Inhibition of NF-κB cellular function via specific targeting of the IκB-ubiquitin ligase

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Activation of the transcription factor NF-κB is a paradigm for signal transduction through the ubiquitin–proteasome pathway: ubiquitin-dependent degradation of the transcriptional inhibitor IκB in response to cell stimulation. A major issue in this context is the nature of the recognition signal and the targeting enzyme involved in the proteolytic process. Here we show that following a stimulus-dependent phosphorylation, and while associated with NF-κB, IκB is targeted by a specific ubiquitin-ligase via direct recognition of the signal-dependent phosphorylation site; phosphopeptides corresponding to this site specifically inhibit ubiquitin conjugation of IκB and its subsequent degradation. The ligase recognition signal is functionally conserved between IκBα and IκBβ, and does not involve the nearby ubiquitination site. Microinjection of the inhibitory peptides into stimulated cells abolished NF-κB activation in response to TNFα and the consequent expression of E-selectin, an NF-κB-dependent cell-adhesion molecule. Inhibition of NF-κB function by specific blocking of ubiquitin ligase activity provides a novel approach for intervening in cellular processes via regulation of unique proteolytic events.
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Introduction

NF-κB is a ubiquitous inducible transcription factor that is primarily involved in immune, inflammatory and stress responses (Baeuerle and Baltimore, 1996; Baldwin, 1996). It is retained in a latent form in the cytoplasm of non-stimulated cells by inhibitory molecules collectively termed IκBs. The IκB proteins are structurally related, distinguished by six homologous ankyrin repeats (Baeuerle and Baltimore, 1996). Four members of the family, IκBα, IκBβ, IκBε and *cactus* are 'professional inhibitors' specializing in NF-κB inhibition (Baeuerle and Baltimore, 1996; Whiteside *et al.*, 1997). They fulfill their task by a dual

action: masking the nuclear localization signal of the NF-κB proteins, thereby retaining the factor in the cytoplasm, and inhibiting both its DNA binding and transactivation capacity (Naumann and Scheidereit, 1994; Arenzana-Seisdedos *et al.*, 1995; Baldwin, 1996; Zhong *et al.*, 1997). The latter property might have evolved as a double safety mechanism to prevent incidental activity of NF-κB, which escaped into the nucleus along with its inhibitor, as well as a possible termination event for stimulus-induced NF-κB activity.

Other members of the IκB family share with the 'professional' ones the capacity for either retaining NF-κB in the cytoplasm and/or inhibiting its DNA binding, but have other functions as well, and their physiological role in the process of stimulus-induced NF-κB activation is not entirely clear (Verma *et al.*, 1995). With a few rare exceptions, all stimuli which induce NF-κB activation target the 'professional IκBs' to degradation through a phosphorylation-regulated process; site–specific phosphorylation directs the inhibitors to degradation via the ubiquitin-proteasome pathway (Baeuerle and Baltimore, 1996; Whiteside *et al.*, 1997). Following IκB degradation, NF-κB is translocated into the nucleus as an active factor, able to induce its target genes (Verma *et al.*, 1995; Baeuerle and Baltimore, 1996; Baldwin, 1996).

Ubiquitin-mediated degradation of key regulatory short-lived proteins plays an important role in basic cellular processes such as regulation of cell cycle and division, modulation of the immune system and inflammatory response, biogenesis of subcellular organelles and differentiation of tissues, modulation of cell membrane receptors, ion channels and the secretory pathway, DNA repair and control of transcription. Tight regulation of all these processes must involve timed and specific recognition of the many different substrates at the appropriate physiological and pathophysiological conditions. It is accepted now that a key element in the recognition process is a member of the ubiquitin–substrate ligase family of enzymes, E3. Following binding of the substrate via a specific structural motif, the enzyme transfers activated ubiquitin moieties from a ubiquitin-conjugating enzyme, E2, to a Lys residue in the target protein to generate a polyubiquitin tree. The tagged substrate is proteolyzed by a 26S proteasome complex with the release of free and reutilizable ubiquitin (reviewed recently in Deshaies, 1995; Jentsch and Schlenker, 1995; Ciechanover, 1996; Coux *et al.*, 1996). Recognition of a few substrates appears to be mediated via primary, constitutive signals, such as a specific exposed N-terminal residue (N-end rule; Varshavsky, 1992). Many other proteins are targeted via secondary signals such as post-translational modification(s) or association with ancillary proteins. Activation of enzymatic components of the ubiquitin system can also render their substrates susceptible to conjugation and

subsequent degradation. Despite the central role the ubiquitin system plays in the modulation of many key cellular proteins, the identity of the targeting signals in most of these cases is still elusive. Degradation of the T cell receptor ζ chain is triggered by binding of the appropriate ligand followed by Tyr phosphorylation of the receptor chain (Cenciarelli *et al.*, 1996). p53 is targeted for rapid degradation by Mdm2 (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997), whereas certain other proteins are conjugated and degraded following their association with the molecular chaperone Hsc70 (Lee *et al.*, 1996; Bercovich *et al.*, 1997). The E3 recognition domain in these cases has not been described either. Programmed degradation of mitotic cyclins involves at least two signals, a destruction box in the N-terminal domain of the molecule (Glotzer *et al.*, 1991) and phosphorylation-dependent activation of the cyclosome, the E3-containing complex (or Anaphase Promoting Complex; APC) which is initiated by the protein kinase cdc2/cyclin B (Lahav-Baratz *et al.*, 1995). Here too, the role of the destruction box, the identity of the cyclosome component that is phosphorylated and the structure of the phosphorylation domain are not known (King *et al.*, 1996).

To understand how IκB is targeted by the ubiquitin-proteasome pathway following the stimulus-dependent phosphorylation, we developed a cell-free system that reproduces certain steps of the *in vivo* inducible degradation of the NF-κB inhibitor. This was the basis for our investigation of the mode of IκB recognition by the ubiquitin system, as well as a lead for developing specific means for modulating cellular NF-κB activity.

Results

A high molecular weight cellular complex restricts the ubiquitination of IκBα to the post-stimulation phase

It has been previously shown that phosphorylation of Ser32 and 36 and ubiquitination at Lys21 and 22 are essential for targeting IκBα for signal-induced degradation by the ubiquitin–proteasome system (Baldwin, 1996). It has remained unclear whether the phosphoserine and lysine residues constitute the direct targeting signal through which the ubiquitin system recognizes the modified inhibitor. To investigate the mode of phosphorylated IκBα (pIκBα) recognition, we developed an *in vitro* ubiquitination assay which faithfully reproduces the *in vivo* fate of pIκBα. *In vivo*, the ubiquitination of IκBα appears to be coupled to its inducible site-specific phosphorylation (Alkalay *et al.*, 1995; Z.J.Chen *et al.*, 1995; DiDonato *et al.*, 1996). Therefore, for studying the ubiquitination step *per se*, it was necessary to separate the two IκBα modification steps: prepare a phosphorylated substrate separately and submit it to a ubiquitination assay. Cellular extracts from stimulated Jurkat or non-stimulated HeLa cells were incubated in the presence of *in vitro* translated [35S]IκBα, ATP and the phosphatase inhibitor okadaic acid. This procedure results in specific phosphorylation of [35S]IκBα at Ser32 and 36 and its association with the cellular-derived NF-κB complex, but does not promote pIκBα ubiquitination (Figure 1A, lane 3). Following the *in vitro* phosphorylation step, [35S]IκBα was coimmunoprecipitated with anti-p65 antiserum. Specific
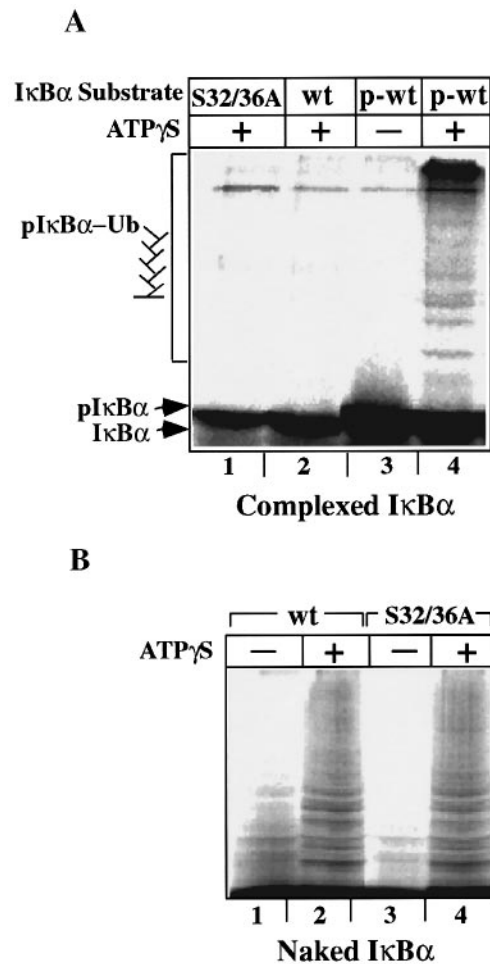
Fig. 1. Specific ubiquitination of NF-κB-associated pIκBα. (**A**) *In vitro* ubiquitination of NF-κB-associated [35S]IκBα in HeLa cell lysate (see Materials and methods). Lanes 1 and 2, control IκBα protein-substrates incubated in the presence of ATPγS. Lane 1, S32/36A mutant IκBα; lane 2, non-phosphorylated wild-type-IκBα (wt). Lanes 3 and 4, phosphorylated IκBα (p-wt), incubated in the absence or presence of ATPγS, respectively. pIκBα-ubiquitin tree is marked. (**B**) Ubiquitination of naked recombinant [35S]IκBα in HeLa cell extract in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of ATPγS.

[35S]IκBα phosphorylation is evident in the slower gel-migration of the immunopurified inhibitor (lane 3), compared with the appearance of a serine-mutant (lane 1) or of wild-type [35S]IκBα complex prepared in the absence of okadaic acid (lane 2). The immunopurified [35S]IκBα species were subjected to *in vitro* ubiquitination in a HeLa cell lysate supplemented with ubiquitin and ATPγS, which supports ubiquitination, but not IκBα phosphorylation (unpublished data) or proteasome-mediated degradation (Johnston and Cohen, 1991). Under these conditions, only wild-type [35S]pIκBα generated multiple ubiquitinated species that were not detected in the absence of ATPγS (Figure 1A, compare lanes 3 and 4). This result indicates that the observed ubiquitin adducts were generated exclusively during the ubiquitination assay and were not carried over from the previous phosphorylation step. It therefore appears that conditions specific for IκBα phosphorylation do not support ubiquitination of the inhibitor. Neither 35S-labeled S32/36A mutant of IκBα (lane 1) nor the non-phosphorylated wild-type [35S]IκBα (lane 2) were ubiquit-
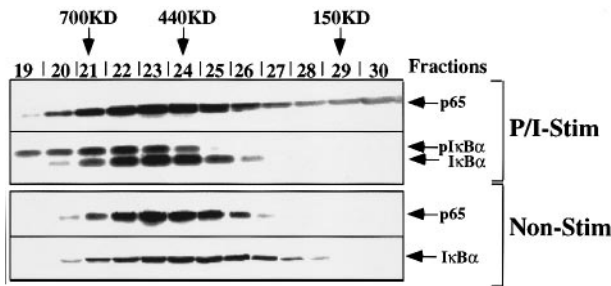
Fig. 2. Phosphorylated IκBα is preferentially associated with high molecular mass complexes. Cellular extracts from PMA and Ca^{2+} ionophore stimulated (P/I-Stim) or non-stimulated Jurkat cells were fractionated on a Superdex 200 gel filtration column and analyzed by Western blot for the presence of pIκBα, IκBα and p65. Fractions 19–21 are size-excluded by this column. Molecular mass markers corresponding to different fractions are indicated. The apparent up-shift of the p65 signal in stimulated cell extracts is a consequence of gel-running conditions and is not due to any modification of the protein.

inated. The physiological relevance of this assay was further corroborated by comparing the *in vitro* ubiquitination of naked [^{35}S]IκBα with that of a complex-associated, phosphorylated substrate. Whereas a complex-associated S32/36A mutant and non-phosphorylated wild-type species were not ubiquitinated (Figure 1A, lanes 1 and 2) in accordance with their *in vivo* fate (DiDonato *et al.*, 1996), their corresponding naked forms were readily conjugated (Figure 1B, lanes 2 and 4). Similarly, only naked, but not a complex-associated K21/22R mutant could be ubiquitinated *in vitro* (data not shown). Thus, it appears that whereas naked IκBα is non-discriminately recognized by the ubiquitin system, potential ubiquitination sites of the complexed inhibitor are masked by its associated proteins prior to IκBα phosphorylation.

The differential ubiquitination properties of IκBα might have been determined by the associated NF-κB complex, post-translational modifications or by other cellular factors. To study some of these properties, we fractionated IκBα from stimulated and non-stimulated cell-lysates on a sizing column and compared their profiles. Surprisingly, the non-phosphorylated IκBα is part of a cellular assembly (~440 kDa) that is larger than would be expected from an IκBα/NF-κB complex (Figure 2). Yet the phosphorylated IκBα, which is only detectable in proteasome-inhibited, stimulated cells, is associated with much larger complexes, mostly excluded from the sizing column. Rel A (p65), as well as other components of NF-κB (not shown), is segregated, along with pIκBα, in the high molecular weight fractions of stimulated cells (Figure 2). Therefore, it appears that a distinct property of the pre-degradation complex (pIκBα/NF-κB complex) is its large size, possibly the consequence of the recruitment of additional cellular proteins following cell stimulation.

Specific inhibition of pIκBα and pIκBβ ubiquitination by Ser 32/36 phosphorylated peptides

To further study the mode of pIκBα recognition, we used a series of peptides spanning the N-terminal signaling domain of the protein, phosphorylated at either one or both serine residues 32 and 36, as well as unmodified and serine-substituted peptides (Figure 3A). The peptides were

tested for inhibition of pIκBα ubiquitination in a cell-free assay. Only IκBα peptides that were phosphorylated at both serine 32 and 36 (pIκBα peptides) inhibited ubiquitination effectively (Figure 3B). Neither a c-Fos phosphopeptide (lane 2) nor a non-phosphorylated peptide (lane 3) had any detectable effect on the ubiquitination of pIκBα. Singly phosphorylated peptides (lanes 4 and 5) inhibited pIκBα conjugation at an IC$_{50}$ of 300 μM. In contrast, doubly phosphorylated IκBα peptides inhibited pIκB conjugation (lanes 6–9) at an IC$_{50}$ of 5–15 μM. The minimal size peptide tested (pp7, lane 9 and see Figure 3A), which virtually spans the signaling phosphorylation site, is sufficient to effectively inhibit ubiquitination, although at somewhat higher IC$_{50}$ (15 μM, not shown). Lys 21 and 22 (excluded from all pIκBα peptides except pp21), which constitute the conjugation site of IκBα both *in vivo* and *in vitro* (Scherer *et al.*, 1995; Baldi *et al.*, 1996; DiDonato *et al.*, 1996) are not essential for the inhibitory effect of the peptides.

The specificity of the IκBα phosphopeptides was tested in a random ubiquitin-conjugation reaction: conjugation of the general population of proteins in HeLa extract (detected by conjugation of [^{125}I]ubiquitin). The pIκBα peptide did not inhibit this non-specific conjugation reaction (Figure 3C), suggesting that the peptide does not have a general inhibitory effect on ubiquitination. Likewise, the pIκBα peptide did not inhibit the ubiquitin-conjugation of several other substrates such as p53 and MyoD, nor did it affect the ubiquitination of naked wild-type or S32/36A mutant IκBα (data not shown), indicating the selectivity of the peptide effect. Importantly, pIκBα peptides were found to abolish the ubiquitination of the pIκBα related substrate pIκBβ (Figure 3D). Similar to the conjugation of pIκBα, specific ubiquitination of IκBβ required an associated NF-κB complex and prior phosphorylation at the homologous residues Ser19 and 23 (Thompson *et al.*, 1995; DiDonato *et al.*, 1996). IκBβ complex prepared in the absence of okadaic acid was not modified by ubiquitination (Figure 3D, lane 1). pIκBα inhibitory peptides (lanes 4 and 6) affected pIκBβ ubiquitination at a similar IC$_{50}$, whereas non-phosphorylated IκBα peptide (lane 7) or a control phosphopeptide (lane 5) had no effect. Hence, it is likely that the same enzyme(s) target both pIκBα and pIκBβ for ubiquitin-dependent degradation.

IκBα-phosphorylated peptides block the specific *in vitro* degradation of pIκBα

The inhibitory peptides were tested in a complementary ubiquitin-dependent *in vitro* degradation assay. Using this assay, we have previously shown that only pIκBα (derived from stimulated cells) is degraded *in vitro* in a ubiquitin-dependent manner, whereas the non-phosphorylated IκBα from the same cell extract remains stable (Alkalay *et al.*, 1995). The pIκBα signal was abolished in the presence of ATP, with no effect on the non-phosphorylated inhibitor (Figure 4, compare lanes 1 and 2). The absence of the pIκBα signal could be attributed to ubiquitination *per se*, i.e. the formation of high molecular mass IκBα adducts, or to degradation that follows ubiquitination (Alkalay *et al.*, 1995). Yet a prolonged exposure of the entire blot did not reveal any high molecular mass IκBα species (data not shown), thus the elimination of pIκBα must be
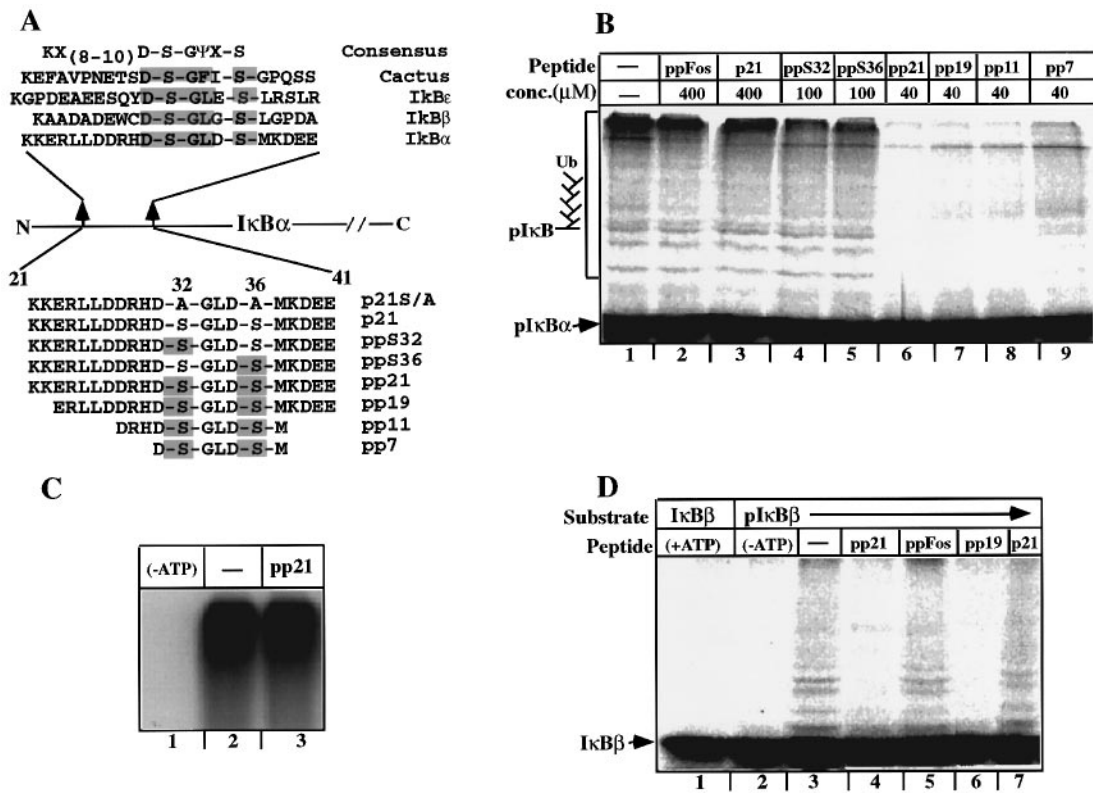
Fig. 3. pIκBα peptides inhibit the specific ubiquitination of pIκBα and pIκBβ. (**A**) Schematic representation of the conserved N-terminal region of IκBs (see Whiteside *et al.*, 1997) and corresponding peptides. Shaded characters above the IκBα line represent residues that are conserved among all IκBs. Shaded S, below the IκBα-line, represents phosphoserine. Ψ represents a hydrophobic amino acid. (**B**) *In vitro* ubiquitination of NF-κB-associated [35S]IκBα in HeLa cell extract in the presence of various synthetic peptides (see Materials and methods). Lane 1, no peptide; lane 2, ppFos; lanes 3–6, peptides corresponding to IκBα residues 21–41. Lane 3, p21 (non-phosphorylated); lane 4, ppSer32; lane 5, ppSer36. Lanes 6–9, ppSer 32,36 peptides of decreasing length. p denotes peptide; pp denotes phospho-peptide. (**C**) Ubiquitination of random HeLa proteins in the presence of pIκBα peptide. Ubiquitination was performed in complete HeLa cell extract in the presence of [125I]ubiquitin. Lane 1, without ATPγS; lane 2, with ATPγS; lane 3, with ATPγs and pp21 (400 μM). (**D**) pIκBα peptides inhibit pIκBβ ubiquitination. NF-κB-associated [35S]IκBβ was subjected to ubiquitination in the presence of various peptides. Lane 1, non-phosphorylated [35S]IκBβ; lanes 2–7, phosphorylated substrate (prepared in the presence of okadaic acid). Lane 2, without ATPγS; lanes 3–7, with ATPγS and the following peptides: lane 3, no peptide; lane 4, pp21 (40 μM); lane 5 ppFos (400 μM); lane 6, pp19 (40 μM); lane 7, p21 (400 μM).

attributed to degradation. The addition of the conjugation-inhibitory phosphopeptides to the degradation assay resulted in stabilization of pIκBα (lanes 3 and 4), whereas the non-phosphorylated IκBα peptide or a control phospho-Fos peptide had minimal or no effect on the specific degradation of pIκBα (lanes 5 and 6). Shorter peptides lacking Lys residues 21/22 were fully effective as well (lane 4 and data not shown), implying that the peptides do not abolish pIκBα degradation by exhausting the ubiquitin–proteasome system components as conjugatable substrates.

pIκBα interacts with a specific component of the ubiquitin system

pIκBα-ubiquitin conjugation and degradation requires a full complement of the ubiquitin system enzymes: E1, a specific E2 (E2-F1) and the as yet unidentified ubiquitin ligase (E3) (Alkalay *et al.*, 1995; Chen *et al.*, 1996) which is likely to bind specifically to the substrate prior to tagging it with ubiquitin (Hershko and Ciechanover, 1992; Ciechanover, 1994). The effect of the pIκBα peptides suggest that they interact with a specific component of the ubiquitin–system. To show that this is indeed the case, we fractionated HeLa lysate over different IκBα peptide columns and assayed the flow-through fractions for pIκBα
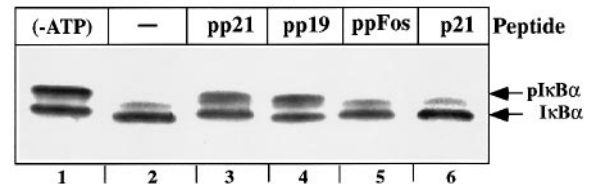
Fig. 4. Phosphorylated IκBα peptides inhibit *in vitro* degradation of pIκBα. pIκBα was immunopurified from stimulated Jurkat cells and subjected to ATP-dependent degradation in reticulocyte extract (Alkalay *et al.*, 1995). Lane 1, without ATP; lane 2, with ATP; lanes 3–6, degradation in the presence of ATP and the indicated peptides (400 μM). IκBα was detected by Western blot analysis with a specific rabbit antiserum (Alkalay *et al.*, 1995). See legend to Figure 3 (panel B) for peptide abbreviations. The origin of the faint band above the IκBα band (lanes 2, 5 and 6) is unknown.

conjugation (Figure 5A). Whereas flow-through fractions from a S32/36A peptide column (lane 2) or a non-phosphorylated peptide column (data not shown) maintained full pIκBα conjugation capacity, a flow-through fraction from a pIκBα peptide adsorbent lost most of its pIκBα-specific conjugation capacity (lane 3). The depleted conjugating activity could be complemented by reticulocyte Fraction II (lane 4), which contains all known species of E3 enzymes (A.Ciechanover, unpublished data), but

A

B
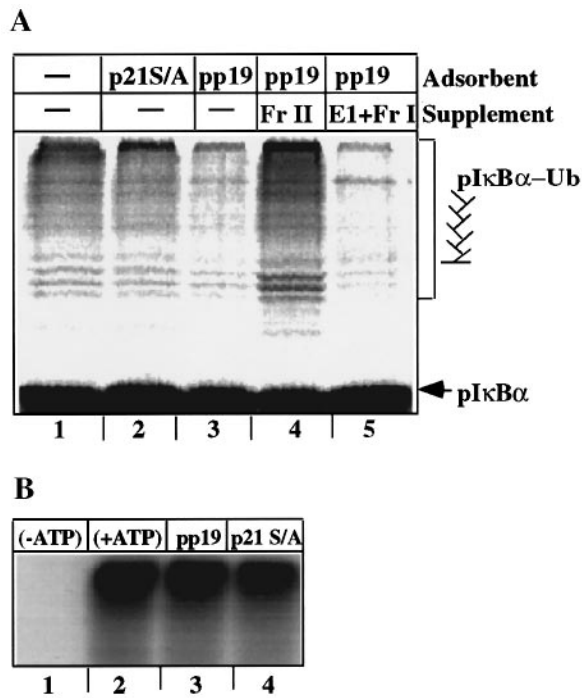
Fig. 5. Specific binding of pIκB-ubiquitin ligase to pIκBα peptides.
(**A**) pIκB-E3 activity is specifically adsorbed onto immobilized pIκBα peptide. HeLa cell extract was fractionated over different peptide columns and the flow-through fractions were assayed for pIκBα-ubiquitination activity (see Figure 3, panel B). Lane 1, non-fractionated HeLa extract; lanes 2–5, flow through fractions from the different indicated peptide adsorbents. pp19-flow-through fractions were supplemented with purified E1 (Hershko and Ciechanover, 1992) and reticulocyte Fraction I (lane 5) (Blumenfeld *et al.*, 1994) or Fraction II (lane 4). The pIκBα ubiquitination tree is marked.
(**B**) pp19 and p21 (S/A) flow-through fractions retain conjugating activity towards random HeLa proteins (see Figure 3C). Lane 1, without ATPγS; lanes 2–4, with ATPγS; lanes 3–4, flow-through fractions from the indicated adsorbents.

not by purified E1 and Fraction I, which contains E2-F1 (Blumenfeld *et al.*, 1994) (lane 5), indicating that the pIκBα peptide column depleted a specific E3 activity. Again, IκBα Lys 21 and 22 were dispensable for retaining the E3, emphasizing the distinction between the substrate recognition and conjugation sites. The peptide column depletion was found to be specific for the pIκBα-E3, as both column flow-through fractions maintained full activity in random HeLa protein conjugation (Figure 5B). These results indicate that a specific component of the ubiquitin system is responsible for recognition of the pIκBs at the identified site.

Microinjection of pIκBα peptides abolish NF-κB activation in stimulated cells

The inhibitory effect of specific peptides on pIκB degradation suggest a potential for specific modulation of cellular NF-κB functions by targeting the pIκB-ubiquitin ligase, E3. To assess this possibility, we tested the effects of the inhibitory peptides in intact HeLa cells. Cells were microinjected with a pIκBα or a control phospho-peptide and immediately stimulated with TNFα. TNFα induces rapid nuclear translocation of NF-κB, evident by p65 nuclear staining of 90% of the cells (Figure 6, panels A–C and G). The pIκBα peptide abolished TNFα-stimulated NF-κB activation in most of the microinjected cells (Figure

6, panel G; see representative fields in panels A and B). In contrast, the control peptide had no effect on the rate of NF-κB-induced nuclear translocation (panels C and G). To rule out the possibility that NF-κB inhibition was due to competition for the IκBα-kinase rather than for the E3, we compared the kinase inhibitory capacity of the pIκBα peptide pp21 with that of the non-phosphorylated peptide p21 in an *in vitro* assay. Whereas at a concentration corresponding to the microinjection content p21 inhibited nearly 50% of the signal-specific IκBα phosphorylation, pp21 had no effect (Figure 6H).

To evaluate the functional consequences of NF-κB inhibition, the IκBα-E3 inhibitory peptide was microinjected into primary human vascular endothelial cells (HUVECs). These cells respond to TNFα by surface expression of NF-κB-regulated adhesion proteins such as I-CAM-1, V-CAM-1 and E-selectin (C.C.Chen *et al.*, 1995; Read *et al.*, 1995). E-selectin expression is particularly NF-κB dependent (C.C.Chen *et al.*, 1995), and is the major inducible endothelial adhesion molecule for initial neutrophil attachment and rolling on activated endothelium (Read *et al.*, 1995). 70% of the HUVECs were intensely stained for E-selectin following stimulation (Figure 6, panels D, F and G). Microinjection of the pIκBα peptide resulted in the inhibition of E-selectin expression (panels D, E and G): only 20% of the microinjected cells were stained by the E-selectin antibodies. Microinjection of the control phospho-peptide had no effect on the rate of E-selectin expression (panels F and G). The inhibitory effect of the pIκBα peptide was specific for NF-κB-dependent expression, since there was no effect on expression of the NF-κB-independent adhesion molecule ICAM-2 (data not shown).

Discussion

NF-κB activation represents a striking example of the targeting of a regulatory protein, IκB, by the ubiquitin–proteasome system as an integral step of a signal transduction process. Following cell stimulation, IκB is modified at a specific site in the N-terminal region that is well conserved among the three mammalian IκBs and their *Drosophila* homolog *cactus* (Baeuerle and Baltimore, 1996; Whiteside *et al.*, 1997 and see Figure 3A). Two serine residues of this site must be phosphorylated to allow degradation of the inhibitor. Mutation of either serine residue is sufficient to render IκB resistant to degradation (Brockman *et al.*, 1995; Brown *et al.*, 1995; Traenckner *et al.*, 1995; Z.J.Chen *et al.*, 1995; Sun *et al.*, 1996).

Several steps of the signal-dependent IκBα degradation can now be faithfully reproduced in a cell-free system. Although it was previously reported that a ubiquitination step is involved in IκBα phosphorylation (Chen *et al.*, 1996), we observed no need for ubiquitin in the IκBα phosphorylation step (data not shown). Neither was there evidence for IκBα ubiquitination throughout this step (Figure 1). It should be noted that the evidence for ubiquitin-modification in the activation of the IκB-kinase was indirect, as the enzyme was not purified to homogeneity (Chen *et al.*, 1996). Later studies from the same laboratory indicated that ubiquitin-activation of the IκB-kinase may not be exclusive, as the kinase can alternatively
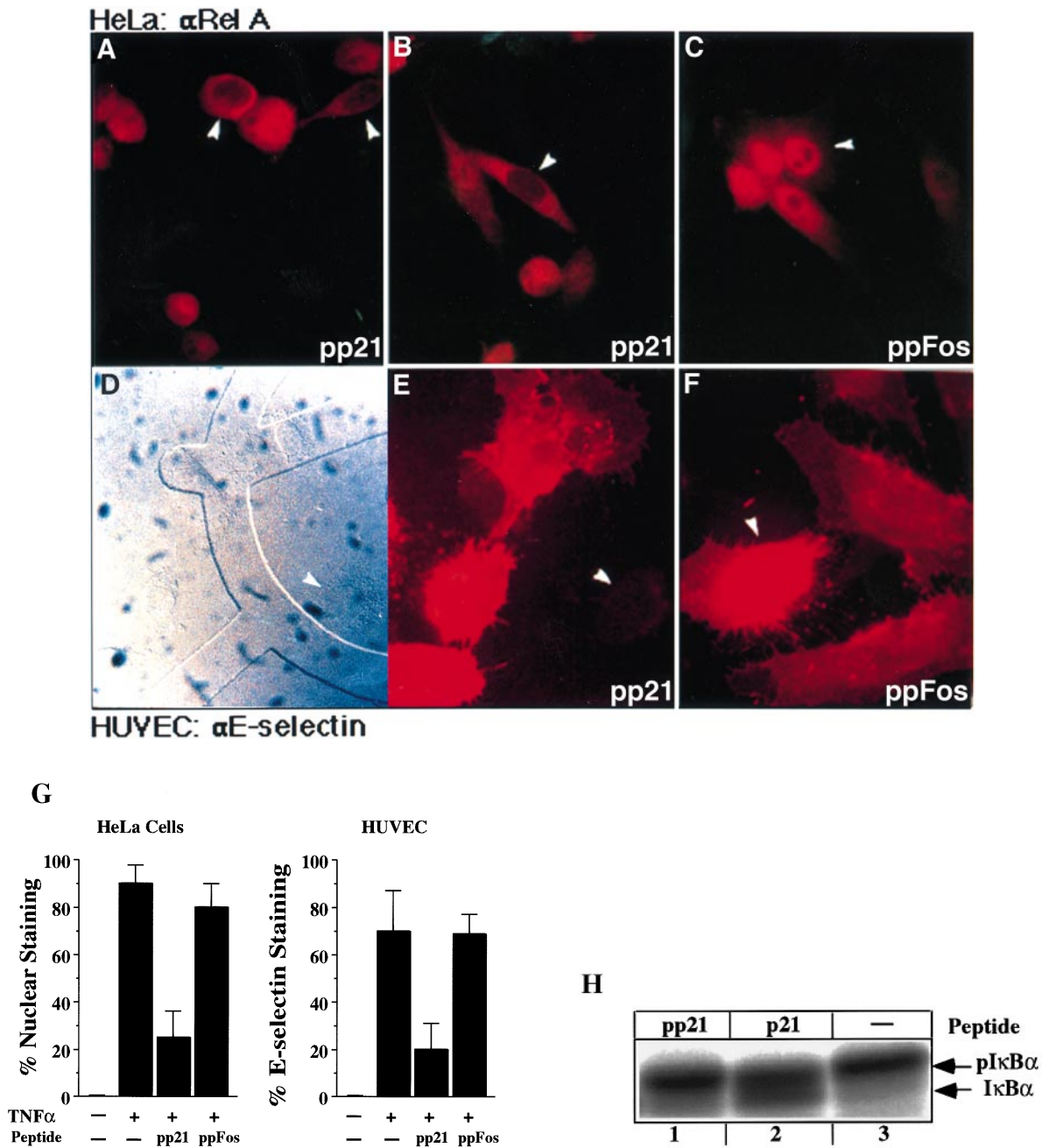
Fig. 6. Microinjection of a specific E3-binding peptide abolishes nuclear NF-κB translocation and the consequent expression of E-selectin. Panels A–C, microinjection into HeLa cells of an E3-binding peptide pp21 (**A** and **B**) or a ppFos control peptide (**C**), followed by anti-p65 staining. Injected cells are marked by arrows. Panels D–F, microinjection of pp21 (**E**) or ppFos (**F**) into human vascular endothelial cells (HUVECs), followed by E-selectin staining. Panel **D** is a phase-contrast photograph of panel E. Injected cells are marked by arrows. Panel **G** represents graphic analysis of the microinjection experiments: Left, percent cells displaying nuclear p65 staining of HeLa cells. 90 and 42 cells were microinjected with pp21 and ppFos, respectively. Right, percent cells displaying E-selectin staining of HUVECs. 160 and 36 cells were microinjected with pp21 and ppFos, respectively. Panel **H**, the IκBα peptide, but not the phosphorylated peptide, inhibits IκBα phosphorylation. [^{35}S]IκBα was subjected to an *in vitro* kinase assay (see Materials and methods) in the absence (lane 3) or presence of 200 μg/ml p21 (lane 2) or pp21 (lane 1). The concentration of the tested peptides correspond to the estimated concentration of the microinjected peptides in the cells.

be regulated by the JNK signaling pathway (Lee *et al.*, 1997). Recently, the IκB-kinase was identified as CHUK, a kinase that is part of a pathway distinct from the JNK cascade. Again, no involvement of the ubiquitin system was evident in the CHUK activation pathway (DiDonato *et al.*, 1997; Regnier *et al.*, 1997). Hence, it appears that the two IκB modification events, phosphorylation and ubiquitination, are carried out sequentially by independent enzymatic systems.

Whereas *in vivo*, the NF-κB-associated, non-phosphorylated inhibitor is relatively stable, exogenously-expressed IκBα is rapidly degraded (Rice and Ernst, 1993; Scott *et al.*, 1993). Indeed, our *in vitro* data show that naked IκBα is non-discriminately recognized and conjugated, most probably at lysine residues that are bared in the naked form, but are masked in the complex-associated inhibitor (Figure 1). This feature restricts IκB degradation to the post-stimulation phase when it is exposed to the

ubiquitin system following the site-specific phosphorylation. Particularly interesting in this respect is the nature of the pIκBα-associated complex. The IκBα complex from resting cells is larger than the expected mass of an NF-κB/IκBα complex and must, therefore, contain other proteins, some of which may have a role in shielding it from incidental ubiquitination. Indeed, it has recently been reported that IκBα and IκBβ are associated with another regulatory protein, protein kinase-A (Zhong *et al.*, 1997). Upon cell stimulation, we noticed that pIκBα, but not the non-phosphorylated inhibitor, is associated, along with NF-κB, with much larger complexes (Figure 2). It is therefore likely that following the site-specific phosphorylation, other cellular proteins, perhaps some components of the ubiquitin system, associate with pIκB, a subject worthy of further investigation.

How then is IκB recognized by the ubiquitin system? Is it due to a phosphorylation-induced conformational change, e.g. the exposure of a concealed binding and/or conjugation site, or is it the consequence of direct recognition of the modification site? Our results indicate that the ubiquitin system directly recognizes the signal-induced phosphorylation site: (i) Short peptides spanning the phosphorylation sites effectively inhibit the *in vitro* ubiquitination of pIκB provided that they are phosphorylated on both serine residues, 32 and 36. (ii) Lysine residues 21 and 22, which are essential for the *in vivo* (Baldi *et al.*, 1996) and *in vitro* (Scherer *et al.*, 1995) ubiquitination of pIκBα, are not required for the inhibitory activity of the pIκBα peptide implying that the pIκB recognition site is distinct from the conjugation site. This observation argues against the conjugation-site exposure model, as if the latter was correct, the lysine residues would have to be part of the conjugation-site competitors. (iii) Doubly phosphorylated, immobilized pIκBα peptides retain the specific pIκBα-conjugating activity of a HeLa lysate, pointing to the direct binding of a specific ubiquitin system component to the pIκB phosphorylation site.

In general, the basis for substrate specificity in ubiquitin-mediated degradation processes is poorly understood. Both E2 and E3 enzymes have been implicated in specific ubiquitination events (Ciechanover, 1994). *In vitro* ubiquitination and degradation of pIκBα involve a particular E2, E2-F1 or the related enzymes Ubc4 and Ubc5 (Alkalay *et al.*, 1995; Chen *et al.*, 1996). Yet our results suggest that the recognition of pIκB is mediated by a specific E3. The pIκB-conjugating activity that is specifically depleted by the peptide column is complemented by reticulocyte Fraction II which contains all the known E3s (unpublished results), but not by the E2-F1-containing Fraction I. Preliminary experiments suggest that the Fraction II component that binds the modified IκB site is not one of the few known E3 species (unpublished data). The same component appears to recognize both pIκBα and pIκBβ, as pIκBα peptides effectively inhibit the ubiquitination of pIκBβ. Therefore, while it appears that the E3s represent a large, mostly unfamiliar family of enzymes, it is unlikely that each enzyme targets a single substrate. Rather, it is conceivable that different cellular proteins are recognized by a single ligase via similar but not necessarily identical structural elements, such as the one shared by pIκBα and pIκBβ.

With the exception of the 'N-end rule' (Varshavsky,

1992), for which no physiological substrate has been assigned, the mode of substrate recognition by the ubiquitin system remains obscure. Certain substrates besides IκB must be phosphorylated prior to ubiquitination. The few known examples include G_1 cyclins and the Cdk inhibitor Sic1, both recognized by F-box proteins or by their associated cullins (CDC53-related proteins) (Jackson, 1996; Willems *et al.*, 1996). Whereas it is not evident whether a phosphate group or a phosphorylated residue is part of the F-box/cullins recognition signal, it is unlikely that the recognition of pIκB is determined by phosphorylation *per se* for the following reasons: (i) Doubly phosphorylated peptides that are not related to pIκB are non-inhibitory. (ii) Singly phosphorylated IκB peptides are extremely poor inhibitors of pIκB ubiquitination, in contrast to the doubly phosphorylated peptides. (iii) Mutation at the conserved IκB residue Asp31, adjacent to the phosphorylated serine, abolishes the inducible degradation of IκBα (unpublished data). Similarly unlikely is the notion that the recognition of pIκB is dominated by simple ionic interactions, as substitution of Ser32 and 36 by glutamic acid does not promote pIκBα ubiquitination (Chen *et al.*, 1996), nor are Ser/Glu substituted peptides inhibitory in our *in vitro* ubiquitination assay (data not shown).

Rather, it appears that the recognition of IκBs is mediated by a specific recognition motif. This motif is probably composed of six amino acids, DS(PO_3)GΨXS-(PO_3), conserved among all 'professional' IκBs (α, β, ε and cactus) (Figure 3A). Interestingly, a similar motif occurs among the β catenins (DSGΨXS) which, like the IκBs, are serine-phosphorylated and are probably subject to degradation via the ubiquitin-proteasome pathway (I.Alkalay, unpublished results; Aberle *et al.*, 1997). Both in IκBs and catenins, mutations at either serine residue of the putative ubiquitin-recognition site result in stabilization of the protein *in vivo* (Baldwin, 1996; Morin *et al.*, 1997, Rubinfeld *et al.*, 1997). A lysine residue, located 9–12 amino acids N-terminal to the recognition site, is also conserved among the two protein families. Our data indicate that this residue, which is the conjugation site in IκBs, is not part of the recognition motif. This finding implies that if a single enzyme mediates the recognition and ubiquitin ligation of IκB, it bears two distinct sites: a recognition site and a catalytic site that accommodates the lysine residue. Alternatively, the two sites could reside on two distinct proteins that might be part of a single complex, and the inhibitory peptide would affect the recognition protein. The latter would then resemble the ubiquitination of G_1 and mitotic cyclins, which is mediated by distinct large E3 complexes (King *et al.*, 1996). These are composed of several different proteins, the individual function of which is not well specified (King *et al.*, 1996). The observed high molecular weight predegradation complex of pIκBα (Figure 2) may function in a similar way to these cyclin-degradation complexes.

The identification of the putative pIκB-E3 recognition motif has important practical implications. Introduction of short peptides based on this motif into intact cells abrogates a specific cellular process, nuclear translocation of NF-κB in response to cytokine stimulation. Consequently, we showed that the microinjected peptides abolish an important function of NF-κB, the expression of an

inducible cell-adhesion molecule. Using a similar strategy, it may be possible to alleviate inflammatory processes mediated by NF-κB. A particularly attractive example is the rheumatoid arthritis model, where NF-κB is activated in synoviocytes of the inflamed joints (Marok *et al.*, 1996). Introduction of inhibitory peptide derivatives into such joints may help to induce remission. Obviously, the capacity to modulate specific cellular processes via the targeting of a ubiquitin-ligase opens up new perspectives for research and drug development.

Materials and methods

Synthetic peptides
HPLC-purified synthetic peptides (doubly, singly and non-phosphorylated peptides) were purchased from SynPep (Dublin, CA). All peptides were analyzed by mass-spectrometry, verified for the predicted structure and proved to be over 85% pure. Peptides used for microinjection were highly purified (over 95%).

In vitro kinase and ubiquitination assays
HA-tagged IκBα or HA-tagged IκBβ cDNAs were translated *in vitro* in wheat germ extract in the presence of [^{35}S]methionine according to the manufacturer's instructions. To phosphorylate IκBα or IκBβ, 1 μl of the *in vitro* translated protein was incubated in a reaction mixture containing (in a final volume of 30 μl) 100 μg HeLa or Jurkat cell extract (prepared as previously described; Alkalay *et al.*, 1995), 50 mM Tris (pH 7.6), 2 mM MgCl$_2$, 2 mM ATP, 0.3 mg/ml creatine phosphokinase, 10 mM phosphocreatine and 1 μM okadaic acid. During this incubation, the labeled inhibitor associates with the endogenous NF-κB complex. Following incubation for 90 min at 30°C, 1 μl of anti-p65 serum was added, and the NF-κB immune complex was immobilized to protein A–Sepharose as described (Alkalay *et al.*, 1995). The heterotrimeric p50–p65–IκB immobilized complex was subjected to either conjugation or degradation (Alkalay *et al.*, 1995) in a cell-free system, in either HeLa or reticulocyte extracts. For the conjugation assay, the immobilized substrate was agitated in a Thermomix (Eppendorf) for 90 min at 37°C in reaction buffer containing 100 μg hypotonic HeLa cell lysate, 50 mM Tris (pH 7.6), 2 mM MgCl$_2$, 1 mM DTT, 20 nM okadaic acid, 1 mg/ml bovine ubiquitin (Sigma) and 5 mM ATPγS (Sigma). Following the assay the IκB beads were boiled in SDS-buffer and the sample was analyzed by SDS–PAGE (9%), followed by fluorography. To identify the IκB–ubiquitin ligase recognition motif, various peptides were added at the indicated concentrations to the reaction mixtures in the presence of the peptidase inhibitor Bestatin (40 μg/ml). When conjugation of free IκBα was monitored, the translated protein was added directly to the conjugation reaction mixture. Measurement of ubiquitin conjugation to the bulk of cellular proteins was followed by incubating [^{125}I]ubiquitin in the conjugation reaction mixture (Alkalay *et al.*, 1995).

Fractionation of Jurkat cytoplasmic extracts and Western blot analysis
Jurkat cells (10^7/ml) were preincubated in complete RPMI medium in the presence of the proteasome inhibitor ALLN (150 μM Calpain Inhibitor 1, Calbiochem) for 2 h and stimulated for 12 min with PMA (10 ng/ml) and the Ca^{2+} ionophore A23187 (1 μM). Hypotonic cellular extracts were prepared as described (Alkalay *et al.*, 1995), supplemented with phosphatase inhibitors (20 mM *p*-nitrophenyl phosphate, 20 mM glycerol 2-phosphate) and separated by size exclusion chromatography on a Superdex 200 column in buffer containing 50 mM Tris–HCl (pH 7.5), 1 mM DTT and 150 mM NaCl. The fractions were analyzed by Western blot as described (Alkalay *et al.*, 1995).

IκB-E3 binding assay
Peptides were coupled to NHS-Sepharose (Pharmacia) according to the manufacturer's instructions at a concentration of 2 mg/ml. 100 μg of HeLa extract were incubated with 2.5 μl coupled resin in the presence of 0.1% NP40 and 3% ovalbumin for 1 h at 4°C. The resin was discarded and the unbound material tested in the ubiquitination assay. Reticulocyte fractions I and II were prepared by anion-exchange chromatography as described (Blumenfeld *et al.*, 1994).

Microinjection and immunofluorescence
HeLa or HUVEC cells (Read *et al.*, 1995) were plated on a grid coverslips (Cellocate, Eppendorf) 18 h before microinjection. Microinjection was performed by a semi-automated apparatus (Eppendorf). Peptides were injected into the cell cytoplasm at a concentration of 2 mg/ml in 100 mM KCl, 5 mM Na$_2$HPO$_4$ (pH 7.2), and immediately activated with TNFα [200 U/ml (Chiron, Emeryville, CA)]. HeLa and HUVEC cells were stimulated for 20 min and 3 h, respectively, fixed and antibody-stained as described (Mercurio *et al.*, 1993).

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