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Activation of NF-κB *in vivo* is regulated by multiple phosphorylations

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The activation of nuclear factor κB (NF-κB) in intact cells is mechanistically not well understood. Therefore we investigated the modifications imposed on NF-κB/IκB components following stimulation and show that the final step of NF-κB induction *in vivo* involves phosphorylation of several members of the NF-κB/IκB protein families. In HeLa cells as well as in B cells, TNF-α rapidly induced nuclear translocation primarily of p50–p65, but not of c-rel. Both NF-κB precursors and IκBα became strongly phosphorylated with the same kinetics. In addition to the inducible phosphorylation after stimulation, B lymphocytes containing constitutive nuclear NF-κB revealed constitutively phosphorylated p65 and IκBα. Phosphorylation was accompanied by induced processing of the precursors p100 and p105 and by degradation of IκBα. As an *in vitro* model we show that phosphorylation of p105 impedes its ability to interact with NF-κB, as has been shown before for IκBα. Surprisingly, even p65, but not c-rel, was phosphorylated after induction *in vivo*, suggesting that TNF-α selectively activates only specific NF-κB heteromers and that modifications regulate not only IκB molecules but also NF-κB molecules. In fact, cellular NF-κB activity was phosphorylation-dependent and the DNA binding activity of p65-containing NF-κB was enhanced by phosphorylation *in vitro*. Furthermore, we found that the induction by hydrogen peroxide of NF-κB translocation to the nucleus, which is assumed to be triggered by reactive oxygen intermediates, also coincided with incorporation of phosphate into the same subunits that were modified after stimulation by TNF-α. Thus, phosphorylation appears to be a general mechanism for activation of NF-κB *in vivo*.

Key words: H_2O_2/IκB/NF-κB transcription factor/precursor/processing/tumor necrosis factor

Introduction

The transcription factor NF-κB is constitutively active in mature B lymphocytes (Sen and Baltimore, 1986; Kawakami *et al.*, 1988) and in some monocytes/macrophages (Griffin *et al.*, 1989) and is inducible by a number of agents in a broad spectrum of cell lines (reviewed in Lenardo and Baltimore, 1989; Baeuerle, 1991; Blank *et al.*, 1992; Grilli *et al.*, 1993). For several inducing agents it has been shown that activation of NF-κB by a post-translational mechanism involves the release of NF-κB from cytosolic IκB proteins (Baeuerle and Baltimore, 1988). NF-κB consists of dimeric complexes of several transcription factors belonging to a family whose conserved members include the *rel* (proto)oncogene product. A remarkable feature of NF-κB is that its p50 and p52 subunits are encoded by larger precursors that require processing as one essential activation step (reviewed in Blank *et al.*, 1992). The C-terminal domains of the precursors, which are removed after processing, have structural similarity to IκBα (for review see Beg and Baldwin, 1993). Both p105 (NF-κB1, the precursor for p50) and p100 (NF-κB2, the precursor for p52), form cytoplasmic complexes with other NF-κB subunits and have IκB-like functions (Rice *et al.*, 1992; Mercurio *et al.*, 1993; Naumann *et al.*, 1993a,b). The interaction of IκBα or p105 with NF-κB subunits is mediated by a similar mechanism involving ankyrin-like repeats and a short acidic sequence (Hatada *et al.*, 1993). These structural similarities suggested that the regulation of NF-κB complexes containing the precursors or IκBα should share mechanistic aspects regarding their activation.

It has been demonstrated that phosphorylation with several kinases leads to the dissociation of NF-κB–IκB complexes *in vitro* (Shirakawa and Mizel, 1989; Ghosh and Baltimore, 1990). Furthermore, avian IκBα is a phosphoprotein and its interaction with NF-κB can be blocked *in vitro* by phosphorylation with PKA (Kerr *et al.*, 1991). Human IκBα is inactivated by phosphorylation at a site residing in the repeat domain (D.Krappmann and C.Scheidereit, unpublished data).

The activation of NF-κB by several inducers, including interleukin 1, lipopolysaccharide (LPS), phorbol myristate acetate (PMA), tumor necrosis factor α (TNF-α) and double-stranded RNA, is accompanied by degradation of IκBα (Beg *et al.*, 1993; Brown *et al.*, 1993; Cordle *et al.*, 1993; Henkel *et al.*, 1993; Mellits *et al.*, 1993; Sun *et al.*, 1993). IκBα is unstable, when not bound to NF-κB, as demonstrated for ectopically expressed IκBα (Scott *et al.*, 1993) and is rapidly turned over even in unstimulated cells (Rice and Ernst, 1993). The activation of NF-κB seems to be subject to a stimulus-dependent proteolysis event, as it can be blocked by the protease inhibitors tosylphenylalanine-chloromethyl ketone (TPCK) or tosyllysine-chloromethyl ketone (TLCK) (Henkel *et al.*, 1993; Mellits *et al.*, 1993). The hypothesis that NF-κB is directly activated by phosphorylation has been challenged by the absence of direct supportive *in vivo* data and by recent reports showing that NF-κB induction by many effectors tested was blocked by oxygen-radical scavenger compounds and that hydrogen peroxide can activate NF-κB (Staal *et al.*, 1990; Schreck *et al.*, 1991, 1992). The exact mechanism by which reactive oxygen intermediates (ROIs) can activate NF-κB remains unknown.

NF-κB is activated by TNF-α, a fast and strong physiological inducer, at a post-translational level, but also at the transcriptional level by increased expression of the p105 and p100 genes (Hohmann *et al.*, 1991b; Meyer *et al.*, 1991; Mercurio *et al.*, 1992; Ten *et al.*, 1992). In addition, TNF-α induces transcription of the IκBα gene (de Martin *et al.*, 1993; Le Bail *et al.*, 1993). The rapid induction of NF-κB requires only a few minutes and does not depend on *de novo* protein synthesis (Hohmann *et al.*, 1991a,b). It has been proposed that TNF-α activates NF-κB via ceramide as a second messenger (Schütze *et al.*, 1992). Ceramide may, in turn, activate specific protein kinases (Dressler *et al.*, 1992). It has been shown that in certain cell types TNF-α increases the activity of PKA and PKC (Chen *et al.*, 1992). In contrast, PKA and PKC seem not to be involved in the induction of NF-κB by TNF-α in HL-60 or Jurkat cells (Feuillard *et al.*, 1991; Hohmann *et al.*, 1991a). In addition to the activation of kinases, TNF-α has been proposed to induce NF-κB through the generation of ROIs (Schreck *et al.*, 1991). The activation of NF-κB by TNF-α can be strongly reduced either when the mitochondrial electron transfer system is blocked by chemical antagonists or when cells lack a functional respiratory chain (Schulze-Osthoff *et al.*, 1993).

The aims of this study are to investigate the mechanism of NF-κB activation *in vivo* in B cells and HeLa cells. We used TNF-α as a strong and fast physiological inducer, and hydrogen peroxide, which activates with a delay in time. Our data demonstrate that NF-κB is activated by a mechanism involving protein phosphorylation, as well as proteolytic processes. Phosphorylation appeared to trigger not only the response to cellular inducers, but also constitutive NF-κB activity in B cells. We found that there are different levels of control by phosphorylation. After stimulation, the p65 subunit of NF-κB is phosphorylated and exhibits strongly increased DNA binding activity. On the other hand, the p65-associated molecules p100, p105 and IκBα became phosphorylated with similar kinetics. As an *in vitro* model we show that phosphorylation with PKA abrogates the ability of p105 to interact with p65, but enhances the DNA binding activity of p65.

Results

TNF-α activates NF-κB by inducing rapid concerted processing and degradation events, involving the two NF-κB precursors and IκBα

Both the precursor molecules p100 and p105, and IκBα form cytoplasmic complexes with NF-κB proteins. We intended to investigate how the modification state and the stability of these protein complexes is affected in the course of NF-κB activation by cellular stimulation.

Namalwa or HeLa cells were treated with TNF-α for various times, fractionated into cytoplasmic and nuclear components and subjected to Western blot analysis for NF-κB/IκB proteins (Figure 1). p105, p100 and IκBα were observed exclusively in the cytoplasm, in agreement with the localization of these proteins determined by indirect immunofluorescence labelling of intact cells (Beg *et al.*, 1992; Rice *et al.*, 1992; Naumann *et al.*, 1993b). In both cell types stimulation led to rapid loss of IκBα (Figure 1A and B), confirming other reports (see Introduction), and to simultaneous processing of p100 and p105

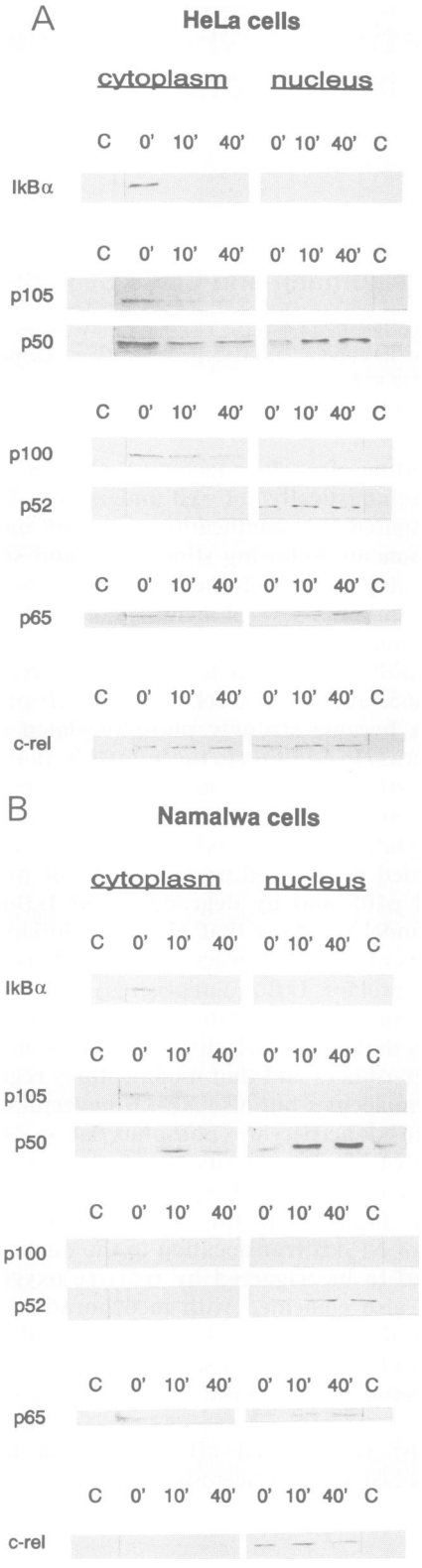

Fig. 1. Cellular distribution of NF-κB/rel factors in HeLa and Namalwa cells after TNF-α stimulation. Cytoplasmic (100 μg) and nuclear extracts (25 μg) from (**A**) HeLa and (**B**) Namalwa cells were prepared, separated by SDS–PAGE and analysed by immunoblotting. The cells were either induced with TNF-α (10 or 40 min) or left untreated (0 min), as indicated. The different panels represent immunoblots performed with anti-IκBα, anti-p105N, anti-p100N, anti-p65 and anti-c-rel antisera. As a control, the appropriate preimmune sera (C) were used.

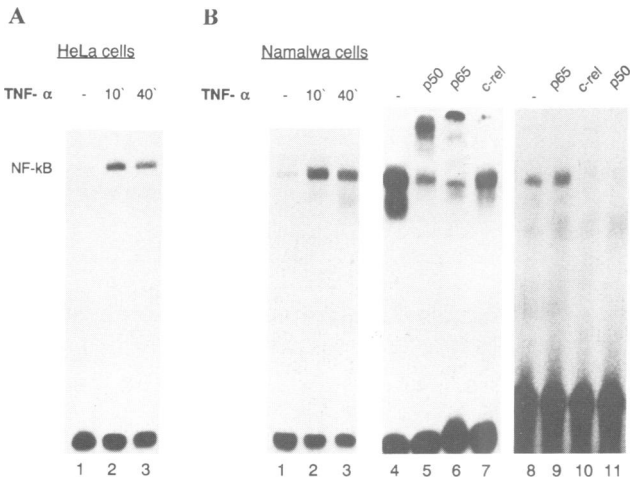

Fig. 2. TNF-α-induced nuclear NF-κB activity consists primarily of p50−p65, whereas constitutive NF-κB is composed of p50−c-rel. Nuclear extracts (5 μg) from (**A**) HeLa or (**B**) Namalwa cells were analysed in a gel retardation assay using an H2-K gene NF-κB binding site oligoprobe. Nuclear extracts were prepared before stimulation (lane 1 in A and lanes 1 and 8–11 in B) or after 10 min (lane 2 in A and lanes 2 and 4–7 in B) or 40 min (lanes 3 in A and B) of TNF-α treatment. The composition of TNF-α-induced NF-κB in Namalwa cells (panel B, lane 4) was investigated by antibody supershifting and inhibition using anti-p50 (lane 5), anti-p65 (lane 6) or anti-c-rel antisera (lane 7). The composition of constitutive NF-κB in Namalwa cells (B, lane 8) was analysed with anti-p65, anti-c-rel or anti-p50 antibodies (lanes 8–11). Note that in panel B, lanes 1–3, a shorter exposure is shown than for lanes 4–11. The NF-κB−DNA complex is indicated.

as well as a concomitant increase of the processing products p50 and, to a lesser extent p52, in the nucleus. Processing of p105 seemed to be more efficient than that of p100. Similarly, p65 translocated to the nucleus, correlating with the time of IκBα degradation and p105 processing. In contrast, c-rel did not change its cellular distribution in response to the inducer in either cell type. We found a higher constitutive amount of c-rel in the nuclear fraction of B cells than in HeLa cells (compare Figure 1A and B).

The correlation of precursor breakdown and IκBα degradation with nuclear translocation of p50 or p65 demonstrates that the cytoplasmic complexes formed between p100, p105 and other NF-κB/rel factors (Rice *et al.*, 1992; Mercurio *et al.*, 1993; Naumann *et al.*, 1993a,b) are indeed subject to cellular regulation. The observation that c-rel was not affected, although it is well documented that c-rel is associated with p100, p105 or IκBα (Mercurio *et al.*, 1993; Rice and Ernst, 1993), suggests that c-rel-containing complexes cannot be directly activated by TNF-α. This also indicates that the identity of the NF-κB/*rel* factor in the complexes with IκBα molecules may affect a recognition mechanism involved in the activation. The observed effect of TNF-α on the NF-κB/IκB system is the same in B cells and non-B cells and hence appears to be a general mechanism.

The observation that TNF-α treatment did not change the intracellular distribution of c-rel tempted us to analyse the components of the TNF-α-induced nuclear DNA binding activity (Figure 2). Even before induction, Namalwa cells displayed a constitutive nuclear binding activity that was absent in HeLa cell nuclear extracts (compare Figure 2B and A, lanes 1). Stimulation with TNF-α led to an equivalent increase of NF-κB activity in both cell types after 10 min, which persisted for at least a further 30 min (Figure 2A and B, lanes 2 and 3). This increase in nuclear binding activity occurred when the amounts of the precursor molecules and of IκBα declined and when protein translocation, except for that of c-rel, was noticed (see Figure 1).

The constitutive NF-κB DNA binding activity seen in unstimulated B-lymphoid cells (Figure 2B, lane 8) was sensitive to antibodies directed against c-rel or p50 (lanes 10 and 11), but not to anti-p65 antiserum (lane 9). In contrast, antibodies directed against c-rel had only a very weak effect on TNF-α-induced NF-κB activity (Figure 2B, compare lane 4 with lane 7), which was strongly reduced and partially supershifted when antibodies against p50 or p65 were used (lanes 5 and 6). Thus, the constitutive NF-κB activity in Namalwa B cells consists mainly of p50−c-rel heterodimers, while TNF-α-induced NF-κB consists primarily of p50−p65 heterodimers. Thus, in agreement with the Western analysis (Figure 1), cytokine induction does not induce immediate translocation of c-rel.

Surveying the steady state amounts of p105 and IκBα by Western analysis showed that the levels of these proteins declined rapidly after cellular stimulation. The very rapid effect makes it very unlikely that induced *de novo* protein synthesis, particularly of p105 or IκBα, would have interfered. To exclude this possibility, HeLa cells were pulse−chased with [^{35}S]methionine and then stimulated with TNF-α, and whole-cell extracts were subjected to immunoprecipitation after various times. As shown in Figure 3A, an antiserum directed against p65 could co-immunoprecipitate p105, p100, p65, c-rel, p50 and IκBα. The identity of these molecules was proven by the use of specific antibodies in a subsequent immunoprecipitation (Figure 3A, lanes 5–9). We observed a rapid decay of p65-associated IκBα following induction (lanes 2–4). An almost complete disappearance of IκBα after 10 min was also observed when total cellular IκBα was directly precipitated from TNF-α-stimulated, [^{35}S]methionine-labelled cells (not shown). Similarly, both precursors, which were found in about equal amounts (lanes 6 and 7), also declined after stimulation with TNF-α (lanes 2–4). In order to confirm that the immediate activation of NF-κB by TNF-α includes induced p105 processing, cells were chased with [^{35}S]methionine, induced for various times and analysed by immunoprecipitation with an antiserum directed against an N-terminal peptide of p105 (Figure 3B). TNF-α administration immediately led to the generation of p50 (compare lane 2 with lanes 3 and 4).

In TNF-α-induced cells, the NF-κB precursors p100 and p105, as well as p65 and IκBα, become phosphorylated with similar kinetics

Since TNF-α treatment induced precursor processing and IκBα degradation, as well as nuclear accumulation of specific NF-κB proteins, we next analysed whether these stimulus-dependent alterations would correlate with changes in the phosphorylation state of these proteins.

Extracts of HeLa cells with incorporated [^{32}P]orthophosphate were subjected to immunoprecipitation after various times of stimulation with TNF-α (Figure 4). Using

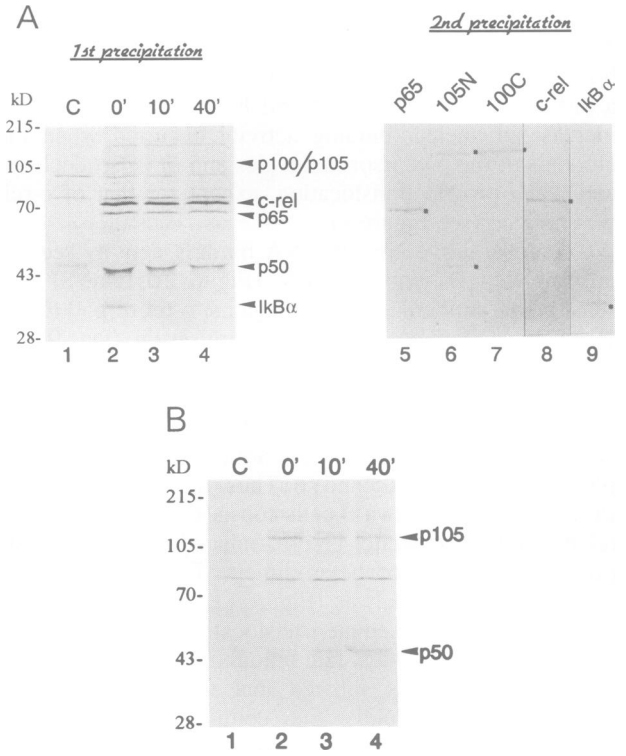

Fig. 3. The amount of NF-κB precursor molecules associated with p65 decreases after TNF-α stimulation. [^{35}S]methionine-labelled HeLa cells were induced with TNF-α and subsequently analysed by immunoprecipitations from whole cell extracts. The cells were either untreated or stimulated with TNF-α for the indicated times (0, 10 or 40 min). (**A**) Immunoprecipitation was performed with an anti-p65 antiserum, and the precipitated proteins were separated by SDS−PAGE and visualized by autoradiography (lanes 2–4). The co-immunoprecipitated proteins are indicated with arrows. As a control, an excess (100 μg) of the p65-specific peptide was added to the sample (lane 1). After the first immunoprecipitation the protein A beads were centrifuged, boiled and diluted and a part was subjected to further immunoprecipitations with the indicated antisera (lanes 5–9). The identified proteins are indicated with small black dots. (**B**) Immunoprecipitation with an antiserum against an N-terminal peptide of p105 (anti-p105N). Excess immunogen was used as a control (lane 1) and cells were TNF-α-treated for the times indicated (lanes 2–4).

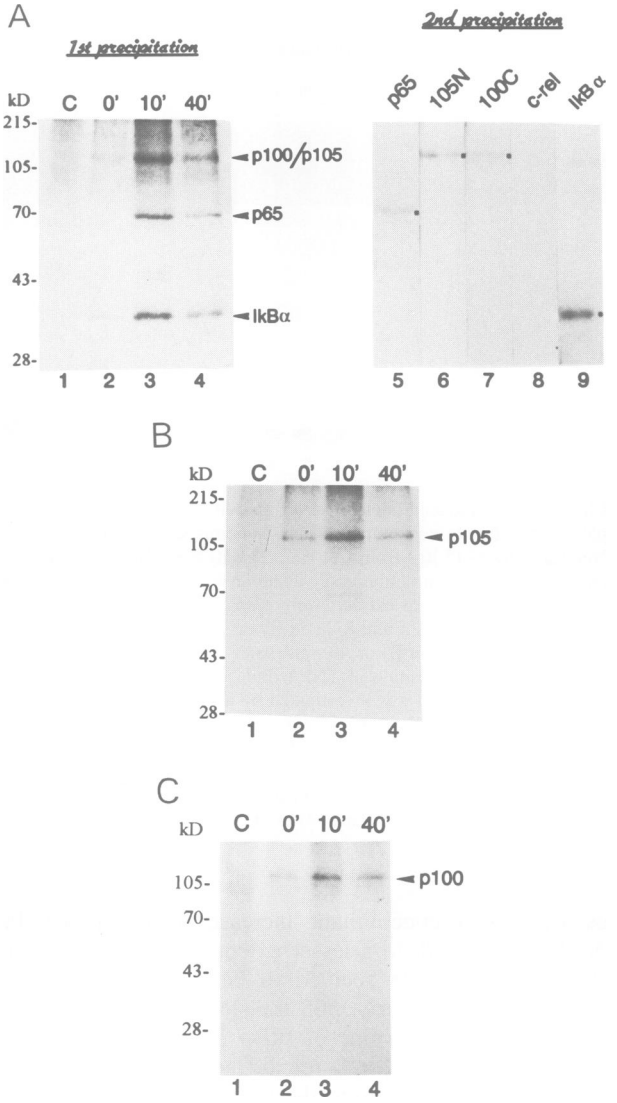

Fig. 4. Similar kinetics of inducible phosphorylation were observed for the NF-κB precursors, for p65 and for IκBα. HeLa cells were labelled with [^{32}P]orthophosphate and whole cell extracts were prepared before or after induction with TNF-α (0, 10 or 40 min). (**A**) Immunoprecipitation with an anti-p65 antiserum (lanes 2–4). As a control, an excess of the p65 peptide, which was used to raise the antibody, was added to the samples (lane 1). A subsequent immunoprecipitation was performed with the indicated antisera to prove the identity of the co-immunoprecipitated molecules (lanes 5–9). The identified proteins are indicated by small black dots. (**B**) Immunoprecipitation with an anti-p105C antiserum (lanes 2–4). As a control the preimmune serum was used (lane 1). (**C**) Immunoprecipitation with an anti-p100C antiserum (lanes 2–4). The control is a precipitation with the preimmune serum (lane 1).

an anti-p65 antibody, three induced phosphorylated species were obtained, corresponding in size to 120, 70 and 35 kDa (Figure 4A, lanes 3 and 4). Similarly, phosphorylated species have been precipitated with the p50 subunit (Mellits *et al.*, 1993). In a second, stringent precipitation the phosphorylated species observed in the first step were unequivocally identified as p100, p105, p65 and IκBα (Figure 4A, lanes 5–9). No phosphorylated c-rel was detected (Figure 4A, lane 8), although c-rel protein was present, as demonstrated by precipitation with p65 from [^{35}S]methionine-labelled cells (Figure 3). Consequently, after stimulation the p65 subunit was engaged in complexes with phosphorylated p105, p100 or IκBα (and non-phosphorylated c-rel, see Figure 3).

Even before the cells were stimulated there was a weak constitutive phosphorylation of p100/p105 and IκBα (Figure 4A, lane 2). The TNF-α treatment then caused a strong increase in the phosphorylation of the precursors, of IκBα and, most notably, of p65 (lane 3). It should be recalled that at the same time point the levels of proteins, particularly of p105, p100 and IκBα, were already strongly diminished (Figures 1 and 3), which demonstrates the drastic increase of phosphorylation of the single molecules. Based on the same reasoning, the signal-dependent specific phosphorylation of the precursor molecules and of IκBα appears to be higher than that of p65. After longer exposure to TNF-α the levels of phosphoproteins declined again (lane 4), paralleled by a further loss of the precursor and IκBα proteins (Figure 1 and 3). Whereas radiolabelled p105 and p100 could be precipitated with p65, no phosphorylated p50 or p52 was detected. The simplest explanation is that both precursors were phosphorylated in their C-terminal domains, which were lost after processing.

This would explain why no labelled p50 or p52 was obtained after processing.

This experiment clearly demonstrates that precursor processing and IκBα degradation are preceded by or accompanied by a phosphorylation event, and it suggests that phosphorylated p100, p105 and IκB can, at least in part, still interact with p65. It is difficult to estimate the absolute amounts of phosphorylated protein and hence we cannot exclude the possibility that only a fraction of the phosphorylated proteins are associated with p65. In addition, p65 itself was found to be the target of signal-dependent modification.

Because both precursors were phosphorylated and co-precipitated with p65 (Figure 4A) we next addressed the question of whether their modification occurred with the same or with distinct kinetics. Therefore, HeLa cells were labelled with [^{32}P]orthophosphate, treated with TNF-α for various times and subjected to immunoprecipitation using antibodies specific for either p105 or p100 (Figure 4B and C). The two precursor molecules showed indistinguishable time-courses, with phosphorylation peaking 10 min after administration of the inducer (Figure 4B and C, lanes 2–4), suggesting an identical modification event. In addition, a similar fraction of both p105 and p100 was phosphorylated even before stimulation (Figure 4B and C, lanes 2). It is thus likely that the same kinase phosphorylates p100 and p105 and, given the similar incorporation (Figure 4A) for the approximately equal protein amounts (Figure 3), it is even possible that the phosphorylation site is conserved in the two precursors.

TNF-α-mediated activation of NF-κB in B cells

B cells contain a constitutive nuclear NF-κB activity, indicating that a mechanism for the activation of NF-κB operates constitutively in these cells. Yet the response to TNF-α treatment resembled that of HeLa cells with a similar breakdown of the precursors and of IκBα at the same time point after stimulation (Figure 1).

Striking differences from HeLa cells were observed when analysing the phosphorylation state of NF-κB/IκB proteins associated with p65 after stimulation in Namalwa cells (Figure 5). After these cells had been labelled with [^{32}P]orthophosphate, a coprecipitation experiment with an anti-p65 antibody revealed that p105, p100, p65 and IκB-α were phosphorylated (Figure 5A, lanes 1–3), as observed already for HeLa cells (Figure 4). The identity of the single proteins was again proven by a second precipitation with specific antibodies (Figure 5A, lanes 5–9). A fainter band migrated with the mobility expected for c-rel (lanes 2 and 3) but could not be detected after the second precipitation (lane 8).

In remarkable contrast to HeLa cells, IκBα was observed as two differently migrating forms which were already strongly phosphorylated before stimulation (Figure 5A, lane 1). After stimulation the faster form became fainter, while the other form displayed enhanced phosphorylation which did not decline after stimulation (lane 3), in contrast to the decrease observed in HeLa cells. Similarly, both precursor proteins were constitutively phosphorylated (lane 1). After stimulation a continuous increase of phosphorylation was observed for proteins in the size range 100–120 kDa (lanes 2 and 3). The same phenomenon was observed for p65, which was phosphorylated constitutively, in striking contrast to HeLa cells (compare Figure 4A, lane 2, with Figure 5A, lane 1). No difference in TNF-α-dependent phosphorylation was seen for p105 and p100, both of which were already constitutively phosphorylated (Figure 5B and C). We also established that there is very little, if any, phosphorylated c-rel after stimulation in B cells (Figure 5A, lane 8). p65 co-immunoprecititated with another phosphorylated protein running below the p105/p100 signal (which migrates at ~115–120 kDa) (Figure 5A). We do not know yet whether this species is a degradation product of p100 or p105 or whether it represents a newly discovered interacting

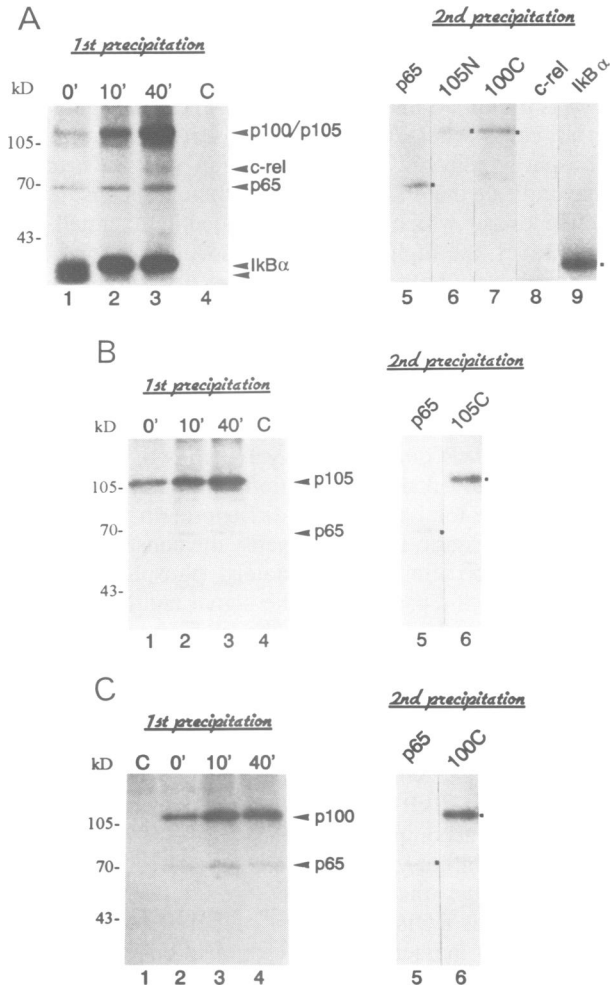

Fig. 5. The NF-κB precursors, p65 and IκBα are constitutively phosphorylated in B cells. Namalwa cells were labelled with [^{32}P]orthophosphate and induced with TNF-α in the same manner as HeLa cells. (**A**) Immunoprecipitation from whole cell extracts with an anti-p65 antiserum (lanes 1–3). As a control, an excess of the p65 peptide antigen was added to the sample beforehand (lane 4). The pellets were solubilized and a part re-precipitated with the indicated antisera, to prove the identity of the co-immunoprecipitated molecules (lanes 5–9). (**B**) Precipitation with the anti-p105C antiserum (lanes 1–3) and with the preimmune serum as a control (lane 4). A subsequent re-precipitation was performed with antibodies against p65 or p105 (lanes 5 and 6). (**C**) An anti-p100C antiserum was used for p100 co-immunoprecipitations (lanes 2–4) with the preimmune serum as a control (lane 1). Further immunoprecipitations were performed from the first solubilized precipitate with anti-p65 or anti-p100C antiserum, respectively (lanes 5 and 6). The immunoprecipitated proteins are indicated with arrows. Identified proteins from the second immunoprecipitations are indicated by small black dots.

polypeptide. We did observe a protein in this size range, but distinct from p100 and p105, which was associated with p65 (data not shown).

It is important that p65 is constitutively phosphorylated in B cells, whereas in HeLa cells it is detected as a phosphoprotein only after stimulation with TNF-α. This may indicate that in Namalwa cells there is a constitutively active kinase, which has to be activated in other cell types. In parallel, the high constitutive phosphorylation both of IκBα and of the precursor proteins in B cells suggests that the constitutive NF-κB activity in these cells is due to an increased turnover of the precursors and of IκBα following continuous phosphorylation. In accordance with this, a faster turnover of IκBα in B cells has also been noted by Rice and Ernst (1993). Fast and continuous turnover of IκBα in B cells may explain why radiolabelled IκBα is observed after stimulation for 40 min in Figure 5A, although the total amounts of IκBα protein were strongly decreased (Figure 1B). Presumably the constantly *de novo* translated protein is immediately phosphorylated and degraded in the presence of the signal and the difference from HeLa cells just reflects the high turnover of IκBα in B cells.

Inducible phosphorylation appears to be a general activation mechanism for NF-κB

Next, we asked whether phosphorylation of components of the NF-κB/IκB system would be a general *in vivo* mechanism accompanied by the release of active NF-κB from its inhibitory cytoplasmic structures and whether inducers other than cytokines would utilize the same modification. ROIs have been proposed to be potential intracellular messengers for the induction of NF-κB and it has been shown that hydrogen peroxide activates NF-κB in a dose-dependent manner (Schreck *et al.*, 1991). Extracellular exposure of HeLa cells to hydrogen peroxide led to the expected nuclear accumulation of NF-κB (Figure 6A). Surprisingly, immunoprecipitation with anti-p65 antibodies revealed that NF-κB induction was again paralleled by phosphorylation of the NF-κB precursors, of p65 and of IκBα (Figure 6B, compare lanes 2 and 3), as observed for the rapid induction by TNF-α. It is striking that the same subunits were modified and that p50, p52, c-rel and other components were not phosphorylated. The effects of cellular stimulation by TNF-α or hydrogen peroxide on NF-κB/IκB are summarized in Table I.

We have also found that NF-κB induction by phorbol ester (PMA) similarly coincides with phosphorylation *in vivo* (data not shown). For all different inducers tested, phosphorylation always occurred at the time of nuclear translocation, no matter how fast the induction was. This may hint at convergent pathways and at the involvement of perhaps only one or very few kinases which finally activate NF-κB.

Nuclear NF-κB is phosphorylated at the p65 subunit, leading to enhanced DNA binding

The striking observation that the NF-κB p65 subunit becomes phosphorylated after stimulation with TNF-α or hydrogen peroxide and is constitutively phosphorylated in B cells tempted us to analyse the effect of phosphorylation on the DNA binding activity. The TNF-α-induced nuclear NF-κB DNA binding activity, which consisted mainly of

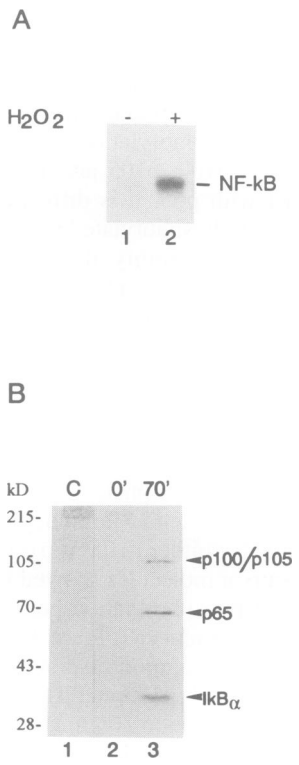

Fig. 6. NF-κB induction by H_2O_2 involves transfer of phosphoryl groups onto p105/p100, p65 and IκBα. (**A**) Nuclear extracts of HeLa cells which were either untreated or stimulated with 250 μM H_2O_2 for 70 min were assayed by EMSA. (**B**) HeLa cells were labelled with [^{32}P]orthophosphate. Subsequently, immunoprecipitations were performed from whole cell extracts before (lane 2) or after (lanes 1 and 3) incubation of the cells with 250 μM H_2O_2 for 70 min using the anti-p65 antibody. In lane 1, the peptide antigen was added as a control.

heterodimers containing p65 (Figure 2) was therefore tested for sensitivity to phosphatases (Figure 7A). Interestingly, treatment with shrimp alkaline phosphatase (lane 3) or potato acid phosphatase (lane 5) strongly reduced the TNF-α-induced NF-κB DNA binding activity (lane 2), whereas heat-inactivated phosphatase had no effect (lane 4). To rule out indirect effects of the phosphatases, p65-containing NF-κB was purified from a nuclear extract of TNF-α-stimulated HeLa cells with an anti-p65 antibody column. The DNA binding activity of purified NF-κB (Figure 7B, lane 1) was again reduced by treatment with phosphatase but not by heat-inactivated phosphatase (lanes 2 and 3).

To determine whether NF-κB subunits can be phosphorylated *in vitro* and whether they can display different DNA binding properties, we used PKA and PKC as modifying kinases. As shown in Figure 7C, p105 was efficiently labelled by PKA and p100 less efficiently labelled, whereas their processing products, p50 and p52, were refractory to modification under the same conditions (compare lanes 2 and 4 with lanes 1 and 3). p65 was also efficiently phosphorylated (lane 5). Similar data were obtained using PKC (data not shown). The results are also in agreement with the observation that the isolated C-terminal precursor domain (Hatada *et al.*, 1992) can be phosphorylated by both PKA and PKC (unpublished data). As shown in Figure 7D (lanes 1–6) with recombinant proteins, DNA binding of p50 is unaffected by phosphoryl-

Table I. Alterations imposed on NF-κB/IκB proteins following cellular stimulation with TNF-α or H_2O_2

	p105	p100	p50	p52	p65	c-rel	IκBα
Association with p65	+	+	+	+	n.d.	+	+
Phosphorylation[a]	+	+	−	−	+	−	+
Constitutive phosphorylation in B cells	+	+	−	−	+	−	+
Processing	+	(+)	−	−	−	−	−
Degradation	−	−	−	−	−	−	+
Nuclear translocation	−	−	+	(+)	+	−	−

[a]Phosphorylation was observed for p65-associated proteins and also for total p100, p105, or IκBα (Figure 4 and data not shown). For H_2O_2 only phosphorylation data are shown.

ation with protein kinase A (lanes 1 and 2), whereas p65 homodimers or p50−p65 heterodimers displayed increased DNA binding activity upon phosphorylation (compare lane 3 with lane 4 and lane 5 with lane 6). The effect of phosphorylation on p65 by PKA *in vitro* may mimic a cellular kinase which may interact with the same site (although we do not exclude the possibility that even PKA may be active on p65 *in vivo*).

These results, which showed that the DNA binding activity p65 is phosphorylation-dependent, are significant because until now only phosphorylation of IκB molecules was known as a potential mechanism of NF-κB activation. In fact, there is this additional level of control which directly affects the DNA interaction of the transcriptionally most potent activator of the NF-κB/rel family (Schmitz and Baeuerle, 1991).

p105 is regulated by phosphorylation in vitro in the same fashion as IκBα

Because both NF-κB precursors are phosphorylated while being processed, and since the modification apparently occurs at the C-terminal domains which mediate the interaction of both p105 and p100 with p65 (Naumann *et al.*, 1993a,b), we decided to analyse the effect of modification on the precursor−p65 interaction *in vitro*.

Phosphorylation of a site in the repeat domain of IκBα by PKA abrogated the interaction of NF-κB and IκBα (D.Krappmann and C.Scheidereit, unpublished data) and phosphorylation of the isolated C-terminal half of p105 inhibited its interaction with p50 (Gerondakis *et al.*, 1993 and data not shown). This suggests that interaction of the entire precursor with NF-κB/rel factors might be regulated by phosphorylation. In Figure 8B we show with recombinant proteins that the ability of p105 to interact with p65 is abrogated by PKA phosphorylation: p65 forms complexes with p105, which are unable to bind to DNA (compare lanes 2 and 3). To demonstrate the effect of phosphorylation on complex formation, the increase of p65 DNA binding activity after kinase treatment (compare lanes 1 and 2), which can be blocked by the PKA inhibitor H7 (compare lanes 2 and 4), has to be considered. Therefore, p65 was treated with PKA, then H7 was added and incubated with unmodified p105 (lane 5). This led to p65−p105 complex formation despite the phosphorylation of p65. When p65 and p105 were instead incubated with PKA in separate reactions, followed by the addition of H7 and further incubation after mixing the two reactions, p65−p105 complex formation no longer occurred (lane 6). The observed p65 DNA binding activity corresponded to that of phosphorylated p65 alone (compare lanes 1 and 6). We conclude from this experiment that, upon phosphorylation, p105 loses its ability to interact with p65, whereas phosphorylated p65 can still interact with unmodified p105. Thus, p105 is regulated by phosphorylation *in vitro* as is IκBα (Ghosh and Baltimore, 1990).

Discussion

In B cells, some nuclear NF-κB activity is constitutive whereas in other cells nuclear activity is obtained only after cellular stimulation. In order to unravel the mechanisms of inducible and constitutive NF-κB activation, we have investigated alterations in the intracellular localization of NF-κB/rel factors. We have followed precursor processing and IκBα degradation after cytokine stimulation in HeLa cells and B cells. Secondly, we have analysed the phosphorylation state of these proteins following stimulation and found that protein phosphorylation appears to trigger the activation of NF-κB in intact cells.

We observed that in both cell types rapid induction of NF-κB involved activation of IκBα complexes as well as of NF-κB−precursor complexes as it was accompanied by a concomitant loss of IκBα and processing of p105. Processing of p100 was induced less efficiently. The very fast induction of precursor processing, within 10 min, suggested an immediate effect of TNF-α, in contrast to the slow induction of p105-processing observed hours after induction with PMA or LPS (Cordle *et al.*, 1993; Mercurio *et al.*, 1993). All factors analysed were present in roughly the same relative amounts in HeLa cells and Namalwa cells and induction led to a nuclear accumulation of p50 and p65, and of minor amounts of p52 but not of c-rel. The predominance of p50 and p65 as the major TNF-α-induced factors was also evident when analysing the induced nuclear DNA binding activity. It is possible that c-rel is induced with a longer lag period than p65 or p50, as has been demonstrated in other cell types (Tan *et al.*, 1992; Doerre *et al.*, 1993).

Our co-precipitation experiments were in agreement with the notion that both cell types contained p65 in complexes either with p105 or p100, or as heteromers with c-rel or p50 and IκBα, in agreement with previous studies (see Introduction). From the results of earlier work (Hatada *et al.*, 1993; Naumann *et al.*, 1993a) we also expect that p65, like other NF-κB/rel factors, forms heterodimers with p105 or p100, and that p50−p65 heterodimers form heterotrimers with IκBα. It thus appears that TNF-α specifically affects the activation of both p65−p105 and p65−p50−IκBα complexes by inducing processing and proteolysis (see Figure 9). This conclusion

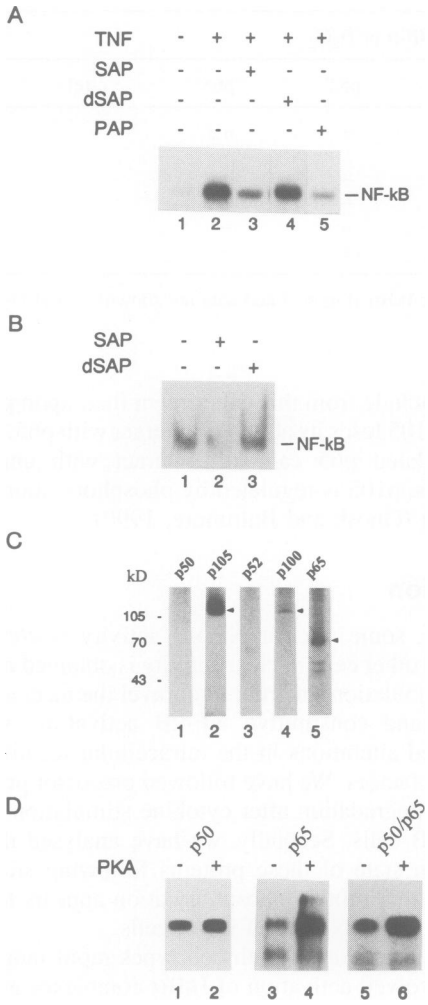

Fig. 7. Phosphorylation of p65 affects its DNA binding activity. (**A**) HeLa cells were either untreated (lane 1) or stimulated with TNF-α (lanes 2–5). Nuclear extracts were prepared and, prior to loading on the gel, treated with shrimp alkaline phosphatase (SAP, lane 3), heat-inactivated SAP (dSAP, lane 4) or potato acid phosphatase (PAP, lane 5) and assayed by EMSA using an Igκ probe. (**B**) p65-containing NF-κB was purified from nuclear extracts of TNF-α-induced HeLa cells by immuno-adsorption to an anti-p65 antibody column and elution with the peptide antigen. Eluted immunopurified NF-κB was assayed by EMSA before (lane 1) or after (lanes 2 and 3) addition of SAP or dSAP using an Igκ probe. (**C**) Recombinant NF-κB proteins were phosphorylated with PKA and [γ-^{32}P]ATP, separated by SDS–PAGE and visualized by autoradiography. The phosphorylated protein species are indicated with arrows. (**D**) Recombinant p50 (lanes 1 and 2), p65 (lanes 3 and 4) or p50–p65 heterodimers (lanes 5 and 6) were phosphorylated *in vitro* with PKA (lanes 2, 4 and 6) or incubated with control buffer (lanes 1, 3 and 5). Subsequently, the proteins were tested by EMSA, using the Igκ enhancer probe. Only sections of the autoradiograms are shown.

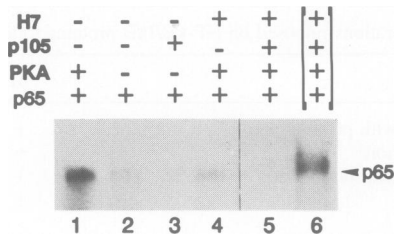

Fig. 8. The p65–p105 association is affected by phosphorylation with protein kinase A *in vitro*. Band shift analysis of the influence of phosphorylation on p105–p65 complex formation, using the H2-K site as a probe (lanes 1–6). As indicated, recombinant p65 was assayed without modification (lane 2), after phosphorylation with PKA (lane 1), after treatment with PKA in the presence of the PKA inhibitor H7 (lane 4) or after complex formation with p105 (lane 3). p65 was phosphorylated with PKA, H7 was added and p65 was further incubated with p105 before adding the DNA probe (lane 5). In separate reactions, both p65 and p105 were phosphorylated with PKA, followed by the addition of H7; then the reactions were mixed and incubated together before adding the DNA probe (lane 6). Note that only the protein–DNA complex is shown and that p65–p105 complex formation is reflected by the absence of p65–DNA complexes.

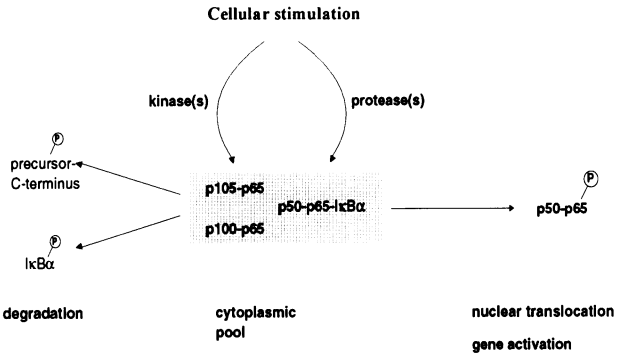

Fig. 9. Model for the activation of NF-κB. Signal-induced kinases phosphorylate IκBα, the C-terminal halves of the precursors and p65. After degradation of phosphorylated IκBα and processing of the precursors, active phosphorylated p50–p65 is released.

was also strongly supported by the fact that, at the time of TNF-α-induced precursor processing and IκBα degradation, these molecules became heavily phosphorylated in both cell types. Unexpectedly, p65 was also phosphorylated at the same time point, whereas neither c-rel, nor p50 nor p52 was modified. At present, we cannot determine whether phosphorylation of p65 is another prerequisite for processing and/or IκBα degradation, or a parallel phenomenon, by which the later nuclear activity of this factor is enhanced (see below).

The intracellular distribution of c-rel did not change despite the complete disappearance of IκBα after stimulation. An interesting possibility is that the identity of the rel/NF-κB components, and not only that of the IκB moieties, could determine which complexes can be activated through contributing to the recognition by modifying enzymes. In any case, inducible phosphorylation of the precursors and of IκBα strongly suggests that stimulus-dependent kinases act on these structures to induce the action of proteases.

Activation of NF-κB by numerous inducers, including TNF-α, can be blocked with oxygen-radical scavenger compounds (Staal *et al.*, 1990; Schreck *et al.*, 1991), which led to the proposal that ROIs are crucial second messengers. This was supported by the observation that the oxygen-radical forming compound hydrogen peroxide also induces NF-κB (Schreck *et al.*, 1991). Our demonstration that rapid incorporation of phosphoryl groups into NF-κB–IκB complexes is the final modification imposed by TNF-α stimulation on NF-κB–IκB suggests that ROIs might instead act upstream of a kinase or protease. Induction of NF-κB by hydrogen peroxide in fact led to phosphorylation of the same subunits which were modified after stimulation with TNF-α. Nuclear translocation of NF-κB after phorbol ester (PMA) stimulation also co-

incided with protein phosphorylation of cytoplasmic complexes (data not shown). Importantly, for each stimulating agent the (different) time points of nuclear translocation coincided with the time points of maximal phosphate incorporation, which strongly supports the notion that phosphorylation is the modifying signal which triggers activation of NF-κB by proteolysis.

A striking difference between HeLa cells and B cells was noted when comparing the phosphorylation state of the NF-κB/IκB proteins. Whereas in HeLa cells phosphorylation of p65 was strictly dependent on stimulation with an inducing agent, in Namalwa cells it appeared to be constitutive, with only a weak increase after cytokine addition. Furthermore, IκBα appeared in B cells as two constitutively and strongly phosphorylated species, one of which persisted after initial increase and one of which disappeared rapidly after stimulation. In addition, B lymphocytes revealed a stronger constitutive phosphorylation of both precursors than observed in HeLa cells. Obviously, in B cells kinases are constitutively active, which may explain the constitutive NF-κB activity in lymphoid cells. The constant phosphorylation of IκBα is in agreement with the observation that IκBα is more unstable in B cells, with a half-life of ~0.5 h, as compared with 1 h in Jurkat or HeLa cells (Rice and Ernst, 1993). The continuous *de novo* synthesis of IκBα is apparently accompanied by ongoing phosphorylation needed to maintain the high turnover. Collectively, these observations strongly support the model that kinases which are cell-specifically active or which are inducible, trigger the activation of NF-κB by directly phosphorylating the precursors, IκBα and even p65.

Several *in vitro* studies showed that phosphorylation of IκB abolishes its interaction with NF-κB (see Introduction). We showed here that PKA phosphorylates p105 *in vitro* at its C-terminal domain and that, as observed for IκBα, upon phosphorylation *in vitro*, p105 lost its ability to interact with p65. Similarly, IκBγ showed impaired interaction with either p50 or c-rel homodimers after modification with PKA (Gerondakis *et al.*, 1993). Phosphorylation of IκBα at a single site in the ankyrin-like repeat domain likewise led to a dissociation of recombinant IκBα–NF-κB complexes *in vitro* (unpublished data). The site which is phosphorylated in IκBα by both PKA and PKC is also conserved in p105 (unpublished observation), making it probable that it is also utilized by the kinases in p105. The effects are thus highly specific and not due to a mere hyper-phosphorylation; this is also supported by our observation that phosphorylation of p65 did not abolish its interaction with p105, while phosphorylation of p105 interfered with its interaction with p65 (see Figure 8). The biochemical experiments thus suggest that phosphorylation of complexes containing IκBα or p105 leads to their spontaneous dissociation.

In apparent contrast, we found that in intact cells phosphorylated precursor molecules or phosphorylated IκBα were still co-precipitated with p65, consistent with the observations made for IκBα by Beg *et al.* (1993) and Brown *et al.* (1993). This might indicate that phosphorylation does not abrogate protein–protein interactions prior to the action of proteases. In this context it must be taken into account that at the time of TNF-α-dependent hyperphosphorylation, the amounts of IκBα and precursor proteins were already strongly diminished. It is likely, therefore, that only a small fraction of the originally phosphorylated proteins were still associated and that the vast majority was dissociated and degraded or processed. The off-rate for the dissociation could be slow enough to co-precipitate some fraction of the phosphorylated proteins. Alternatively, phosphorylation of the complexes could strongly enhance recognition by proteases and degradation or processing of IκBα or p105/p100 could occur within their protein complexes, without dissociation of the inhibitory domains. Therefore, we propose that phosphorylation activates NF-κB by inducing the action of proteases, which recognize their phosphorylated substrates either in the complexes or after dissociation. Future experiments will address the exact mechanism of phosphorylation-induced proteolysis. For this purpose it will be important to map the sites which are modified *in vivo* and to identify the kinases and proteases. Our precipitation experiments with anti-p65 antibodies revealed further molecules in the size range of the precursors, which were phosphorylated constitutively and which interacted with p65, but apparently not with the precursors. These might be specific kinases or proteases or other functionally important components. We are currently attempting to isolate these proteins.

A surprising finding of our study was that the phosphorylation of p65 was constitutive in Namalwa cells but inducible in HeLa cells. We showed that cellular nuclear NF-κB containing p65 is phosphorylated and that dephosphorylation leads to a marked decrease in its DNA binding affinity. These experiments uncover a novel mechanism of NF-κB activation by kinases. In addition to the induction of nuclear translocation, kinases directly affect the nuclear activity of NF-κB by enhancing the affinity towards the target genes. This level of control has also been described for other transcription factors (for reviews see Hunter and Karin, 1992; Nichols *et al.*, 1992). The identification of the sites modified both *in vivo* and *in vitro* will have to be performed in order to understand how p65 is regulated by phosphorylation, whether it is by enhancing the activation of cytoplasmic complexes, improving nuclear uptake, increasing the dimerization efficiency or directly elevating the DNA binding affinity. Our data provide evidence for the last possibility, but we cannot exclude the possibility that p65 phosphorylation has effects at different levels.

Materials and methods

Cell culture

Namalwa and 70Z/3 cells were grown in RPMI 1640 (BRL/Gibco), and HeLa cells were grown in minimum essential medium (S-MEM). The media were supplemented with 4 mM glutamine, 1% non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum (FCS) with the exception that for HeLa cells 5% newborn calf serum (NCS) was used. The cells were stimulated with 10 ng/ml TNF-α (Gibco/BRL) or 250 μM H_2O_2 (Merck) for the indicated times.

Recombinant proteins

Expression plasmids pETp50 (amino acids 1–368), pETp105 (amino acids 18–970), p52pAR (ΔRI) (amino acids 1–373), pRSETCp100 (amino acids 1–900) and pETp65 (amino acids 1–550) have been described elsewhere (Naumann *et al.*, 1993a,b). The constructs were used for transformation of BL21[DE3]pLysS cells and bacterial proteins were gel purified and renatured as described (Hatada *et al.*, 1993).

Immunoblots

Cytoplasmic and nuclear extracts were prepared using a non-ionic detergent method. Briefly, the cells were washed and resuspended in buffer A (Dignam *et al.*, 1983) and 0.15% NP-40 was added. The cells were left for 10 min on ice and subsequently centrifuged at 1000 *g* for 10 min. The supernatant was used as cytoplasmic extract and the pellet was treated with buffer C (Dignam *et al.*, 1983) for 10 min to yield the nuclear extract. The disruption of the cytosolic membrane appeared to be more efficient than in the Dignam *et al.* (1983) protocol, without affecting the nuclear envelope. The cellular extracts were separated by SDS–PAGE and blotted onto PVDF Immobilon membranes (Millipore) in blotting buffer (25 mM Tris–HCl pH 8.3, 0.01% SDS, 0.1 mM dithioerithritol, 20% methanol) as described recently (Naumann *et al.*, 1993a).

Electrophoretic mobility shift assay

Gel retardation assays were performed with either an H-2K or an Igκ oligonucleotide probe. The DNA binding reactions were performed with the indicated fraction or recombinant protein in 20 µl binding buffer [2 µg poly(dI–dC), 1 µg BSA, 5 mM DTT, 20 mM HEPES (pH 8.4), 60 mM KCl and 8% Ficoll] for 20 min at 30°C. The HeLa cell nuclear extract fraction was dephosphorylated with 0.05 U acid phosphatase (Sigma) or 2.5 U shrimp alkaline phosphatase for 30 min at 37°C in the above reaction buffer prior to the DNA binding reaction. As a control heat denatured shrimp alkaline phosphatase was used. Purified NF-κB was obtained from nuclear extracts of TNF-α-stimulated HeLa cells. Affinity purification was performed with an anti-p65 antibody column from which NF-κB was eluted by competition with the epitope-containing peptide. The gels were run in 12.5 mM Tris and 96 mM glycine pH 8.3.

In vitro phosphorylation

In vitro phosphorylation of recombinant proteins (50 ng each) was carried out with protein kinase A (PKA) (Promega) in PKA buffer (40 mM Tris–HCl pH 7.4, 20 mM MgOAc, 0.2 mM ATP, with or without 40 µCi [γ-^{32}P]ATP) for 15 min at 30°C. The proteins were separated by SDS–PAGE and visualized by autoradiography. For testing PKA-dependent interactions of p65 and p105 by EMSA, the PKA inhibitor H7 [1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride] (Calbiochem) was used at a concentration of 3 mM. The indicated components or the appropriate buffers were subsequently added, followed by the probe in DNA binding buffer. The total incubation time was 1 h, with 15 min for each step at 30°C, before loading to the EMSA gel.

Antisera

The anti-p105C and the anti-p100N antisera have been described elsewhere (Hatada *et al.*, 1992; Naumann *et al.*, 1993b). The anti-IκBα antibody was raised against recombinant, gel-purified IκBα (amino acids 51–317). The anti-p100C antiserum was raised against a C-terminal part of the p100 molecule comprising amino acids 445–900. The anti-p105N antibody was raised against a peptide comprising amino acids 273–290. Anti-p65 antibody was raised against a peptide comprising amino acids 6–20 of human p65. The anti-c-rel antiserum was raised against a peptide comprising residues 572–586 of human c-rel.

In vivo labelling of cells and immunoprecipitation

HeLa or Namalwa cells (~10^7 cells/ml) were washed once with methionine-free EMEM (ICN) and incubated for 4 h with 300 µCi/ml [^{35}S]methionine in methionine-free EMEM containing 2% glutamine in the presence of dialysed serum. Prior to induction with TNF-α the cells were chased with cold methionine (2 mM). After induction the cells were washed in PBS and lysed in ice-cold RIPA buffer (20 mM Tris pH 8.0, 150 mM NaCl, 1% deoxycholate, 1% NP-40, 0.15% SDS, 5% glycerol, 1 mM EGTA, 10 mM NaF, 10 mM K_2HPO_4, 100 µM PMSF, 1% Trasylol and 10 µM pepstatin A). The cells were disrupted by passing the lysate several times through a 21 gauge syringe. The cell debris was centrifuged and the supernatant subsequently incubated with antisera coupled to protein A beads. The preimmune sera or antisera, 5 µl of each, were coupled to the protein A beads in PBS for 1 h at 4°C. In a first preadsorption step the supernatants were incubated with protein A beads coupled to preimmune serum for 1 h at 4°C. These immunoprecipitations were used as a control. After this preadsorption the supernatants were incubated with the appropriate antisera for 2–3 h. When using anti-peptide antisera an excess of the appropriate peptide (100 µg) was included as a control. In a second immunoprecipitation the samples from the first precipitation were boiled in 1% SDS for 10 min, then diluted with RIPA buffer and incubated with the other antibody. The precipitated proteins, coupled to protein A beads, were washed several times with 5 ml ice-cold RIPA buffer, boiled, separated by SDS–PAGE and exposed to X-ray film overnight.

For *in vivo* labelling with [^{32}P]orthophosphate the cells were washed once with phosphate-free EMEM (ICN) and subsequently incubated for 2 h with phosphate-free EMEM containing 2% glutamine in the presence of 10% dialysed FCS. Afterwards the cells were labelled with 1 mCi/ml for 45 min and subsequently induced at the indicated times. Further handling of the cells was performed as described for [^{35}S]methionine labelling.

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