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Hypo-phosphorylation leads to nuclear retention of NF- κ B p65 due to impaired I κ B α gene synthesis

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Abstract

Subcellular localization guided by I κ B α is crucial for regulation of NF- κ B function. Here we show that p65 RHD phosphorylation mutants are transported into the nucleus after I κ B α degradation, but as a consequence of lower I κ B α levels their relocation to the cytosol is blocked. We demonstrate that phosphorylation of residues S205, S276 and S281 of p65 is not required for interaction between p65 and I κ B α , but is pivotal for regulating cellular I κ B α levels by positively affecting gene synthesis. Our findings indicate that reduction of phosphorylation leads to nuclear retention of p65, which might be partly responsible for altered transcriptional behavior of p65 serine mutants.

Keywords

NF- κ B; I κ B α ; protein phosphorylation; transcriptional activity; nuclear localization

1. INTRODUCTION

NF- κ B is a key regulator of inflammatory and immune responses [1]. Pathological conditions including inflammation and cancer are linked to dysfunction of NF- κ B, therefore a tight regulation of its activity is mandatory [2]. The mammalian NF- κ B family consists of five members, NF- κ B1 (p50), NF- κ B2 (p52), c-Rel, RelA (p65), and RelB, which all can form homo- or heterodimers, the p50/p65 heterodimer being the predominant form in most cell types. All NF- κ B subunits contain a N-terminal RHD, which confers DNA-binding, dimerization, and inhibitor-binding activity, but only p65, RelB and c-Rel contain potent C-terminal TADs and can therefore activate transcription. NF- κ B activity is controlled by its intracellular localization, which is achieved by the family of I κ B proteins. In resting cells the NF- κ B dimer forms a complex with I κ B α , which masks its NLS thereby preventing nuclear translocation [3]. Upon activation, I κ B α is phosphorylated by the IKK complex, leading to rapid ubiquitination and proteolysis via the 26S proteasome [4,5]. This results in nuclear import of NF- κ B dimers and transcription of target genes, among them I κ B α [6], which is part of a negative feedback loop indispensable for terminating the activity of NF- κ B. Newly synthesized I κ B α translocates to the nucleus, where it binds to NF- κ B, inhibiting its DNA binding and transporting it out of the nucleus [7]. Therefore, I κ B α is crucial for ensuring a rapid and transient NF- κ B transcriptional response.

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In addition to nuclear translocation, post-translational modifications, particularly phosphorylation, are critical for full activation of NF- κ B. Both the RHD and the TAD of p65 contain key phospho-acceptor sites that are specifically targeted by different kinases [8]. p65 transcriptional activity is markedly increased through phosphorylation of S529 and S536, both located within the C-terminal TAD [9,10]. Differential phosphorylation of p65 at S276 and S311 in the RHD influences p65 DNA-binding and oligomerization properties, as well as its association with CBP/p300 [11,12]. We have previously shown that the p65 subunit of NF- κ B is phosphorylated on multiple residues within the RHD and that phosphorylation at S205, S276 and S281 is essential to retain p65 transcriptional activity [13].

In this work we investigate the consequences of hypo-phosphorylation of p65 on its subcellular localization. We show that p65 phospho-serine mutants S205A, S276A and S281A exhibit abnormal localization, which is a consequence of defective I κ B α synthesis.

2. MATERIALS AND METHODS

2.1. Plasmids

Episomal and retroviral vectors expressing human p65 and p65 mutants have been described [13]. HA-I κ B α was obtained by transferring a BamHI/EcoRI fragment from pKSII/ECI-6 (kind gift from Rainer de Martin, Medical University of Vienna, Austria) to a modified pcDNA3 vector harboring a N-terminal HA-tag.

2.2. Cell culture

HEK293 and RelA^{-/-} MEF [14] were cultured as described [13] and transiently transfected with calcium phosphate or Lipofectamine (Invitrogen), respectively. For stimulation 10 ng/ml human recombinant TNF α (Biosource) was used. RelA^{-/-} MEF lines stably expressing p65 wt or mutants were developed by exposing cells to retrovirus-containing supernatants as described elsewhere [13].

2.3. Immunofluorescence

MEF were fixed with 3% formaldehyde, permeabilized with 0.5% Triton X-100 and blocked in PBS/0.05% Tween20/0.1% bovine serum albumin (Sigma-Aldrich). Cells were incubated with p65 (C-20; Santa Cruz Biotechnology) and HA antibodies (12CA5; Roche Applied Science) in blocking buffer for 1 h. After incubation with Alexa488-labeled anti-rabbit IgG and/or Alexa568-labeled anti-mouse IgG_{2b} (Molecular Probes) in PBS/0.05% Tween20 for 1h, they were mounted with SlowFade Gold antifade reagent containing DAPI (Invitrogen). Subcellular distribution of p65 and I κ B α was analyzed using a Nikon Eclipse TE2000 microscope equipped with a 40x objective. Pictures were acquired with a charge-coupled device and images were processed with IPLab software (Scanalytics, Vers. 3.9.3).

2.4. Co-Immunoprecipitation

Cells were harvested in RIPA buffer containing 0.5% deoxycholate and protease inhibitors (Roche Applied Science) and the resulting homogenate was cleared by centrifugation. The cleared lysate was subjected to immunoprecipitation with either anti-myc or anti-HA agarose (Sigma-Aldrich). After 2 h incubation at 4 °C, samples were washed, and bound proteins were eluted in Laemmli buffer. Immunoprecipitated proteins were detected by Western blotting with specific antibodies: c-myc 9E10, HA F-7, I κ B α C-21 (Santa Cruz Biotechnology) and β -actin AC-15 (Sigma-Aldrich). Protein bands were quantified with Kodak 1D V3.6 image software on a Kodak Image Station 2000R. Cellular I κ B α levels were calculated relative to β -actin levels. I κ B α protein level in resting p65 wt expressing cells was set to 1. Levels of precipitated I κ B α were calculated as a fraction of cellular I κ B α levels.

2.5. Preparation of nuclear extracts

Cells were lysed in 10 mM Tris-HCl pH 8.0, 320 mM sucrose, 3 mM CaCl₂, 2 mM Mg (OAc)₂, 0.5% Triton X-100, 5 mM β -glycerophosphate, 1 mM sodium orthovanadate, 1 mM DTT, protease inhibitors. Nuclei were collected by centrifugation, washed, and lysed in 20 mM HEPES pH 7.9, 25% glycerol, 1.5 mM MgCl₂, 800 mM NaCl, 0.2 mM EDTA, 1% Triton X-100, 5 mM β -glycerophosphate, 1 mM sodium orthovanadate, 1 mM DTT, protease inhibitors. Nuclear debris was removed by high-speed centrifugation. Western blots were probed with β -actin AC-15 as loading control, histone H1 FL-219 (Santa Cruz Biotechnology) and GAPDH (Chemicon) antibodies to ensure compartmental separation.

2.6. Gene expression analysis

qPCR was carried out as described [13]. Primers used to amplify a 96-bp fragment specific for the murine I κ B α gene are: forward, 5-CAG CTG ACC CTG GAA AAT-3 and reverse, 5-ATA GGG CAG CTC ATC CTC TGT-3. Primers for the housekeeping gene *hypoxanthine guanine phosphoribosyl transferase* have been described elsewhere [13].

3. RESULTS

3.1. Hypo-phosphorylation leads to nuclear retention of p65

p65 S205A, S276A and S281A exhibit altered phosphorylation status and transcriptional activity compared to the wt protein [13]. As changes in activity of NF- κ B often correlate with altered subcellular distribution, we investigated the cellular localization of p65 wt and mutants. In the absence of stimulus p65 wt is exclusively located in the cytosol, whereas mutants also exhibit a small amount of nuclear staining (Fig. 1A). After 30 min TNF α treatment, p65 proteins translocate to the nucleus regardless of their phosphorylation status. After 120 min stimulation however, p65 wt is relocated to the cytosol, whereas phospho-mutants are retained in the nucleus. Similarly nuclear extracts prepared from sister cultures showed higher nuclear retention of p65 phospho-mutants (Fig. 1B), with nuclear accumulation more pronounced for p65 S276A and S281A, than for p65 S205A. To exclude any temporal shift in the response to TNF α , one of the mutants (S281A) was followed up to 360 min when it still showed nuclear localization (Fig. 1C). Thus, mutation of S205, S276, or S281 severely affects p65 nuclear export.

3.2. I κ B α binding to p65 is not affected by SA mutations

I κ B α is the key molecule regulating NF- κ B activity by controlling nuclear import and export [7]. As the binding site for I κ B α to p65 was narrowed down to the RHD [15], it seemed possible that mutation of certain residues within the RHD could negatively influence interaction. To investigate this possibility we performed co-immunoprecipitation of p65 wt or mutants with I κ B α derived from HEK293 over-expressing both proteins. p65 wt and mutants were equally pulled down by precipitation of I κ B α (Fig. 2A). Confirming this result, there was also no difference in co-precipitated I κ B α when precipitating p65 wt, S205A, S276A or S281A (Fig. 2B). Moreover, stimulation with TNF α did not affect interaction between p65 and I κ B α . Thus, p65 S205A, S276A and S281A retain their binding affinity to I κ B α .

3.3. Cellular I κ B α levels are decreased due to impaired I κ B α mRNA synthesis in cells expressing p65 S205A, S276A or S281A

Protein expression levels of I κ B α are insensitive to TNF α treatment when both proteins are over-expressed (Fig. 2) indicating that a physiologic balance of I κ B α and NF- κ B is not given. Therefore we extended our experiments using either HEK293 only over-expressing p65 or RelA^{-/-} MEF stably expressing p65. p65 proteins were precipitated and endogenous I κ B α was detected. Confirming results from over-expression experiments, in HEK293 no differences in

I κ B α association were observed, however, I κ B α protein levels were profoundly affected by expression of mutants compared to the wt protein (Fig. 3A). As a consequence less I κ B α was found to be associated with p65 mutants. Similarly, p65 wt expression in MEF induced I κ B α protein levels, which was more pronounced after TNF α stimulation (Fig. 3B). In contrast, p65 mutants failed to enhance post-induction I κ B α protein expression. Similar to HEK293, binding affinities of p65 wt and mutants to I κ B α remained unaltered as evidenced by an unchanged ratio of p65 associated and total cellular I κ B α (Fig. 3B).

Considering the significance of phosphorylation of p65 for NF- κ B transcriptional activity, the decreased I κ B α levels in cells expressing phospho-serine mutants could emerge from defective transcriptional regulation. To test this hypothesis we stimulated RelA^{-/-} MEF stably expressing either p65 wt or mutants with TNF α and monitored I κ B α mRNA expression by qPCR. Cells transduced with empty vector were used as control. I κ B α mRNA expression was readily induced by p65 wt, peaking 90 min after TNF α stimulation (Fig. 3C). In contrast, p65 phospho-mutants could induce I κ B α gene expression only up to 50% of the wt protein, which is consistent with observed I κ B α protein levels (Fig 3B). These results indicate that I κ B α mRNA expression is dependent on phosphorylation status of p65.

Newly synthesized I κ B α translocates to the nucleus and terminates the NF- κ B response by binding to NF- κ B and shuttling it to the cytoplasm [7]. To determine the amount of nuclear I κ B α in p65 mutant-expressing cells we transfected HEK293 with p65 constructs and monitored endogenous I κ B α protein expression in cytosolic and nuclear fractions after stimulation with TNF α for 2 h. As shown in Fig. 3D, nuclear I κ B α was barely detectable in cells expressing p65 mutants. While p65 SA mutants lead to 50% reduction in overall I κ B α mRNA and protein levels, the effect on nuclear I κ B α protein levels seem to be even more significant.

3.4. Re-introduction of I κ B α results in restored cytosolic relocation of p65 phosphomutants

The lack of I κ B α protein out of reduced I κ B α mRNA production in p65 mutant-expressing cells could account for the observed atypical p65 localization pattern. To prove this hypothesis, we performed indirect immunofluorescence in RelA^{-/-} MEF stably expressing p65 either with or without co-transfection of I κ B α . As shown in Fig. 4, re-introduction of I κ B α led to cytosolic localization of p65 after 30 min TNF α stimulation regardless of p65 phosphorylation status. This result finally confirms that the predominant nuclear localization of p65 S205A, S276A and S281A after TNF α stimulation is caused by reduced I κ B α expression.

4. DISCUSSION

The biological activity of NF- κ B is tightly regulated by post-translational modifications and subcellular localization, which is mostly governed by the family of I κ B proteins. I κ B α is responsible for a strong negative feedback rapidly turning off NF- κ B responses and accordingly absence of I κ B α results in nuclear accumulation of NF- κ B [7,16]. NF- κ B/I κ B α complexes shuttle continuously between nucleus and cytosol, and dominance of nuclear export over nuclear import and masking the NLS of NF- κ B by I κ B α contribute to the largely cytosolic localization of NF- κ B/I κ B α complexes in absence of stimuli [17].

Considering the importance of I κ B α in driving p65 localization we contemplated that change in phosphorylation status of p65 could influence interactions between the two proteins and therefore leading to predominant nuclear localization of p65. The crystal structure of NF- κ B/I κ B α complexes reveal that p65 contacts I κ B α through its RHD domain [15], therefore mutations within the RHD could negatively affect binding properties. Although no specific serine residue of p65 was shown to directly contact I κ B α , binding between the two proteins is maintained by long-range electrostatic interactions involving charged residues within both

proteins [15]. As crystal structures were obtained from bacterially expressed proteins that lack phosphorylation, it was possible that additional charges within p65 RHD introduced by phospho-serines might change the electrostatic surface of p65 and therefore alter I κ B α binding.

Phosphorylation has also been shown to regulate acetylation of p65, which in turn can affect I κ B α binding [18]. Whereas acetylation of K221 increases DNA binding affinity and prevents the association of p65 with I κ B α , K122/K123 acetylation leads to reduced DNA binding but facilitates p65-I κ B α interaction [19,20]. However, our studies reveal that neither mutation of S205, S276 nor S281 of p65 influences I κ B α binding. This result is in line with data published for the *Drosophila* p65 homologue Dorsal where mutation of six highly conserved serines in the RHD does not eliminate binding of the I κ B α homologue Cactus [21].

Having established that p65-I κ B α interactions were not changed, we considered the possibility that reduced p65 phosphorylation might negatively influence I κ B α gene expression. Indeed, we could show that mutation of potential phospho-acceptor serines within p65 RHD decreases endogenous I κ B α protein synthesis after TNF α stimulation. This finding is supported by results obtained earlier in our laboratory which showed that transcription from NF- κ B DNA-binding elements found in the I κ B α promoter is dependent on p65 phosphorylation status [13].

Lack of stimulus-induced I κ B α neo-synthesis is responsible for augmented nuclear localization of hypo-phosphorylated p65 species, which however is not associated with increased transcriptional activity. Unlike I κ B α binding, alteration in phosphorylation status of p65 could impact binding of other factors, which are not required for nuclear import, but indispensable for transcriptional activation. It was shown that phosphorylation of p65 on S276 promotes enhanced interaction with p300 which accelerates acetylation and transcriptional activity of p65 [18]. In contrast, dephosphorylation of p65 leads to inactivation of NF- κ B by binding to HDAC-1 [22]. Alternatively, cytosolic-nuclear shuttling of p65 might be necessary to maximize NF- κ B transcriptional activity by facilitating nuclear IKK α import, which is linked to histone H3 phosphorylation at S10, a modification associated with efficient gene transcription [23]. In summary, lack of phosphorylation due to mutations of serines in the RHD of p65 has a great impact on NF- κ B transcriptional activity. Our data show that I κ B α synthesis is negatively regulated by hypo-phosphorylation of p65. The resulting I κ B α deficiency leads to dysregulation of natural cytosolic to nuclear NF- κ B balance, which in turn could account for the altered transcriptional activity of hypo-phosphorylated p65. However, the cellular events primarily leading to depleted NF- κ B transcriptional activity as a consequence of missing phosphorylation are still unknown and further investigations will be necessary to identify involved factors.

5. ACKNOWLEDGEMENTS

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Abbreviations

A, alanine; CBP, CREB-binding protein; DAPI, 4',6-diamidino-2-phenylindole; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, hemagglutinin; HEK, human embryonic kidney; I κ B, inhibitor of NF- κ B; IKK, I κ B kinase; K, lysine; MEF, mouse embryonic fibroblast; NF- κ B, nuclear factor- κ B; NLS, nuclear localization signal; PBS, phosphate-buffered saline; qPCR, Real-time quantitative polymerase chain reaction; Rel, Reticuloendotheliosis viral oncogene; RHD, Rel homology domain; SDS, sodium dodecyl sulfate; S, serine; TAD, transcription-activation domain; TNF α , tumor necrosis factor alpha; wt, wild type.

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Fig. 1. p65 mutants are preferentially located in the nucleus. (A, C) RelA^{-/-} MEF expressing p65 wt, S205A, S276A or S281A were treated with TNF α for indicated time points, and processed for immunostaining with p65 antibody. Nuclei were counterstained with DAPI. (B) Subcellular fractions were analyzed by Western Blotting with p65, β -actin, GAPDH, and histone H1 antibodies.

Fig. 2. p65 mutants associate with I κ B α . (A, B) HEK293 transfected with p65 and I κ B α constructs were stimulated with TNF α for 0.5 and 2 h. After lysis, cellular extracts were either incubated with HA agarose to precipitate I κ B α (A) or c-myc matrix to pull down p65 (B). Co-precipitation of I κ B α and p65 was determined by Western Blotting using respective antibodies. HC, heavy chain.

Fig. 3.

Impaired IκBα mRNA expression results in lower cellular IκBα protein levels in p53 mutant-expressing cells. (A) HEK293 lysates exogenously expressing p53 wt and mutants were used to pull down endogenous IκBα. (B) RelA^{-/-} MEF reconstituted with p53 wt, S205A, S276A or S281A were stimulated with TNFα for 0.5 and 2 h. For precipitation of p53 cell extracts were incubated with c-myc agarose and presence of IκBα was determined by Western Blotting. Bands in immunoblots were quantified. Error bars represent mean ± SEM of two independent experiments. HC, heavy chain. (C) RelA^{-/-} MEF expressing p53 wt or mutants were stimulated with TNFα for 30, 90, 180 and 360 min, total RNA was isolated, and mRNA levels were analyzed by qPCR. Fold induction of experimental groups was calculated relative to expression level of unstimulated, vector-transfected cells, which was set to 1. Error bars represent mean ± SEM of triplicates from 3 independent experiments. (D) HEK293 were transfected with p53 expression plasmids and stimulated with TNFα for 2 h. Cell lysates were separated into cytoplasmic and nuclear fractions, which were analyzed by Western Blotting for the presence

of I κ B α . Purity of the nuclear fraction was shown by absence of GAPDH, β -actin was used as loading control for both fractions.

Fig. 4.

Reconstitution of I κ B α leads to cytosolic relocation of p65 regardless of phosphorylation status. RelA^{-/-} MEF stably expressing p65 wt and mutants were transfected with HA-I κ B α and stimulated with TNF α for 30 min. Cells were immunostained with p65 (green) and HA (red) antibodies. Arrows indicate double-labeled cells.