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CORRECTION

Efficient nuclear export of p65-IκBα complexes requires 14-3-3 proteins

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The GST-14-3-3 Coomassie stain shown in Fig. 2B was inadvertently duplicated from that shown in Fig. 2A. In Fig. 2C, the left-hand and middle GST Coomassie stains were also inadvertently duplicated, and annotation of the re-ordering of the GST-BD42 and GST-BD42A samples was omitted. Given that the data are more than 10 years old, the original images are no longer available for assembly of a corrected figure; however, the corresponding original membranes were available and have been restained with Ponceau and Coomassie stains, as indicated. The figure below shows the restaining of the membranes for Ponceau and Coomassie. Additionally, a scan of the original print out of the ^{32}P-peptide autoradiograph for Fig. 2C is shown. These errors do not affect the conclusions of the study.

The authors apologise to the readers for any confusion that this error might have caused.

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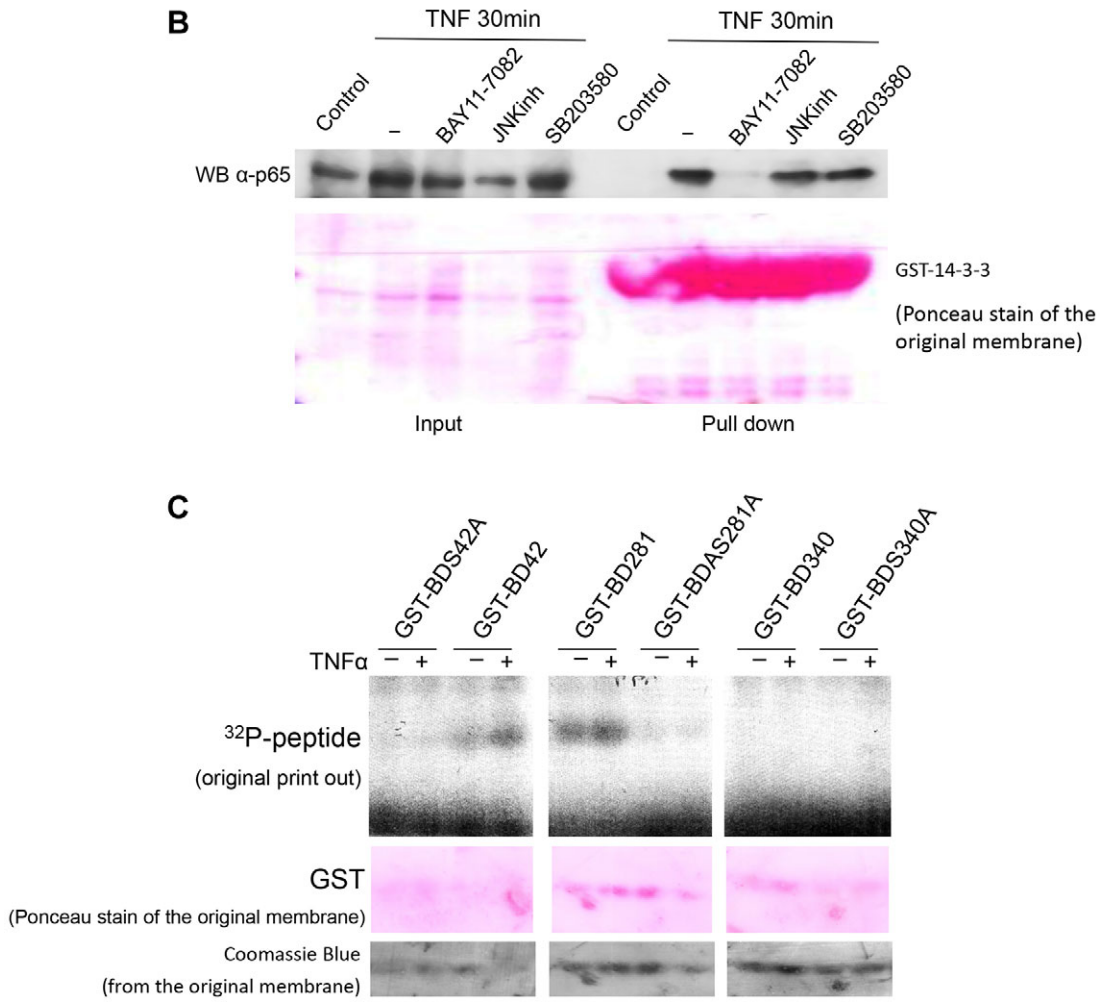

Fig. Original data for Fig. 2 of C. Aguilera et al, 2006.

Journal of Cell Science

Efficient nuclear export of p65-IκBα complexes requires 14-3-3 proteins

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Summary

IκB are responsible for maintaining p65 in the cytoplasm under non-stimulating conditions and promoting the active export of p65 from the nucleus following NFκB activation to terminate the signal. We now show that 14-3-3 proteins regulate the NFκB signaling pathway by physically interacting with p65 and IκBα proteins. We identify two functional 14-3-3 binding domains in the p65 protein involving residues 38-44 and 278-283, and map the interaction region of IκBα in residues 60-65. Mutation of these 14-3-3 binding domains in p65 or IκBα results in a predominantly nuclear distribution of both proteins. TNFα treatment promotes recruitment of 14-3-3 and IκBα to NFκB-dependent promoters and enhances the binding of 14-3-3 to p65. Disrupting 14-3-3 activity by transfection with a dominant-negative 14-3-3 leads to the accumulation of nuclear p65-IκBα complexes and the constitutive association of p65 with the chromatin. In this situation, NFκB-dependent genes become unresponsive to TNFα stimulation. Together our results indicate that 14-3-3 proteins facilitate the nuclear export of IκBα-p65 complexes and are required for the appropriate regulation of NFκB signaling.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/119/17/3695/DC1

Key words: NFκB, IκBα, 14-3-3, Nuclear export, TNFα

Introduction

Altered NFκB activity has been linked to several human diseases such as inflammation and cancer. Several extracellular stimuli such as TNFα lead to IκB phosphorylation (residues 32-36 in IκBα) and degradation, thus releasing NFκB and permitting the transcriptional activation of NFκB target genes. In general, IκB proteins inhibit NFκB by maintaining p65 in the cytoplasm (reviewed by Baldwin, 1996) whereas IκBα plays a unique role in actively removing p65 from the nucleus because of its ability for nucleocytoplasmic shuttling both under basal conditions (Huang et al., 2000; Johnson et al., 1999) or in response to stimuli (Huang et al., 2000; Huang and Miyamoto, 2001; Johnson et al., 1999; Nomura et al., 2003; Tam and Sen, 2001). Cytoplasmic retention of p65 has been classically associated with IκB; however, in cells lacking isoforms α, β and ϵ, p65 is still in the cytoplasm (Tergaonkar et al., 2005). Although p100 and p105 can interact with p65, other unknown proteins may be involved (Moorthy and Ghosh, 2003; Prigent et al., 2000; Tergaonkar et al., 2005).

14-3-3 is a highly conserved family of proteins that regulate a wide variety of signal transduction pathways (Fu et al., 2000; Tzivion et al., 2001) by promoting the cytoplasmic export of prephosphorylated substrates (Brunet et al., 2002; Grozinger and Schreiber, 2000). Many different 14-3-3 targets have already been identified, such as Raf-1 (Thorson et al., 1998), the cell cycle regulator Cdc25 (Lopez-Girona et al., 1999), histone deacetylases (Grozinger and Schreiber, 2000), the proapoptotic factors FKHRL1 (Brunet et al., 2002), Bad (Hsu et al., 1997) and Bax (Nomura et al., 2003), the tumor suppressor p53 (Waterman et al., 1998) or the kinase Par1 (Benton et al., 2002), and most, but not all, require prephosphorylated 14-3-3 binding domains corresponding to the RxxpSxP or RxxxpSxP consensus, where pS represents a phosphorylated serine. Although 14-3-3 are primarily cytoplasmic proteins, it has been shown that protein kinase B-dependent phosphorylation of the 14-3-3 binding sites of FKHRL1, which promote its cytoplasmic translocation, occurs in the nucleus, thus suggesting that the interaction between both proteins takes place in this cellular compartment (Brunet et al., 2002).

Since nucleocytoplasmic shuttling is crucial in the regulation of NFκB activity, we investigated whether 14-3-3 proteins may play a role in this signaling pathway. Here we show that 14-3-3 proteins interact with both p65 and IκB, and play a role in the regulation of the NFκB pathway after TNFα treatment by facilitating the export of IκBα-p65 complexes.

Results

TNFα induces p65 binding to 14-3-3

Using pull-down experiments, we first demonstrated that p65 physically bound to GST-14-3-3η in response to TNFα stimulation and that this interaction was maintained after 60 minutes of chronic treatment in HEK-293T cells (Fig. 1a). We next confirmed this interaction by precipitating endogenous p65 from these cells and detecting 14-3-3 after TNFα treatment in the precipitates (Fig. 1b). By sequence analysis, we identified three putative 14-3-3 binding domains containing the RxxSxP and RxxxSxP (Yaffe, 2002), involving residues 38-44

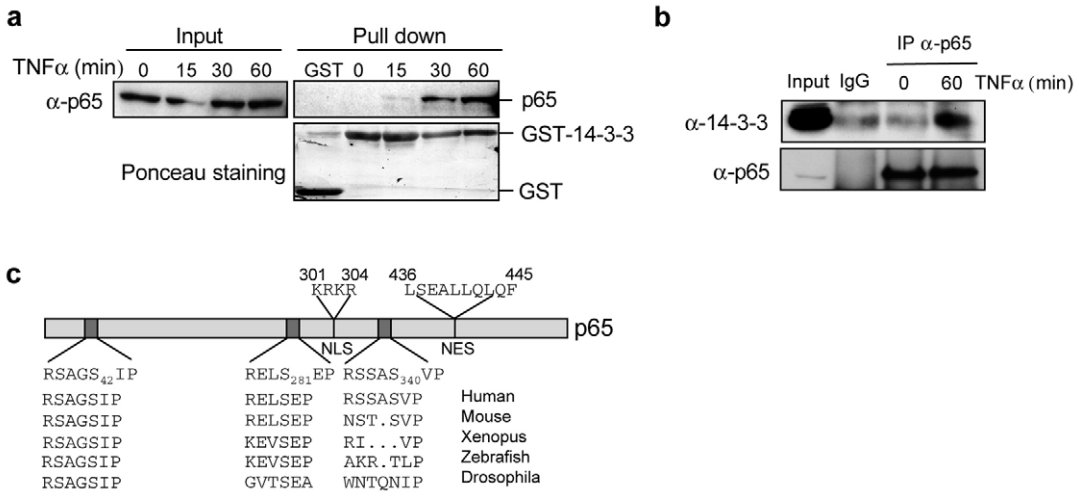

Fig. 1. p65 binds to 14-3-3 proteins in response to TNFα. (a) Pull-down experiment using GST-14-3-3η and lysates from untransfected HEK-293T cells incubated with TNFα at the indicated times. Upper panel shows immunoblot with anti-p65 antibody. Ponceau staining of GST proteins is shown in the lower panel. Inputs represent 1/10 of the lysates. (b) Cell lysates from HEK-293T, untreated or treated with TNFα, were precipitated with anti-p65 antibody or rabbit IgG as a control. The presence of 14-3-3 in the precipitates was determined by immunoblotting with anti-14-3-3β antibody recognizing different 14-3-3 isoforms (α-14-3-3). Inputs represent 1/10 of the lysates. (c) Schematic overview of the p65 protein sequence indicating the putative 14-3-3 binding domains.

(BD42), 278-283 (BD281), and 336-342 (BD340) of human p65. Nevertheless, only two of these domains, 38-44 and 278-283 are evolutionary conserved, suggestive of their functional relevance (Fig. 1c).

In many identified 14-3-3 substrates, phosphorylation is required for 14-3-3 binding (Muslin et al., 1996). To further investigate whether binding of p65 to 14-3-3 was phosphorylation dependent, we incubated protein extracts from TNFα-treated HEK-293T cells with acid phosphatase and performed pull-down assays with GST-14-3-3η. In Fig. 2a, we show that phosphatase treatment completely abolished the TNFα-induced binding of p65 to 14-3-3, indicating that this interaction was phosphorylation dependent. To investigate which kinases were involved in regulating this interaction, we treated HEK-293T cells with different kinase inhibitors such as BAY11-7082 (IKK), JNK inhibitor, SB203580 (p38), wortmannin (PKC and PI3K), PD98059 (ERK) and H89 (PKA and MSK) and used the different cell lysates for pull-down assays with GST-14-3-3η (Fig. 2b and supplementary material, Fig. S1). Our results demonstrated that BAY11-7082 specifically abrogated the interaction between p65 with 14-3-3 suggesting that IKK activity is required for p65 binding to 14-3-3.

We next tested whether the three theoretical 14-3-3 binding domains of p65 were phosphorylated in response to TNFα. With this aim we generated GST fusion proteins that contained these domains from wild-type p65 (GST-BD42, -BD281 and -BD340) and the corresponding Ser to Ala mutants. Wild-type (wt) or mutated GST-p65 peptides were incubated with untreated or TNFα-treated cell lysates in the presence of [^{32}P]ATP. We detected TNFα-dependent phosphorylation in GST-BD42 and GST-BD281 proteins that was abrogated by the point mutations S42A and S281A (Fig. 2c), whereas GST-BD340 was not phosphorylated. To confirm that S42 and S281 were phosphorylated in response to TNFα, an antibody that specifically binds to phosphorylated 14-3-3 binding motifs (α-P-14-3-3BM) efficiently recognized precipitated GFP-p65 from TNFα treated cells and in a minor extent the GFP-p65 mutants S42A and S281A (Fig. 2d).

To test whether these binding domains were functionally involved in the interaction between p65 and 14-3-3, we performed pull-down assays with cell extracts from HEK-293T cells transfected with GFP-p65wt or the point mutants S42A, S281A and S340A. In Fig. 2e, we show that 30 minutes after TNFα treatment, interaction between 14-3-3 and p65 is severely reduced in mutants S42A and S281A. Together, these results indicate that both Ser42 and Ser281 are phosphorylated in response to TNFα to create two functional 14-3-3 binding sites in p65. Although we did not detect phosphorylation of Ser340 in response to TNFα, we observed a decrease in the interaction between S340A mutant and 14-3-3. Based on this result, we cannot exclude the fact that BD340 is also involved in 14-3-3 binding.

IκBα interacts with 14-3-3 in a TNFα-independent manner

We next tested whether other NFκB family members were also associated with 14-3-3 proteins. Using pull-down experiments, we demonstrated that the NFκB inhibitor IκBα binds to GST-14-3-3 in untreated HEK-293T cells, whereas TNFα resulted in a decrease in the total levels of IκBα as expected, and also in the amount of IκBα bound to 14-3-3 (Fig. 3a). However, co-precipitation experiments from HEK-293T cells transfected with the non-degradable IκBα$_{32-36}$ demonstrated that TNFα treatment does not inhibit the binding of IκBα to 14-3-3 (Fig. 3b).

Pull-down assays with phosphatase-treated cell extracts demonstrated that phosphorylation of IκBα was not required for its binding to 14-3-3 (Fig. 3c). Next, we analyzed the IκBα protein sequence for the presence of conserved 14-3-3 binding

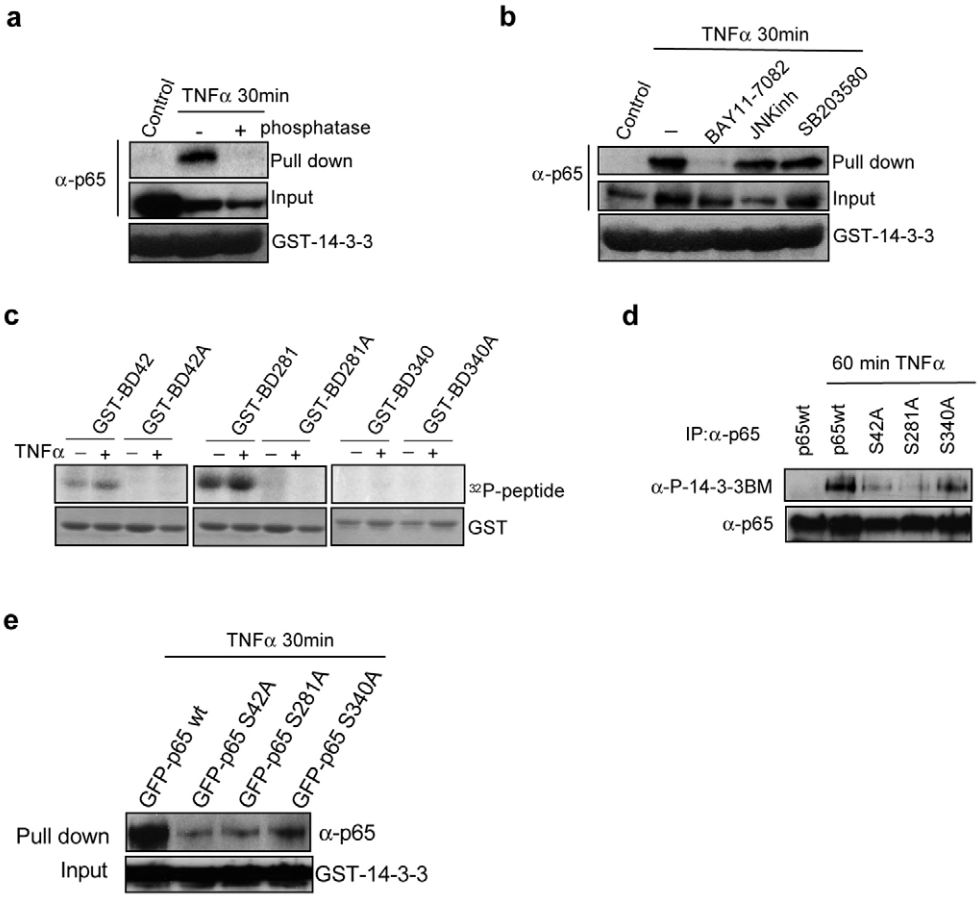

Fig. 2. Interaction between p65 and 14-3-3 depends on phosphorylation. (a,b) Pull-down assay with GST-14-3-3η and cell lysates from TNFα-treated HEK-293T cells incubated with acid phosphatase for 30 minutes (a) or from cells incubated for 2 hours with the indicated inhibitors plus 30 minutes with TNFα (b). The presence of p65 in the precipitates was determined by western blot with anti-p65 antibody. Coomassie staining of GST proteins is shown in lower panel. Inputs represent 1/10 of the lysates. (c) In vitro kinase assay to test the capacity of total cell lysates, from untreated or TNFα-treated HEK-293T cells, to phosphorylate GST-p65 peptides including the putative 14-3-3 binding domains. Upper panels show phosphorylated peptides by autoradiography. Coomassie staining of GST proteins is shown in lower panel. (d) Cell lysates from HEK-293T transfected with the full-length GFP-p65wt and the indicated mutant plasmids, treated for 60 minutes with TNFα were precipitated with the anti-p65 antibody. Western blot analysis with anti-P-14-3-3 binding motif antibody confirmed the presence of phosphorylated 14-3-3 binding domains in p65. Precipitated GFP-p65 protein levels are shown in the lower panel. (e) Pull-down experiment using GST-14-3-3η and lysates from HEK-293T cells transfected with the indicated p65 mutants and treated for 30 minutes with TNFα. Upper panel shows immunoblot with anti-p65. Coomassie staining of GST proteins is shown in lower panel. Inputs represent 1/10 of the lysates.

motifs. As we could not identify any consensus, we generated sequential IκBα deletion mutants (Fig. 3d-f) and tested their ability to bind 14-3-3 in pull-down assays. From these experiments, we identified a unique region in the first ankyrin repeat of IκBα that was required for the interaction of IκBα with 14-3-3. Since IκBαΔ55-59 still bound GST-14-3-3 whereas IκBαΔ55-65 did not (Fig. 3f), we propose that 14-3-3 binding domain of IκBα includes residues AA60-65, that greatly resembles a 14-3-3 binding motif (Fig. 3f).

14-3-3 binding domains of p65 and IκBα are required for their efficient nuclear export

Since 14-3-3 proteins regulate the subcellular distribution of many of their substrates, we determined the subcellular localization of GFP-p65wt compared with GFP-p65 mutants S42A and S281A in RPW1 (Fig. 4a) and HEK-293T (data not shown) cells. As shown in Fig. 4a, transiently transfected GFP-p65 was mainly cytoplasmic, as expected. Single mutation of S42 or S340 to Ala resulted in a moderate increase in the nuclear localization of GFP-p65 (56% and 66% respectively) compared with the wild type (43%) whereas S281A mutation was sufficient to promote the nuclear retention of p65 in almost all the cells (94%) (Fig. 4a). These results indicate that the 14-3-3 binding domains of p65, and particularly BD281, are required for cytoplasmic localization.

To test the functional relevance of the 14-3-3-binding region of IκBα, we determined the subcellular distribution of the 14-3-3-binding-deficient mutant, IκBαΔ55-65, as well as its capacity to induce cytoplasmic localization of p65 in IκBα-deficient MEFs. As shown in Fig. 4b, transfected flag-p65 primarily localized in the nucleus in most of the IκBα$^{-/-}$ cells (86%). Coexpression of the non-degradable IκBα$_{32-36}$ or the IκBαΔ55-59 mutant that binds 14-3-3 leads to the cytoplasmic redistribution of GFP-p65 compared to the control (24% and

19% nuclear, respectively). By contrast, the IκBαΔ55-65 mutant, lacking the 14-3-3 binding site, failed to retain p65 in the cytoplasm (76% nuclear) (Fig. 4b). Of note, localization of this IκBαΔ55-65 mutant, containing an intact NES (see Fig. 3f), was predominantly nuclear, suggesting that 14-3-3 also regulates subcellular distribution of IκBα. As a control, we performed coprecipitation experiments to demonstrate that mutations in the 14-3-3 binding motifs do not affect the interaction between p65 and IκBα (Fig. 4c,d).

14-3-3 activity is required for the appropriate nuclear export of p65-IκBα complexes

To test whether the aberrant nuclear distribution of the p65 and IκBα mutants was due to 14-3-3, we analyzed the subcellular localization of endogenous p65 (Fig. 5a) in control HEK-293T cells transfected with a dominant-negative form of 14-3-3 (DN-14-3-3) (Thorson et al., 1998) that blocks the interaction between p65 and 14-3-3 induced by TNFα (Fig. 5b). Confocal microscopy showed that in unstimulated HEK-293T cells, DN-

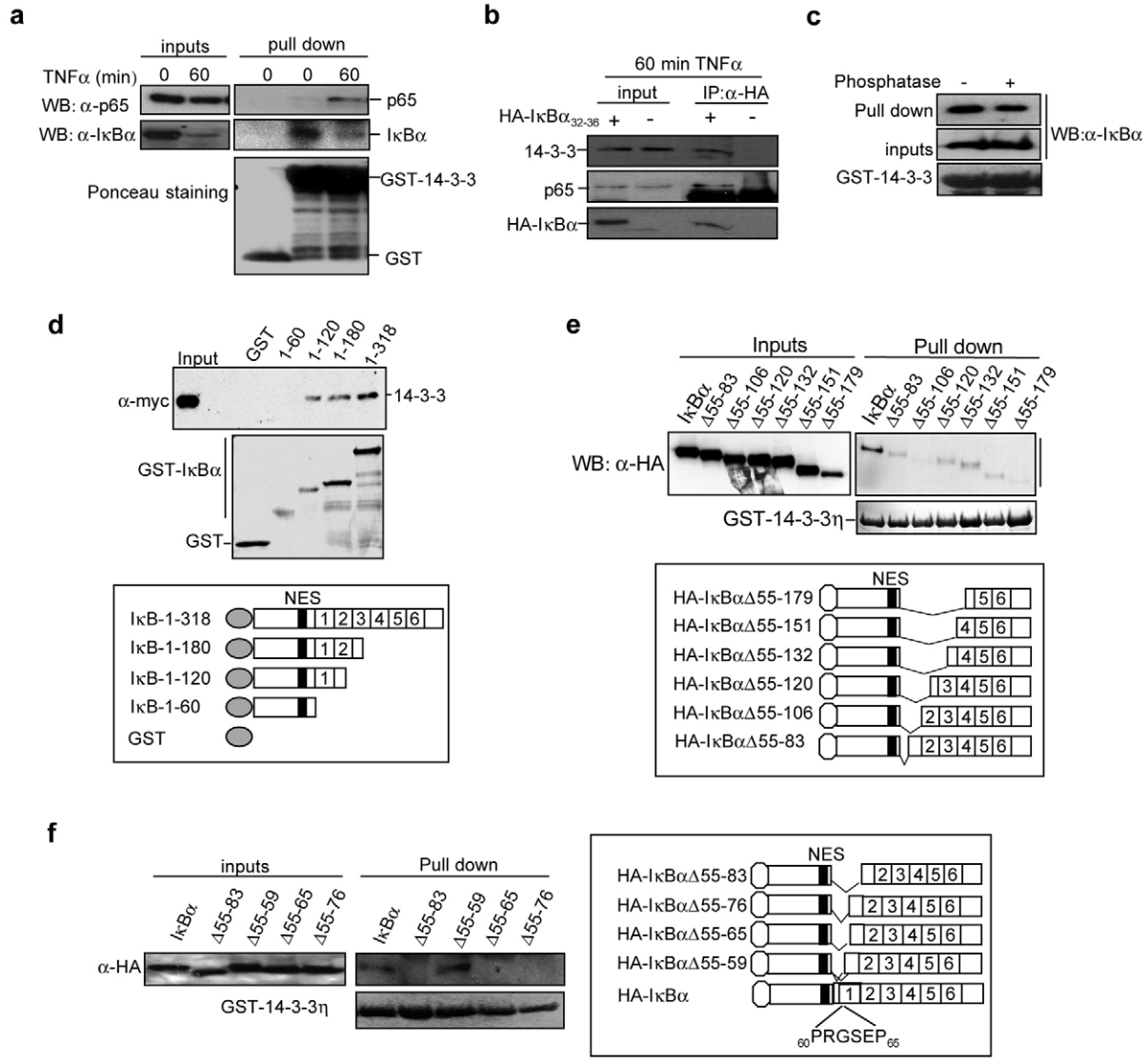

Fig 3. IκBα binds to 14-3-3 proteins independently of TNFα. (a) Pull-down experiment using GST-14-3-3η and lysates from HEK-293T cells incubated with TNFα as indicated. Upper panel shows immunoblot with α-p65 and α-IκBα antibodies. Ponceau staining of GST proteins is shown in lower panel. Inputs represent 1/10 of the lysates. (b) Immunoprecipitation with anti-HA from control HEK-293T cells and cells transfected with HA-IκBα$_{32-36}$, treated for 60 minutes with TNFα. The presence of co-precipitated endogenous 14-3-3 and p65 was determined by western blot with anti-14-3-3γ/η or anti-p65 antibodies. (c) Pull-down assay with GST-14-3-3η and cell lysates from HEK-293T cells untreated or treated with acid phosphatase for 30 minutes. The presence of IκBα proteins in the precipitates was determined by immunoblotting with anti-IκBα antibody. Ponceau staining of GST proteins is shown in the lower panel. Inputs represent 1/10 of the lysates. (d) Pull-down experiment using different GST-IκBα constructs and lysates from HEK-293T cells transfected with myc-14-3-3η. Upper panel shows immunoblot with anti-myc antibody. Coomassie staining of GST proteins is shown in middle panel. (e,f) Pull down experiments using GST-14-3-3η and lysates from HEK-293T cells transfected with the indicated Ha-IκBα deletion mutants. Upper panels show immunoblot with α-HA. Coomassie staining of GST proteins is shown in the lower right panels. Inputs represent 1/10 of the lysates. A schematic overview of the IκBα protein is shown in d-f, indicating the putative 14-3-3 binding domain.

14-3-3 induces a partial redistribution of p65 into the nuclear compartment compared with the exclusively cytoplasmic p65 observed in the control or in cells transfected with wild-type 14-3-3. After 15 minutes of TNFα treatment, nuclear entry of p65 was observed in both control cells and cells expressing myc-14-3-3 constructs, as expected. Interestingly, expression of DN-14-3-3 significantly reduced the nuclear export of p65 after 60 minutes of TNFα treatment (Fig. 5a). By western blot (Fig. 5a) and immunofluorescence (data not shown) analysis with anti-myc antibody, we confirmed that 80-90% of the cells expressed similar levels of the 14-3-3 constructs. Since IκBα is mainly responsible for p65 nuclear export after TNFα activation, we reasoned that nuclear persistence of p65 in the DN-14-3-3-expressing cells in both non-stimulated and 60-minute TNFα-treated cells might be due to (1) the nuclear accumulation of IκBα-p65 complexes or (2) the impairment of nuclear p65 to bind IκBα. To investigate these possibilities we isolated nuclear extracts from HEK-293T cells expressing DN-14-3-3, precipitated endogenous p65 and checked for the presence of IκBα in the precipitates. We demonstrated that nuclear extracts from DN-14-3-3 expressing cells contained increased levels of p65 bound to IκBα compared with almost undetectable levels of this complex in untransfected cells (Fig. 5c), suggesting that 14-3-3 activity is required for the efficient nuclear export of IκBα-p65 complexes.

We did not detect TNFα-dependent interaction of p65 with GST-14-3-3 in IκBα knockout cells (Fig. 5d), further evidence that a ternary complex is formed after TNFα treatment.

To confirm the requirement of 14-3-3 for regulating subcellular distribution of p65, we used specific siRNA to knockdown different 14-3-3 isoforms in HEK-293T cells. As shown in Fig. 5e, treatment with siRNA against both 14-3-3β and 14-3-3γ resulted in the nuclear retention of endogenous p65 compared with HEK-293T control cells and cells treated with the specific 14-3-3ϵ siRNA. Altogether, these results demonstrated that abrogation of specific 14-3-3 activities results in a general redistribution of p65 protein to the nucleus in unstimulated cells and, more interestingly, in response to TNFα activation.

14-3-3 proteins are recruited to the chromatin and modulate NFκB-dependent transcription

Since 14-3-3 can enter the nucleus to bind specific partners (Brunet et al., 2002; Miska et al., 2001; Wakui et al., 1997) and because after TNFα treatment p65 is mainly nuclear, we tested whether the subcellular distribution of 14-3-3 proteins was modulated by TNFα. Immunodetection of endogenous 14-3-3 with an antibody recognizing different isoforms demonstrated that 30 minutes of TNFα treatment induces a partial redistribution of these proteins from a predominantly cytoplasmic localization into a combined cytoplasmic plus nuclear pattern (Fig. 6a). Nuclear entry of 14-3-3 was

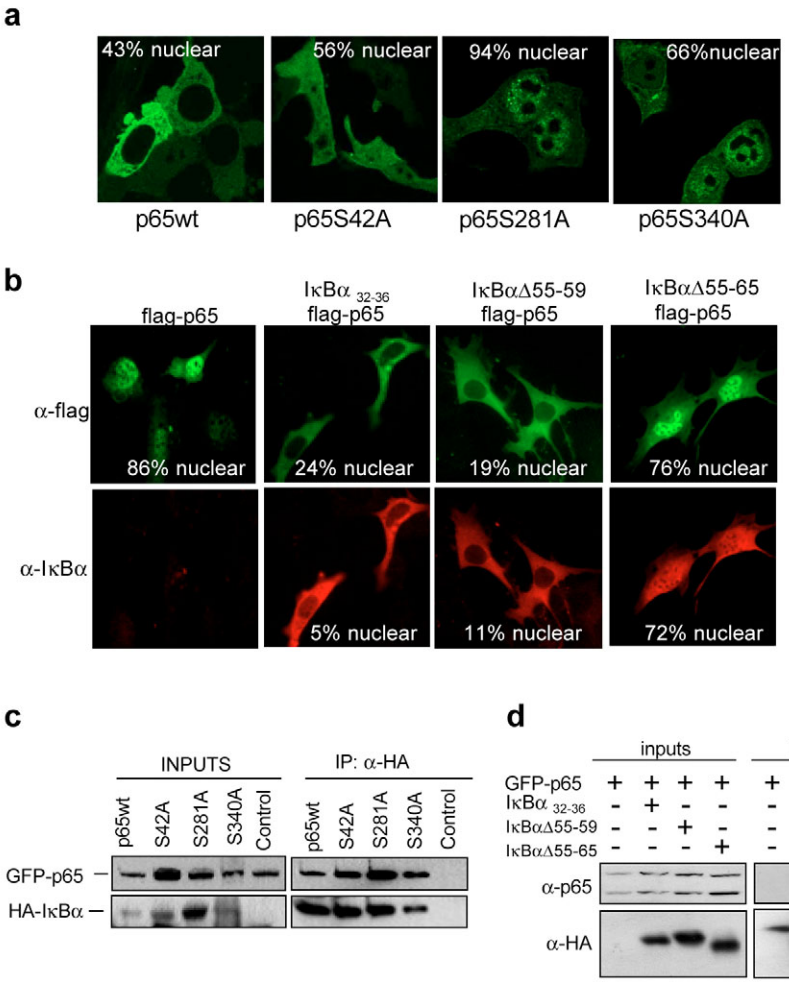

Fig. 4. 14-3-3 binding domains in p65 and IκBα are important to regulate their subcellular distribution. (a) GFP-p65 mutants were transfected in RPW1 cells to determine their subcellular localization. Slides from three independent experiments were counted on the BX-60 microscope and the percentage of cells displaying nuclear p65 is indicated. Representative confocal images are shown (magnification, 630×). (b) Immunolocalization of flag-p65 and the IκBα mutants in IκBα$^{-/-}$ MEF transfected with the indicated plasmids. Slides were counted on the BX-60 microscope and the percentage of cells displaying nuclear p65 and IκBα is indicated. Representative images are shown (magnification, 400×). (c) Cell lysates from HEK-293T cells cotransfected with HA-IκBα and the indicated GFP-p65 mutants were precipitated with anti-HA antibody. The presence of wild-type or mutant GFP-p65 in the precipitates was determined by immunoblotting with anti-p65 antibody. Input represents 1/10 of the lysates. (d) Anti-HA antibody was used to precipitate cell lysates from HEK-293T transfected with GFP-p65 and the indicated HA-IκBα mutants. Western blot α-p65 is shown in the upper panel. Input represents 1/10 of the lysates.

confirmed by western blot analysis with nuclear extracts from HEK-293T cells treated with TNFα at different time points (Fig. 6b). Our results demonstrated that nuclear levels of 14-3-3 gradually increased following TNFα treatment and reached a maximum at 30 minutes. Interestingly, nuclear accumulation of 14-3-3 seemed delayed compared with p65 nuclear entry and both nuclear p65 and 14-3-3 levels decreased after 60 minutes of chronic TNFα treatment.

Recruitment of 14-3-3ε proteins to the chromatin has been shown to occur in both cIAP-2 and IL-8 promoters in response to laminin attachment resulting in the release of chromatin-associated SMRT and facilitating gene activation (Hoberg et al., 2004). We speculated that 14-3-3 could also interact with the chromatin to facilitate the release of chromatin-bound p65 from specific genes, thus participating in the termination of TNFα signaling. Chromatin precipitation from NIH-3T3 cells demonstrated that 14-3-3β, 14-3-3γ and IκBα associated with the *IL6*, TNFα-induced protein 3 (*TNFAIP3* or *A20*) and chemokine (C-C motif) ligand 5 (*CCL5* or *RANTES*) promoters in response to TNFα with slightly delayed kinetics compared

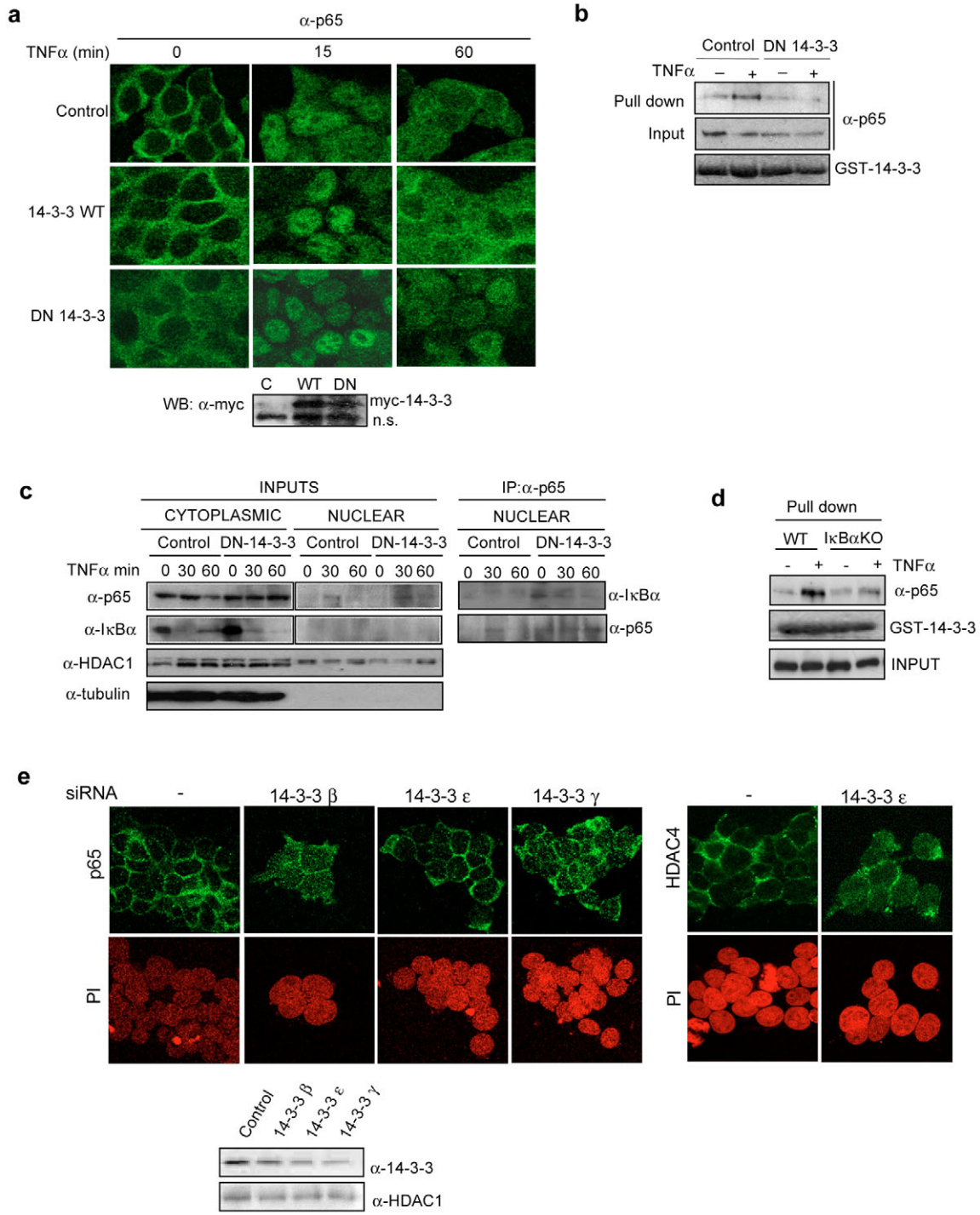

Fig. 5. See next page for legend.

with p65 recruitment (Fig. 6c). By contrast, in HEK-293T cells transfected with DN-14-3-3, p65 was constitutively associated with the NFκB-target promoters in the absence or presence of TNFα (Fig. 6d), suggesting that 14-3-3 proteins play a role in regulating the specific interaction of p65 with the chromatin. To test whether 14-3-3 proteins modulate the transcriptional activity of NFκB, we performed semiquantitative RT-PCR of different NFκB targets from HEK-293T cells transfected with DN-14-3-3. We detected increased basal transcriptional activity from *TNFAIP3* (*A20*) and *CCL5* (*RANTES*) genes, which correlated with the greater association of p65 with the chromatin in unstimulated conditions (Fig. 6d). However, DN-14-3-3-expressing cells became unresponsive to TNFα-dependent transcriptional activation (Fig. 6e).

Altogether, our results fit in a model in which 14-3-3 cooperate with IκBα in the release of p65 from the chromatin of specific promoters and participate in the subsequent nuclear export of IκBα-p65 complexes after TNFα treatment.

Discussion

14-3-3 proteins regulate the subcellular distribution of many proteins that are crucial in both cell cycle control and cell signaling (reviewed by Tzivion et al., 2001). In this study, we show that 14-3-3 proteins bind to both p65 and IκBα, facilitate the nuclear export of IκBα-p65 complexes and are required for the appropriate regulation of NFκB activity.

Part of the complexity of NFκB resides in the regulation of its nucleocytoplasmic shuttling. Although it is clearly established that IκBα is mainly responsible for sequestering NFκB in the cytoplasm, it also participates in the nuclear export of NFκB complexes not only after TNFα stimulation, but also in basal conditions to ensure NFκB-target gene silencing (Huang and Miyamoto, 2001). Nevertheless, it has been recently reported that in the absence of the three classical IκB family members, p65 remains localized in the cytoplasm (Tergaonkar et al., 2005). Although p100 and p105 are overexpressed and interact with p65 in these cells, other proteins such as 14-3-3 could also play a role (Moorthy and Ghosh, 2003; Prigent et al., 2000; Tergaonkar et al., 2005). We have characterized two functional 14-3-3 binding consensus sequences including residues 38-44 and 278-283 in p65 that are highly conserved from *Drosophila* to human.

We also demonstrate that IκBα interacts with 14-3-3 independently of TNFα stimulation. By pull-down assay we characterize a unique functional 14-3-3 binding site in the IκBα protein involving residues 60-65, close to its nuclear export signal (NES). Interestingly, it has been proposed that 14-3-3 might facilitate subcellular redistribution by masking or unmasking the nuclear localization signal (NLS) or NES of their substrates (Muslin and Xing, 2000). By comparative sequence analysis we show that S63, located in the core of this 14-3-3 binding domain, is exclusively found in the human IκBα protein but not in closely related species such as mouse or pig, consistent with the finding that phosphorylation is not required for IκBα to bind 14-3-3.

Although many efforts have been made to identify proteins that interact with the NFκB pathway after TNFα treatment, direct association between p65 and IκBα with 14-3-3 has not previously been detected (Bouwmeester et al., 2004).

It has been proposed that formation of heterodimers between different 14-3-3 isoforms could allow interaction between signaling proteins that do not directly associate (Jones et al., 1995). Although this is not the case for p65 and IκBα, our results suggest that after TNFα treatment, phosphorylated nuclear p65 binds to IκBα and 14-3-3, leading to the formation of a ternary complex that is more efficiently exported from the nucleus. Involvement of 14-3-3 proteins in the export of nuclear factors has been extensively reported, mainly associated with cell differentiation, cell cycle and apoptosis (Brunet et al., 2002; Lopez-Girona et al., 1999; Yoshida et al., 2005), and both altered expression of 14-3-3 and aberrant activation of NFκB have been directly implicated in cancer development (reviewed by Garg and Aggarwal, 2002; Hermeking, 2003). More specifically, 14-3-3σ is considered a tumor suppressor gene in breast carcinomas by promoting the nuclear export of cdc25 thus leading to cell cycle arrest (Urano et al., 2002). However abrogation of NFκB activity is sufficient to inhibit cell proliferation and to induce cell death in MCF-7 and MDA-MB-231 breast cancer cells (Tanaka et al., 2006). Considering our results, it is tempting to speculate that aberrant NFκB activation may result from deficiency of 14-3-3 in breast cancer. To address this question we are currently investigating the precise role of specific 14-3-3 isoforms in regulating the NFκB pathway in cancer cells.

In summary, we have demonstrated that 14-3-3 proteins are required for regulating the appropriate subcellular distribution of p65 and that abrogation of 14-3-3 activity by DN-14-3-3 or specific siRNAs induces the accumulation of p65-IκBα complexes in the nucleus and the constitutive binding of p65 to the chromatin. Since efficient nuclear export of p65-IκBα

Fig. 5. 14-3-3 activity is required to maintain p65 in the cytoplasm. (a) Immunolocalization of endogenous p65 in HEK-293T cells transfected with the indicated 14-3-3 plasmids and incubated with TNFα for 15 and 60 minutes as indicated. Representative confocal images are shown (magnification, 630×). Western blot showing the levels of transfected wild type (WT) and DN-14-3-3 (DN) with anti-myc antibody. (b) Pull-down experiment using GST-14-3-3η and lysates from HEK-293T cells untransfected or transfected with DN-14-3-3 treated for 30 minutes with TNFα as indicated. Upper panels show immunoblot with anti-p65 antibody. Inputs represent 1/10 of the lysates. Ponceau staining of GST proteins is shown in lower panel. (c) Nuclear extracts from HEK-293T cells, untransfected or transfected with DN-14-3-3 and treated with TNFα at different time points, were precipitated with the anti-p65 antibody. Co-precipitated IκBα was detected by western blot with α-IκBα antibody. Tubulin was detected as a fractionation control and HDAC1 as a loading control for nuclear extracts in the left panel. Inputs represent 1/10 of the lysates. (d) Pull-down experiment using GST-14-3-3η and lysates from wild-type or IκBα-knockout MEF cells untreated or treated for 30 minutes with TNFα as indicated. Upper panel show immunoblot with anti-p65 antibody. Ponceau staining of GST proteins is shown in middle panel. Inputs represent 1/10 of the lysates (detected with anti-p65). (e) Subcellular localization of endogenous p65 in HEK-293T cells treated with siRNA against different 14-3-3 isoforms. Right panels show the nuclear entry of HDAC4 in the 14-3-3-ϵ siRNA-treated cells as a control. Representative confocal images are shown (magnification, 630×). PI was used for nuclear staining. Western blot assayed with anti-pan-14-3-3 antibody recognizing 14-3-3 family members shows the levels of 14-3-3 in cell lysates from HEK-293T cells treated with the isoform-specific siRNA. HDAC1 was detected as a loading control.

complexes is required for reestablishing the permissive conditions for NFκB to be rapidly activated in response to specific stimuli (Arenzana-Seisdedos et al., 1997; Huang and Miyamoto, 2001), abrogation of 14-3-3 function results in a deficiency in NFκB target gene activation after TNFα treatment.

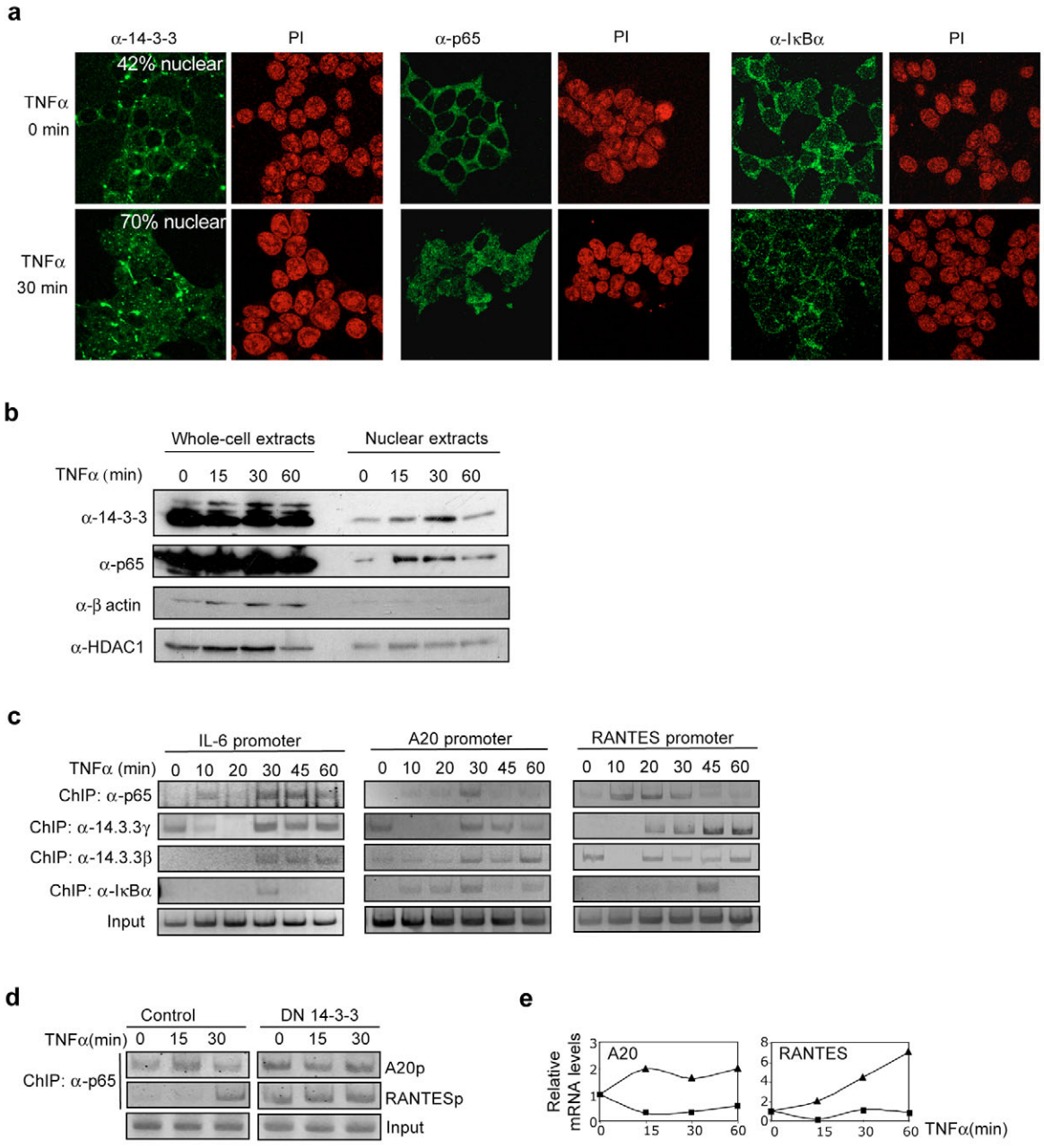

Fig. 6. 14-3-3 proteins associate with the chromatin in response to TNFα to modulate NFκB-dependent transcription. (a) Subcellular localization of endogenous 14-3-3 (left panels), p65 (middle panels) and IκBα (right panels) in control or HEK-293T cells treated for 30 minutes with TNFα. PI was used for nuclear staining. Slides from three independent experiments were counted on the BX-60 microscope and the percentage of cells displaying nuclear 14-3-3 is indicated. Representative confocal images are shown (magnification, 630×). (b) Western blot with the indicated antibodies of total cell lysates (left) and nuclei (right) from HEK-293T cells incubated with TNFα at different time points. Absence of β-actin in the nuclear extracts is shown as a fractionation control whereas HDAC1 was detected as a nuclear loading control. (c) Chromatin from TNFα-treated NIH-3T3 cells was immunoprecipitated with anti-14-3-3β and -γ, anti-p65 and anti-IκBα antibodies. The presence of the indicated NFκB target promoters in the precipitates was determined by PCR. (d) Chromatin from HEK-293T cells untransfected or transfected with DN14-3-3 and treated with TNFα at different time points, was immunoprecipitated with the α-p65 antibody. The presence of the indicated NFκB target promoters in the precipitates was determined by PCR. (e) Semiquantitative RT-PCR to determine the transcriptional activity of the indicated NFκB target genes in HEK-293T cells untransfected (▲) or transfected with DN-14-3-3 (■) treated with TNFα at the indicated time points. Graphs represent the relative amounts of mRNA, as measured by densitometric analysis, from one of two equivalent experiments.

Materials and Methods

Plasmids

Expression vectors for pCMV-HA-IκBα$_{32-36}$ (Ser32 and Ser36 to Ala mutations), GFP-p65, Flag-p65, myc-14-3-3η, myc-14-3-3η$_{R56A-R60A}$ (DN-14-3-3), and GST-14-3-3η have been previously described (DiDonato et al., 1995; Thorson et al., 1998). IκBα and p65 constructs were obtained by PCR and primer sequences are available upon request. PCR products were cloned in-frame into the pGEX-5.3 vector (Pharmacia) or into the pcDNA3.1-HA. GFP-p65 mutants were generated with the QuickChange Site-Directed Mutagenesis Kit (Stratagene). All construct sequences were confirmed by automated sequencing.

Antibodies and inhibitors

Antibodies recognizing IκBα (sc-1643 and sc-371G), p65 (sc-109), HDAC1 (sc-7872), 14-3-3β (sc-629) and 14-3-3γ (sc-731) were purchased from Santa Cruz Biotechnology. Anti-14-3-3β antibody (recognizing different 14-3-3 isoforms and referred as α-pan-14-3-3) (KAM-CCO13) was from Bioreagents, anti-flag (clone M2) and anti-α-tubulin antibodies were from Sigma, anti-HA from Babco and anti-HDAC4 (07-040) and anti-IκBα (06-494) antibodies were from Upstate and anti-phospho-14-3-3 binding motif (9601) was from Cell Signaling. Secondary antibodies conjugated to horseradish peroxidase (HRP) were purchased from DAKO. Fluorescein-conjugated goat anti-mouse or Cy3-conjugated goat anti-rabbit antibodies were from Amersham and Alexa Fluor 594-conjugated goat anti-mouse antibody was from Molecular Probes.

BAY 11-7082, JNK inhibitor II and SB203580 were used at 100 μM, 20 μM and 12 μM, respectively, and purchased from Calbiochem. TNFα was purchased from Preprotech and used at 40 ng/ml.

Cell culture and transfection

HEK-293T, RWP1 pancreatic cancer cells, NIH-3T3 murine embryonic fibroblasts (MEF), p65$^{-/-}$ MEF and IκBα$^{-/-}$ MEFs were cultured in DMEM with 10% FBS. Cells were plated at subconfluence and transfected with calcium phosphate or Lipofectamine Reagent (Invitrogen). Cells were harvested after 24 hours for immunofluorescence, coimmunoprecipitation, ChIP or western blot analysis.

Cell fractionation

Trypsinized cells were incubated in 500 μl of 0.1% NP-40/PBS for 5 minutes on ice and the reaction was stopped by adding 3 ml of cold PBS. Nuclei were isolated by centrifugation at 2,000 rpm and washed with 3 ml of cold PBS twice. After washing, nuclei were lysed for 30 minutes in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 5 mM EGTA, 5 mM EDTA, 20 mM NaF and complete protease inhibitor cocktail (Roche).

siRNA transfection

siRNAs against 14-3-3β (sc-29186), 14-3-3γ (sc-29582), 14-3-3ϵ (sc-29588) or control siRNA (sc-36869) were transfected in HEK-293T cells with the siRNA reagents from Santa Cruz Biotechnology, according to the manufacturer's instructions (sc-36868).

Pull-down assays

Pull-down assays have been previously described (Espinosa et al., 2003). Briefly, GST fusion proteins were purified and incubated with 400 μg of cell lysates for 2 hours at 4°C in lysis buffer and extensively washed. Pulled down proteins were analyzed by western blot.

Coimmunoprecipitation assays

Cells were lysed for 30 minutes at 4°C in 500 μl of PBS containing 0.5% Triton X-100, 1 mM EDTA, 100 μM sodium orthovanadate, 0.25 mM PMSF and complete protease inhibitor cocktail (Roche). After centrifugation, supernatants were incubated for 3 hours at 4°C with 1 μg of the indicated antibody coupled to Protein A-Sepharose beads. Beads were extensively washed with the precipitation buffer and samples were assayed by western blot.

Immunofluorescence

Cells were grown on glass slides and transfected with the indicated plasmids. After 48 hours, cells were fixed in 3% paraformaldehyde and permeabilized with 0.3% Triton X-100, 10% FBS and 5% non-fat dry milk in PBS. After incubation with the appropriate antibodies, slides were mounted with Vectashield plus DAPI or propidium iodide (PI) (Vector) and staining was visualized in an Olympus BX-60 microscope or a Leica TCS-NT laser-scanning confocal microscope equipped with a 63× Leitz Plan-Apo objective (NA 1.4). Representative images were edited using Adobe Photoshop. For each experiment a minimum of 200 cells was counted in the Olympus BX-60 by two independent researchers.

Protein kinase assays

Cells were lysed for 30 minutes at 4°C in 500 ml PBS containing 0.5% Triton X-100, 1 mM EDTA, 100 mM sodium orthovanadate, 0.25 mM PMSF and complete protease inhibitor cocktail (Roche). Cell lysates were assayed for kinase activity on GST-p65 peptides in the presence of [γ-^{32}P]ATP.

Chromatin immunoprecipitation assay

Chromatin from crosslinked cells was sonicated, incubated overnight with the indicated antibodies in RIPA buffer and precipitated with protein G/A-Sepharose. Crosslinkage of the co-precipitated DNA-protein complexes was reversed, and DNA was used as a template for semiquantitative PCR. Primer sequences are available upon request.

RT-PCR

Total RNA from HEK-293T cells was isolated using Trizol Reagent (Invitrogen), and cDNA was obtained with RT-First Strand cDNA Synthesis kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Densitometric analysis of the PCRs was performed with the Quantity One software from Biorad. Oligonucleotide sequences can be provided upon request.

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