg of RNA were loaded onto a 1% agarose gel cast in 1× MOPS buffer (20 mM MOPS, pH 7.0, 5mM NaOAc, 1mM EDTA) containing 6.6% formaldehyde. The RNA was transferred onto a nylon membrane (Hybond N+, Amersham) by capillary blotting and fixed by baking at 80°C for 1 h. The blot was hybridized in Church–Gilbert buffer (7% SDS, 0.5 M sodium phosphate, pH 7.2, 1% BSA, fraction V) at 65°C. IκB-α, BiP and GAPDH cDNAs were labelled using the Ready-to-Go labelling kit (Pharmacia) and [α-^{32}P]dCTP (Amersham). The blots were washed twice for 15 min in 2× SSC, 0.1% SDS at room temperature and twice at 65°C for 15 min in 0.2× SSC, 0.1% SDS.

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