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Phosphorylation of human IκB-α on serines 32 and 36 controls IκB-α proteolysis and NF-κB activation in response to diverse stimuli

E.Britta-Mareen Traenckner, Heike L.Pahl, Thomas Henkel[1], Kerstin N.Schmidt, Sherwin Wilk[2] and Patrick A.Baeuerle[3]

Institute of Biochemistry, Albert-Ludwigs-University, Hermann-Herder-Strasse 7, D-79104 Freiburg, Germany and [2]The Mount Sinai Medical Center, Department of Pharmacology, Box 1215, One Gustave Levy Place, New York, NY 10029–6574, USA
[1]Present address: Medigene GmbH, Lochhamerstrasse 11, D-82152 Martinsried, Germany

[3]Corresponding author

Post-translational activation of the higher eukaryotic transcription factor NF-κB requires both phosphorylation and proteolytic degradation of the inhibitory subunit IκB-α. Inhibition of proteasome activity can stabilize an inducibly phosphorylated form of IκB-α in intact cells, suggesting that phosphorylation targets the protein for degradation. In this study, we have identified serines 32 and 36 in human IκB-α as essential for the control of IκB-α stability and the activation of NF-κB in HeLa cells. A point mutant substituting serines 32 and 36 by alanine residues was no longer phosphorylated in response to okadaic acid (OA) stimulation. This and various other Ser32 and Ser36 mutants behaved as potent dominant negative IκB proteins attenuating κB-dependent transactivation in response to OA, phorbol 12-myristate 13-acetate (PMA) and tumor necrosis factor-α (TNF). While both endogenous and transiently expressed wild-type IκB-α were proteolytically degraded in response to PMA and TNF stimulation of cells, the S32/36A mutant of IκB-α remained largely intact under these conditions. Our data suggest that such diverse stimuli as OA, TNF and PMA use the same kinase system to phosphorylate and thereby destabilize IκB-α, leading to NF-κB activation.
Key words: IκB-α/NF-κB/phosphorylation/proteolysis

Introduction

The higher eukaryotic transcription factor NF-κB/Rel comprises a system of at least five distinct DNA binding subunits, called p50, p52, p65 (RelA), c-Rel and RelB (Blank *et al.*, 1992; Grilli *et al.*, 1993; Siebenlist *et al.*, 1995). These can dimerize in various combinations and were also found to associate with members of other transcription factor families, including the glucocorticoid hormone receptor and various basic leucine zipper proteins. A hallmark of the NF-κB/Rel family is their post-translational control by specific inhibitory subunits, called IκB proteins (Schmitz *et al.*, 1991; Beg and Baldwin, 1993; Gilmore and Morin, 1993). At least six species (IκB-α, IκB-β, IκB-γ, Bcl-3, p100, p105), which associate with NF-κB/Rel proteins in the cytoplasm or nucleus, are known. With the exception of a Bcl-3/p52 complex (Bours *et al.*, 1993), IκB interactions prevent DNA binding and nuclear transport of NF-κB/Rel proteins. The central means of regulating the transcription factor is by control of the protein–protein interactions between NF-κB/Rel and IκB proteins. Additional levels of regulation involve transcriptional up-regulation, splicing and cross talk with other transcription factors by protein–protein interaction (Liou and Baltimore, 1993).

p50/p65 (RelA) and p65/p65 dimer combinations of NF-κB/Rel factors are rapidly activated under numerous pathogenic conditions (Baeuerle and Henkel, 1994; Siebenlist *et al.*, 1995). This allows an immediate–early transcriptional response to viral and bacterial infections, UV light, inflammatory cytokines and proliferation stimuli. Numerous pathogen-induced genes are known to contain NF-κB binding motifs in their regulatory regions. In many instances, it has been shown that inducible expression of such genes crucially depends on intact NF-κB DNA binding motifs.

An IκB protein frequently associated with the p50/p65 and p65/p65 dimers is IκB-α. This inhibitor is rapidly degraded in response to diverse stimuli (Brown *et al.*, 1993; Cordle *et al.*, 1993; Henkel *et al.*, 1993; Mellits *et al.*, 1993; Sun *et al.*, 1994a). Loss of IκB-α correlates temporally with the activation of NF-κB. IκB-α degradation and NF-κB activation are prevented by peptide inhibitors of the proteasome (Palombella *et al.*, 1994; Traenckner *et al.*, 1994). Treatment of cells with these inhibitors leads to the accumulation of a phosphorylated IκB-α intermediate which shows a decreased mobility in SDS gels. IκB-α phosphorylation is not sufficient for activation of NF-κB because the modification does not release IκB-α from NF-κB (Finco *et al.*, 1994; Miyamoto *et al.*, 1994; Traenckner *et al.*, 1994; Alkalay *et al.*, 1995; DiDonato *et al.*, 1995).

In the present study, we have identified serines 32 and 36 in IκB-α as the residues whose phosphorylation causes the decreased mobility of IκB-α in response to various stimuli. Substitution of the serines by alanine residues creates an IκB-α mutant protein, which neither decreases in mobility nor rapidly degrades in response to extracellular stimuli. Various Ser32/36 mutants showed a significantly higher dominant negative effect on κB-dependent transactivation than wild-type IκB-α, or other IκB-α mutant proteins. The N-terminal portion of IκB-α, which contains the two phosphorylation sites, was previously found to be dispensable for both p65 binding and inhibition of NF-κB DNA binding and nuclear transport (Ganchi *et al.*, 1992; Inoue *et al.*, 1992; Hatada *et al.*, 1993). Here we show that it constitutes a regulatory domain, which responds to multiple extracellular signals and controls the inducible degradation of IκB-α.

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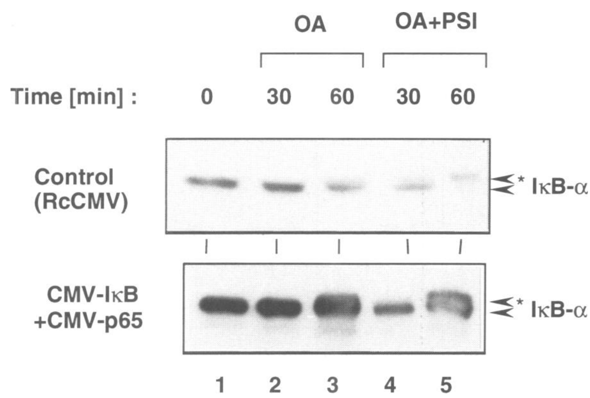

Fig. 1. Overexpressed IκB-α is phosphorylated but not significantly degraded in response to okadaic acid treatment of HeLa cells. HeLa cells were transfected with 5 μg of the control vector RcCMV (upper panel) or 5 μg of the expression vectors CMV-IκB-α and CMV-p65 (Schmitz and Baeuerle, 1991; Zabel *et al.*, 1993) (lower panel) and treated for the indicated times with 500 nM OA and 60 μM CBz-Ile-Glu(tBu)-Ala-Leu-CHO (proteasome inhibitor, PSI). Fifty micrograms (upper panel) and 20 μg (lower panel) of protein were subjected to reducing 10% SDS–PAGE. Lanes 4 and 5 received 50% of these amounts, respectively. After Western blotting, filters were probed with affinity-purified polyclonal anti-IκB-α IgG (Henkel *et al.*, 1993) and the bound IgG visualized by a second antibody and enhanced chemoluminesence labeling. The positions of endogenous and overexpressed IκB-α proteins are indicated by arrowheads. Newly phosphorylated variants are indicated by asterisks.

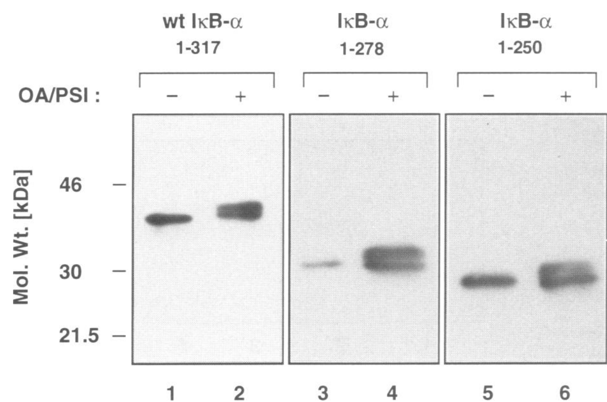

Fig. 2. C-terminally truncated IκB-α variants are inducibly phosphorylated. HeLa cells were transfected with 5 μg of the indicated CMV-controlled expression vectors for full-length and C-terminally truncated human IκB-α variants and either left untreated (lanes 1, 3 and 5) or treated with 500 nM OA and 60 μM PSI (lanes 2, 4 and 6). Sections of Western blots stained with anti-IκB-α are shown. The positions of molecular size standards are indicated at the left (ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 21.5 kDa).

Results

Highly overexpressed IκB-α is phosphorylated but not efficiently degraded in response to OA stimulation

Identification of the IκB-α sites which are phosphorylated in response to extracellular stimuli requires the analysis of mutant forms of the protein. We therefore tested whether transiently overexpressed IκB-α is phosphorylated in okadaic acid (OA)-induced HeLa cells, as was previously observed for the endogenous IκB-α (Traenckner *et al.*, 1994). Inducibly phosphorylated IκB-α is characterized by a decreased mobility in SDS–polyacrylamide gels. This form appears to be highly susceptible to degradation in intact cells because it is stabilized by treatment of cells with PSI, a peptide inhibitor of the proteasome.

Transfected HeLa cells were treated with the inducer OA in the presence of the proteasome inhibitor PSI. Total cell extracts were analyzed by Western blotting using an affinity-purified anti-IκB-α polyclonal IgG (Henkel *et al.*, 1993). After 60 min, all endogenous IκB-α in RcCMV-mock-transfected HeLa cells was present in a newly phosphorylated form (Figure 1, upper panel; lane 5). This variant was not observed in OA-treated cells in the absence of PSI (lane 3). As reported earlier (Traenckner *et al.*, 1994), the variant form can be completely converted into the faster migrating form by phosphatase treatment. When cells were induced with OA in the absence of PSI, ~50% of the endogenous IκB-α was depleted after 60 min, while no degradation was visible after a 30 min treatment (compare lanes 1–3).

In HeLa cells co-transfected with cytomegalovirus (CMV) enhancer-controlled expression vectors for IκB-α (and p65), the IκB-α signal in the Western blots was strongly increased (Figure 1, lower panel). The signal increase is underestimated because less protein was analyzed in the lower panel (see legend to Figure 1). The overexpressed IκB-α was not significantly degraded in response to OA alone (compare lanes 1 and 3). However, ~50% of the overexpressed IκB-α was converted into the more highly phosphorylated variant when a 60 min OA stimulation was performed in the presence of PSI (lane 5). Similar results were obtained when IκB-α was overexpressed in the absence of the CMV-p65 vector (see Figure 2), suggesting that IκB-α phosphorylation did not depend on p65. In conclusion, these data show that overexpressed IκB-α and mutants thereof can be analyzed for their phosphorylation status in response to inducing conditions.

C-terminal sequences of IκB-α are not the target for inducible phosphorylation

The C-terminal portion of human IκB-α contains destabilizing PEST sequences and a potential protein kinase C (PKC) phosphorylation site (Haskill *et al.*, 1991). As shown in Figure 2, overexpressed IκB-α mutants lacking either the 39 (aa 1–278; lane 4) or the 67 C-terminal amino acids (aa 1–250; lane 6) are still as efficiently phosphorylated in response to OA as is the full-length wild-type IκB-α when PSI is present (aa 1–317; lane 2). Because of the high level expression of transfected IκB-α, the endogenous IκB-α was not detectable in the exposure of the Western blot shown. The data indicate that IκB-α is inducibly phosphorylated either within the central ankyrin repeat domain or within its N-terminal portion. An N-terminally truncated form of IκB-α, which starts with the internal Met45, did not yield a more slowly migrating variant upon OA/PSI treatment (data not shown). We have therefore concentrated on analyzing the N-terminal portion by point mutations.

Substitution of serines 32 and 36 by alanines prevents the inducible phosphorylation of human IκB-α in response to OA

There are only three hydroxy amino acid residues within the N-terminal 44 residues of human IκB-α: Ser32, Ser36

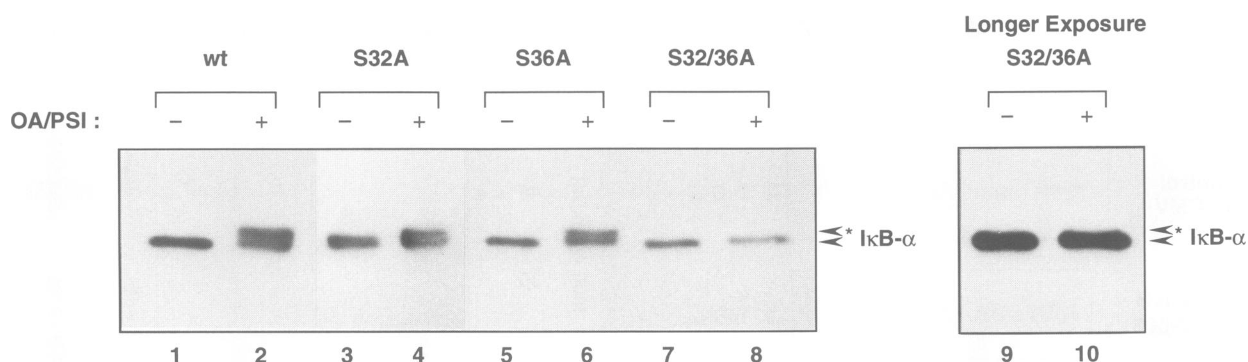

Fig. 3. A Ser32/36A mutant of IκB-α is no longer phosphorylated in response to OA treatment of HeLa cells. HeLa cells were transfected with 4 μg of the indicated CMV-controlled expression vectors for wild-type IκB-α or point-mutated variants and either left untreated (lanes 1, 3, 5 and 7) or treated with a combination of 500 nM OA and 60 μM PSI (lanes 2, 4, 6 and 8). Sections of Western blots stained with anti-IκB-α are shown. Lanes 9 and 10 show a longer exposure of lanes 7 and 8. Arrowheads indicate the positions of overexpressed IκB-α proteins. The asterisks mark more slowly migrating phosphorylated variants.

and Tyr42. These are highly conserved in the IκB-α proteins from various species (Haskill *et al.*, 1991; Davis *et al.*, 1992; Tewari *et al.*, 1992; de Martin *et al.*, 1993) and represent consensus sites for either casein kinase II or tyrosine kinases, respectively. Using site-directed mutagenesis, Ser32 and Ser36 were substituted by non-phosphorylatable alanine residues. Both the S32A and the S36A mutant IκB-α proteins gave rise to more slowly migrating variants following OA/PSI treatment of HeLa cells (Figure 3, lanes 4 and 6). However, the mobilities of their newly phosphorylated forms appeared slightly enhanced compared with that of the overexpressed wild-type IκB-α (lane 2). Slower migrating variants were no longer evident with a S32/36A double mutant (lane 8), even upon longer exposure of the chemoluminescence-stained Western blot (lane 10). This indicates that IκB-α can be newly phosphorylated on both Ser32 and Ser36 in response to OA treatment of cells. Moreover, phosphorylation on either serine residue can take place independently from phosphorylation of the other serine residue.

Cells expressing mutant proteins in which Tyr42 was replaced by phenylalanine, Thr146 by glycine, Thr168 and Thr169 by alanine and glycine, respectively, Tyr251 and Ser252 by aspartic acid and isoleucine, respectively, or Thr291 by isoleucine all showed a more slowly migrating variant following OA/PSI treatment (data not shown).

Phosphorylation of IκB-α on serines 32 and 36 is necessary for NF-κB activation in response to OA, PMA and TNF

The functional significance of Ser32/36 phosphorylation for IκB-α inactivation and NF-κB activation was tested in transient transactivation assays. An initial problem was that (highly) overexpressed IκB-α was not efficiently degraded (see Figure 1) and, hence, did not allow activation of the endogenous NF-κB and transactivation of κB-dependent reporter genes. We therefore performed a titration analysis and found that transient transfection of 100 ng of wild-type CMV-IκB-α vector still allowed a significant induction of transcription from a thymidine kinase (tk) promoter-driven luciferase reporter plasmid controlled by six reiterated κB sites [$(\kappa B)_6$ tk luc]. In a typical experiment, transcriptional induction of $(\kappa B)_6$ tk luc in the absence of CMV-IκB-α was 37-fold for OA, 12-fold for phorbol 12-myristate 13-acetate (PMA) and 8.6-fold for tumor necrosis factor-α (TNF). In the presence of 100 ng CMV-IκB-α it was still 23-fold for OA, 7.2-fold for PMA and 5.3-fold for TNF. The wild-type IκB-α expressed from 100 ng CMV-IκB-α was degraded to a significant extent in response to PMA treatment of HeLa cells (see Figure 6, lane 4). This system allowed us to analyze the inhibitory activity as well as the stability of the IκB-α mutants in comparison with the wild-type IκB-α.

As shown in Figure 4A, the S32/36A double mutant exerted a dominant negative effect on κB-dependent transactivation. While the wild-type IκB-α still allowed a 23-fold induction of the reporter plasmid in response to OA, the induction was reduced by 65% in the presence of the transfected S32/36A mutant. S32A and S36A single mutants reproducibly showed a weaker effect than the double point mutant, suggesting that phosphorylation on both serine residues is necessary in order to optimally relieve the inhibition by IκB-α. Substitution of Ser32 and Ser36 with the phosphoserine-mimetic amino acid glutamate did not abolish the dominant negative effect. There are other examples where glutamate could not mimick the effect of serine phosphorylation (M.Karin, personal communication). The CMV-controlled wild-type and point mutant IκB-α expression vectors showed similar levels of IκB-α protein expression, as tested by Western blotting (see Figures 3 and 6). This shows that the dominant negative effect of the phosphorylation site mutants was not due to an increased expression of the IκB-α mutant proteins relative to the wild-type protein.

TNF and PMA are two other potent inducers of NF-κB in HeLa cells. As shown in Figure 4B and C, the S32/36A double mutant also revealed a significant dominant negative effect when κB-dependent transactivation was induced by PMA or TNF. This indicates that the same phosphorylation sites are used by PMA, TNF and OA to inactivate IκB-α. The S32A and S36A mutants also had a dominant negative effect. The S36A mutant showed the weakest inhibitory potential, suggesting that phosphorylation of Ser32 was slightly more critical for inactivation of IκB-α than phosphorylation of Ser36. Substitution of Ser32 and Ser36 with glutamate again did not prevent the dominant negative effect but showed a slightly enhanced inhibition compared with the alanine substitutions. A

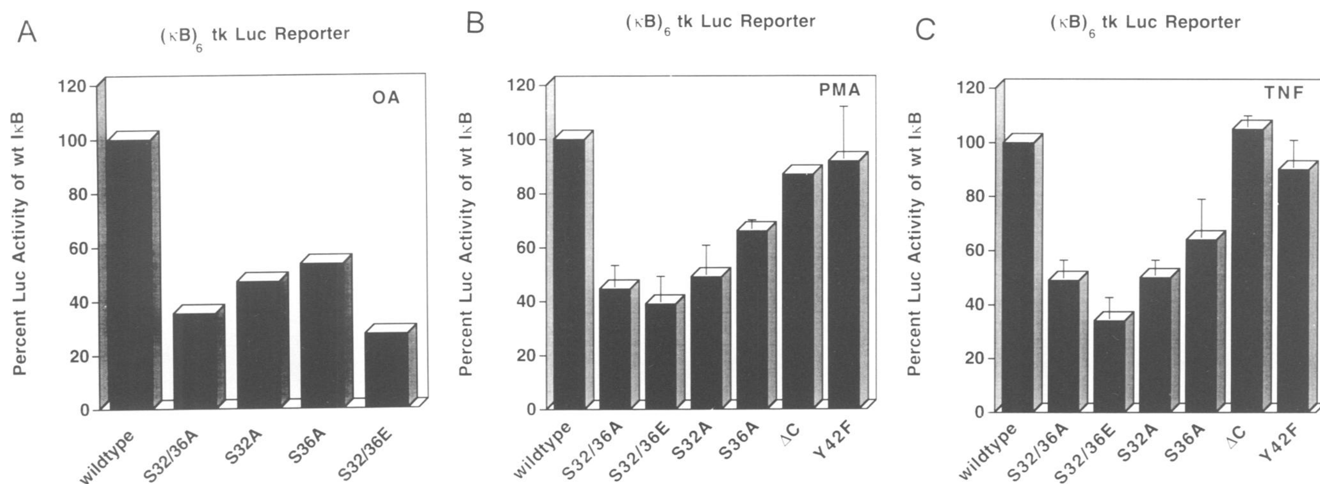

Fig. 4. The effect of S32/36 mutants on the expression of a κB-dependent reporter gene. (**A**) OA stimulation. HeLa cells were transfected with 100 ng of CMV-controlled expression plasmids encoding wild-type, S32/36A, S32A or S36A mutants together with 2.5 μg of a luciferase reporter gene controlled by a minimal tk promoter and six reiterated κB sites ($κB_6$ tk luc). Cells were stimulated for 8 h with 200 nM OA. Overexpression of wild-type IκB-α still allowed a 23-fold induction in response to OA. This value was set to 100%. Data from a typical transfection experiment is shown. (**B**) PMA stimulation. HeLa cells were left untreated or stimulated for 8 h with 100 ng/ml PMA. The 100% value corresponds to an induction of 7.2-fold. (**C**) TNF stimulation. HeLa cells were left untreated or stimulated for 6 h with 200 U/ml recombinant human TNF. The 100% value corresponds to an induction of 5.3-fold. Two additional IκB-α expression plasmids were tested with PMA and TNF as stimuli: Y42F and C-terminally truncated IκB-α (aa 1–278; ΔC). Mean values from 3–5 independent transfection experiments are given and standard deviations of the mean are indicated by error bars.

C-terminally truncated form (aa 1–278) and a Y42F point mutant of IκB-α allowed virtually the same transcriptional induction of the κB reporter plasmid as the wild-type IκB-α, consistent with the finding that all three proteins can still undergo inducible phosphorylation (see above). Overexpression of an IκB variant lacking the 67 C-terminal amino acids (aa 1–250) showed virtually no inhibitory activity when compared with mock-transfected cells (data not shown). This is consistent with its reduced p65 binding activity (Ganchi *et al.*, 1992; Ernst *et al.*, 1995).

To test for the specificity of the dominant negative effect of S32/36A, S32/36E, S32A and S36A IκB-α mutants, the PMA-dependent activity of three other reporter plasmids was tested in the presence of over-expressed IκB-α proteins. As expected from the absence of functional NF-κB sites, the overexpression of wild-type IκB-α did not affect the PMA inducibility of luciferase reporter genes controlled by a basal tk promoter (Figure 5A), a tk promoter driven by two copies of a serum response element from the c-*fos* enhancer (Figure 5B) or an AP-1-dependent collagenase promoter (Figure 5C). However, we were surprised to find that the PMA inducibility of all three reporter constructs was slightly enhanced when either S32/36A or S32/36E mutants were overexpressed. Likewise, S32A and, to a lesser extent, S36A had the same effect. This may be indicative of a weak negative cross talk between the PMA-controlled NF-κB and SRF/TCF/AP-1 pathways. At any rate, these data show that the inhibitory effect of IκB-α phosphorylation site mutants was highly specific for κB-dependent transactivation.

The IκB-α phosphorylation site mutant S32/36A has an increased resistance to PMA- and TNF-induced proteolytic degradation

One likely explanation for the dominant negative effect of the S32/36A mutant on κB-dependent gene expression is that the mutant protein is no longer degraded in response to stimulation of HeLa cells. To test this idea, HeLa cells were transiently transfected with 100 ng of the RcCMV control vector, wild-type CMV-IκB-α or S32/36A CMV-IκB-α vectors and equal amounts of cell extracts analyzed by Western blotting for the stability of IκB-α. The endogenous as well as the overexpressed wild-type IκB-α were significantly degraded in response to PMA treatment of cells (Figure 6, lanes 2 and 4). On the more highly resolving 7.5% polyacrylamide–SDS gel, the over-expressed S32/36A mutant protein could be distinguished from the endogenous wild-type protein by a slightly reduced mobility in SDS gels (lanes 5 and 7). Upon treatment of cells with either PMA (lane 6) or TNF (lane 8), the endogenous IκB-α was degraded, while the transfected S32/36A mutant protein remained largely intact. This shows that phosphorylation of IκB-α on Ser32 and Ser36 promotes the proteolytic degradation of IκB-α and explains the dominant negative effect of the mutant on κB-dependent transactivation by its superior stability.

Discussion

Phosphorylation and the control of NF-κB activity

The activity of many transcription factors is controlled by protein phosphorylation (Hunter and Karin, 1992). In most cases, direct phosphorylation of DNA binding subunits positively or negatively modulates DNA binding, dimerization or the activity of transactivation domains. The DNA binding subunits of NF-κB were also found to be phosphorylated in intact cells (Ostrowski *et al.*, 1991; Neumann *et al.*, 1992; Li *et al.*, 1994; Naumann and Scheidereit, 1994). In the case of p50, this promotes DNA binding (Donald *et al.*, 1995). PMA-inducible phos-phorylation of the TA_2 domain in the p65 C-terminus enhances its transactivating potential (Schmitz *et al.*, 1995). Phosphorylation of the NF-κB homolog dorsal

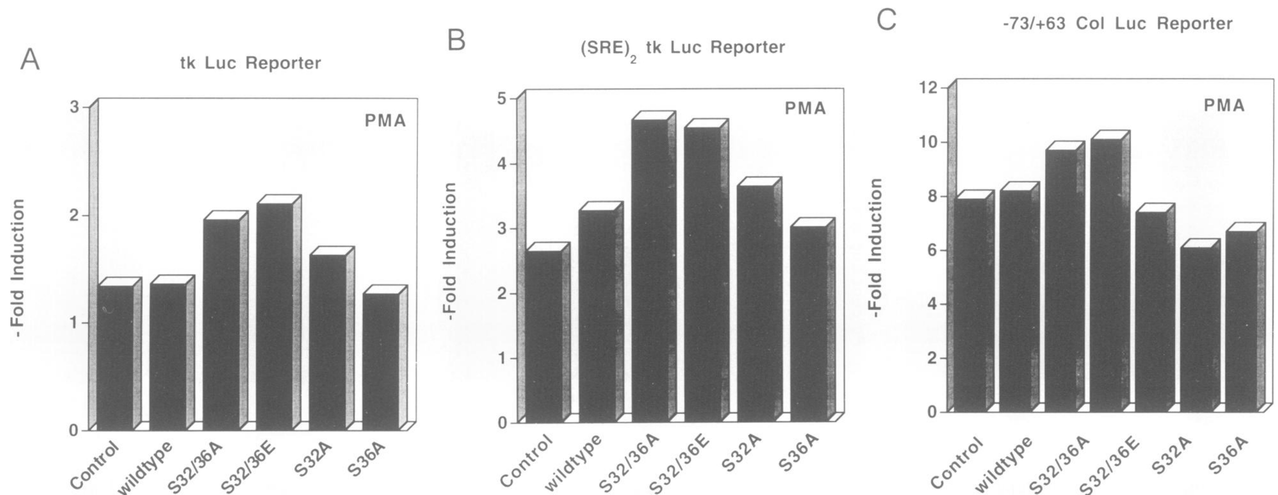

Fig. 5. S32/36 mutants of IκB-α do not inhibit the PMA-inducible expression of various other reporter genes. HeLa cells were transfected with 100 ng RcCMV vector as a control or 100 ng of the indicated amounts of CMV-controlled expression vectors encoding the indicated variants of IκB-α. HeLa cells were left untreated or stimulated for 8 h with 100 ng/ml PMA. Co-transfected luciferase reporter plasmids (2.5 μg) were (**A**) a minimal thymidine kinase (tk) promoter, (**B**) a tk promoter controlled by two copies of the serum response element from the human *c-fos* gene, or (**C**) a portion of the collagenase promoter from –73 to +63 controlled by one AP-1 site. Results from a representative series of transfection experiments are shown.

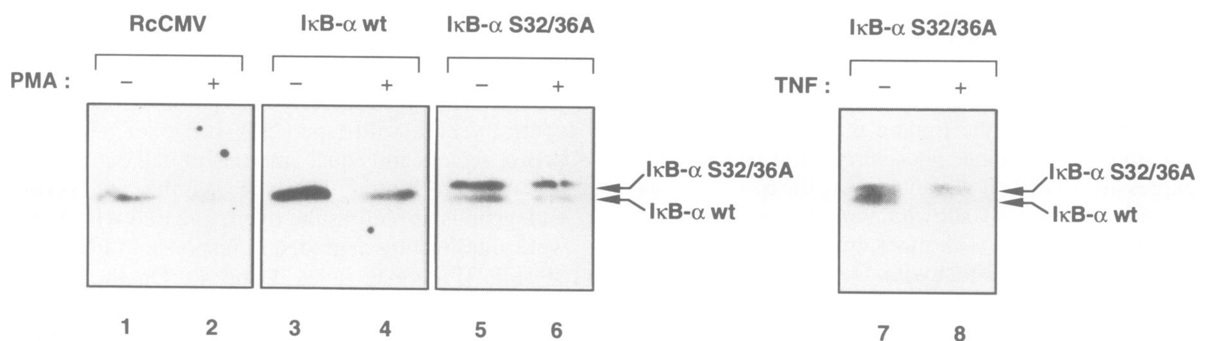

Fig. 6. The effect of a S32/36A mutation on the stability of IκB-α in response to PMA and TNF treatments of HeLa cells. HeLa cells were transfected with 100 ng of a control plasmid (RcCMV), CMV-IκB-α or CMV-IκB-α S32/36A followed by a 20 min treatment with 100 ng/ml PMA (lanes 2, 4 and 6) or 200 U/ml TNF (lane 8). To achieve a higher resolution, proteins were not separated on 10% SDS–polyacrylamide gels as in Figures 1–3, but on a 7.5% SDS gel. A section of an anti-IκB-α-stained Western blot is shown. The positions of endogenous IκB-α (lane 1), co-migrating overexpressed wild-type IκB-α (lane 3) and of the more slowly migrating S32/36A mutant (lanes 5–8) are indicated.

from *Drosophila melanogaster* appears to be involved in releasing the associated IκB homolog cactus in response to ligand binding to toll, an IL-1 receptor-homologous membrane protein (Whalen and Steward, 1993; reviewed in Ingham, 1994). So far, a role for DNA binding subunit phosphorylation in dissociation of the inhibitor could not be demonstrated for vertebrate NF-κB. Rather, phosphorylation and subsequent proteolysis of the inhibitory subunit IκB-α was reported in response to a variety of conditions, including PMA, TNF, OA, H_2O_2 and the viral transactivator Tax from the human T-cell leukemia virus type I (Beg *et al.*, 1993; Brown *et al.*, 1993; Cordle *et al.*, 1993; Menon *et al.*, 1993; Kanno *et al.*, 1994; Naumann and Scheidereit, 1994; Sun *et al.*, 1994b). When IκB-α proteolysis was suppressed in intact cells by proteasome inhibitors (Palombella *et al.*, 1994; Traenckner *et al.*, 1994), a more slowly migrating, hyperphosphorylated IκB variant was detected, suggesting that IκB-α phosphorylation was stoichiometric and responsible for the decreased stability of the protein. We show here that this phosphorylation occurred on both Ser32 and Ser36 and was involved in controlling the stability of the IκB-α protein. Hence, the NF-κB system is quite unique in that phosphorylation controls transcription factor activation primarily by modification of the inhibitory subunit rather than the DNA binding subunits. However, it cannot be excluded at present that phosphorylation of NF-κB DNA binding subunits exerts a modulatory effect on IκB-α phosphorylation, stability or binding affinity. One example may be the constitutively nuclear RelB protein, which is resistant to inhibition by IκB-α only in its B-cell form (Lernbecher *et al.*, 1994).

The N-terminus of IκB-α as a regulatory domain

The phosphorylated serines 32 and 36 are contained within the N-terminal portion of IκB-α, which to date had no known function. Deletion analyses have shown that the N-terminal portion of IκB-α is dispensable for p65 binding, DNA binding inhibitory activity and cytoplasmic retention of NF-κB (Ganchi *et al.*, 1992; Inoue *et al.*, 1992; Hatada

et al., 1993; Ernst et al., 1995; T.Henkel and P.A.Baeuerle, unpublished observations). The central ankyrin repeat domain of IκB-α is absolutely necessary for these activities, while an acidic region in the C-terminal portion was shown to promote the binding to p65 (reviewed in Siebenlist et al., 1995). The C-terminal portion contains PEST sequences and a potential PKC site. Our data indicate that the PKC consensus site in the C-terminus is apparently not crucial for activation of NF-κB by PMA because the N-terminal S32/36 mutants also showed a dominant negative effect when cells were stimulated with PMA. It must therefore be concluded that cell-free phosphorylation experiments, in which PKC could directly activate NF-κB–IκB complexes, did not faithfully reflect the *in vivo* mechanism (Shirakawa and Mizel, 1989; Ghosh and Baltimore, 1990). One function that can now be ascribed to the N-terminus of IκB-α is that of a regulatory domain, which receives signals from distinct stimuli and controls the inducible degradation of IκB-α.

Phosphorylation as the signal for IκB-α degradation

The IκB-α in cytoplasmic NF-κB complexes has a higher turnover than NF-κB itself (Rice and Ernst, 1993). Consequently, the inhibitor is constantly replaced within the NF-κB–IκB complex by newly synthesized IκB-α. Following stimulation of cells with diverse stimuli, the rather short half-life of IκB-α is further reduced by two orders of magnitude (Henkel et al., 1993). This inducible degradation is prevented by various peptide inhibitors of proteasome activity (Palombella et al., 1994; Traenckner et al., 1994) and by depletion of intracellular ATP (Donald et al., 1995), suggesting an involvement of the general protein degradation machinery of the cell. Conjugation of ubiquitin to IκB-α prior to its inducible degradation has not yet been reported. However, we could now observe higher molecular weight forms of IκB-α when the phosphoform of IκB-α was stabilized by a proteasome inhibitor (Traenckner and Baeuerle, 1995). It will be interesting to determine whether the normal turnover of IκB-α is controlled by the same proteolytic pathway as its inducible degradation in response to extracellular stimuli. The pharmacological evidence for an involvement of the constitutively active proteasome in inducible IκB-α degradation suggests that proteolysis is initiated by activation of the substrate and not of the proteas(om)e. The present data support this notion. If the IκB-α substrate cannot be phosphorylated on Ser32 or Ser36, its degradation in response to extracellular stimuli is impaired.

Is casein kinase II the IκB-α kinase?

As suggested from the dominant negative effect of S32/36A mutants, the N-terminal domain of IκB-α is targeted by at least three distinct stimuli: OA, PMA and TNF. These are not known to activate a common kinase system, which phosphorylates acidic sites of the casein kinase II (CKII) consensus sequence SXXD (Pearson and Kemp, 1991). Additional experiments are required to address whether Ser32 and Ser36 are indeed high affinity sites for phosphorylation by CKII and are regulated by this particular kinase in intact cells.

An interesting observation is that transformation of T lymphocytes by the eukaryotic parasite *Theileria parva* involves both activation of CKII (ole-MoiYoi et al., 1993) and activation of NF-κB (Ivanov et al., 1989). T-cell transformation by the parasite can be mimicked by CKIIα overexpression in a transgenic animal (Seldin and Leder, 1995). Pharmacological elimination of *T.parva* normalizes T-lymphocyte growth and inactivates NF-κB (Ivanov et al., 1989). Antisense CKIIα was also found to reverse T-cell transformation (Seldin and Leder, 1995) but has not yet been tested for inhibition of NF-κB activation. Future experiments are necessary to clarify whether these phenomena are causally linked.

The use of S32/36 IκB-α dominant negative mutants

In numerous studies, overexpression of wild-type IκB-α has been shown to prevent κB-dependent transactivation. As was evident from Figure 1, overexpressed IκB-α is phosphorylated but not degraded upon cell stimulation (see Figure 1). Therefore, the rate-limiting step in IκB inactivation, and consequently NF-κB activation, appears to be the protease and not the kinase reaction. Consistent with this notion is the observation that the newly phosphorylated form of IκB is not released from NF-κB and that phosphorylation is not sufficient to allow the appearance of active NF-κB in cells (Finco et al., 1994; Miyamoto et al., 1994; Traenckner et al., 1994; DiDonato et al., 1995). In the present study, we show that low levels of overexpressed IκB-α are inducibly degraded and that the endogenous NF-κB can be activated under these conditions. Comparable amounts of all four S32/36 mutants had a significant dominant negative effect on κB-dependent transactivation. One reason why these mutants did not yield a close to 100% inhibition of κB-dependent transactivation might be that part of the NF-κB was associated with the transfected mutant IκB-α and part with the endogenous wild-type IκB-α. Following stimulation, the latter complex can still release active NF-κB which may escape re-inhibition by S32/36 IκB-α mutant proteins and activate the reporter gene.

S32/36 mutants can now be used in transactivation assays to test which of the numerous other NF-κB-inducing conditions uses S32/36 phosphorylation for NF-κB activation. Another interesting application of the mutants is their use as transgenes. Low expression levels will suffice to interfere with the activition of most members of the NF-κB/Rel family of transcription factors. Cell type-specific expression of mutant IκB-α proteins in thymocytes, neurons, astrocytes, macrophages, B cells, liver cells, endothelial cells and β islet cells can answer many questions concerning the role of NF-κB in autoimmune and inflammatory diseases.

Materials and methods

Cell culture and treatments

HeLa cells were grown in Dulbecco's-modified Eagle's medium (DMEM, GIBCO), supplemented with penicillin/streptomycin and 10% fetal calf serum (FCS, Sigma). For activation of NF-κB, human recombinant TNF-α (Boehringer Mannheim) was added to a final concentration of 200 U/ml in the culture medium. The sodium salt of OA (Paesel+Lorei) was dissolved as a stock solution of 0.5 mM in ethanol. PMA was dissolved in dimethyl sulfoxide (DMSO) and used at a final concentration of 100 ng/ml culture medium. The proteasome inhibitor CBz-Ile-Glu(tBu)-Ala-Leu-CHO (PSI) (Figueiredo-Pereira et al., 1994) was

dissolved in DMSO and diluted to a final concentration of 60 μM in culture medium 50 min prior to stimulation with OA.

Transfections

HeLa cells (10^5) in 60×15 mm culture dishes were used for transfection. Four to five hours prior to transfection, the culture medium was changed to DMEM supplemented with 5% FCS, which gave better transfection efficiencies. The amounts of expression vectors and reporter plasmid used for transfection and the transfection periods are indicated in the figure legends. RcCMV was used both as a control vector and to equalize for the total amount of transfected DNA. The $CaPO_4$ transfection method was used as described (Wigler *et al.*, 1978). Briefly, the DNA was diluted with Tris–EDTA (pH 7.0) to a volume of 90 μl, and 100 μl of 2× HBS buffer (274 mM NaCl, 40 mM HEPES, 1.4 mM Na_2HPO_4, pH 7.06) was added. The precipitate was formed by slowly pipetting 10 μl 2 M $CaCl_2$ into the DNA solution. After 20 min incubation at room temperature, the precipitate was added dropwise to the cells. For luciferase assays, the protein expression was allowed for 18 h prior to stimulation. Luciferase activity was measured 6 h after TNF treatment, and 8 h after OA and PMA stimulation. For treatments with PSI and the various stimuli, the medium was changed again to DMEM supplemented with 10% FCS 14 h post-transfection, i.e. 4 h before stimulation.

Cell extracts

For Western blotting, whole cell extracts were prepared by a high salt/ detergent buffer (Baeuerle and Baltimore, 1988). For luciferase assays, cells were lysed as described by Brasier *et al.* (1989) with slight modifications. HeLa cells were washed once with ice-cold phosphate-buffered saline (PBS) and lysed for 5 min directly on the dish in 300 μl of 1% (v/v) Triton X-100, 25 mM glycylglycine (pH 7.8 adjusted with KOH), 15 mM $MgSO_4$, 4 mM EGTA (pH 8 adjusted with KOH) and 1 mM dithiothreitol (DTT). The lysates were transferred to 1.5 ml Eppendorf tubes and cleared by centrifugation. The supernatants were assayed for protein concentrations and luciferase activity. Protein concentrations were determined by the Bradford assay (Bio-Rad).

Luciferase assay

One hundred microlitres of cell lysates were assayed for luciferase activity by addition of 50 μl reaction buffer (25 mM glycylglycine, pH 7.8, 15 mM $MgSO_4$, 30 mM potassium phosphate, pH 7.6, 4 mM EGTA, 1 mM DTT and 3 mM ATP) and then measuring the light emission in a Microlumat LB96 P luminometer (Berthold). The luminometer was programmed to inject 100 μl of 0.3 mg/ml luciferin (Sigma) and to measure light emission for 30 s after injection.

Antibodies

A polyclonal affinity-purified antibody to human IκB-α was used and prepared as described (Henkel *et al.*, 1993).

Western blotting

Western blotting and detection of immune complexes were performed as described (Traenckner *et al.*, 1994). Exposure times to Kodak XAR-5 films were between 3 s and 20 min.

Expression plasmids

The IκB-α single and double point mutants were generated by the polymerase chain reaction (PCR) technique according to Ho *et al.* (1989). The following oligonucleotides were used as sense primers: S32A, 5′-CGCCACGACGCCGGCCTGGACTCCATGAAAGAC-3′; S36A, 5′-CGCCACGACAGCGGCCTGGACGCCATGAAAGAC-3′; S32/36A, 5′-CGCCACGACGCCGGCCTGGACGCCATGAAAGAC-3′; S32/36E, 5′-GACCGCCACGACGAAGGCCTGGACGAAATGAAA-GACGAG -3′.

The C-terminal deletion mutants were cloned by insertion of a new stop codon with following PCR antisense primers: IκB-α 1–250, 5′-GGATCCTCTAGATTAGCCCTGGTAGGTAAC-3′; IκB-α 1–278, 5′-GGATCCTCTAGATTACTGAAGGTTTTCTAGTG-3′.The primer used for the construction the N-terminally deleted IκB-α variant was IκB-α 45–317: 5′-GCAAGCTTCAGATGGTCAAGGAG-3′.

The mutated PCR fragments were cloned into the eukaryotic expression vector RcCMV (Invitrogen) and verified by sequencing on an automated DNA sequencer (Applied Biosystems).

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