Phosphorylation of Serine 468 by GSK-3β Negatively Regulates Basal p65 NF-κB Activity*  

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The activity of NF-κB is controlled at several levels including the phosphorylation of the strongly transactivating p65 (RelA) subunit. However, the overall number of phosphorylation sites, the signaling pathways and protein kinases that target p65 NF-κB and the functional role of these phosphorylations are still being uncovered. Using a combination of peptide arrays with in vitro kinase assays we identify serine 468 as a novel phosphorylation site of p65 NF-κB. Serine 468 lies within a GSK-3β consensus site, and recombinant GSK-3β specifically phosphorylates a GST-p65 (354–551) fusion protein at Ser468 in vitro. In intact cells, phosphorylation of endogenous Ser468 of p65 is induced by the PP1/PP2A phosphatase inhibitor calyculin A and this effect is inhibited by the GSK-3β inhibitor LiCl. Reconstitution of p65-deficient cells with a p65 protein where serine 468 was mutated to alanine revealed a negative regulatory role of serine 468 for NF-κB activation. Collectively our results suggest that a GSK-3β/PP1-dependent mechanism regulates phosphorylation of p65 NF-κB at Ser468 in unstimulated cells and thereby controls the basal activity of NF-κB.

NF-κB is a dimeric transcription factor that plays an important role in the immune response, cell survival, and cancer. NF-κB activity is controlled at two levels: (i) by proteasome-dependent generation of DNA-binding subunits and (ii) by regulation of its nuclear function. In the recent years, evidence has accumulated that post-translational modifications of the DNA-binding subunits add another level of regulation for the function of NF-κB (1–3).

A number of protein kinases have been shown to phosphorylate the strongly transactivating subunit p65 at Ser276 (4), Ser311 (5), Ser329 (6), and Ser336 (7), and, with the exception of Ser329, phospho-specific antibodies have confirmed phosphorylation of endogenous p65 at these sites. The relevance of regulatory phosphorylations is also evident from the analysis of cells lacking the protein kinases GSK-3β (8), TBK1/NAK (9, 10), IKKe (11), NIK (12), and PKCζ (13), which show an intact IκB phosphorylation but an impaired expression of NF-κB target genes. Nonetheless, the overall number of p65 phosphorylation sites is not yet known as is the number of all potential p65 protein kinases. As an example, we and others (14–17) have recently shown that at least six distinct kinases converge on phosphorylation of p65 at Ser336. The molecular mechanisms and the biological consequences of p65 phosphorylation are currently a focal point of intense research (2, 3). Most p65 phosphorylation sites are located in the COOH-terminal part of the Rel homology domain and in the COOH-terminal transactivation domains (2, 3).

The involvement of GSK-3β in activation of NF-κB as suggested by gene deletion has been a surprising finding, as NF-κB activating stimuli such as IL-1, TNF, or phorbol ester will inactivate GSK-3β by phosphatidylinositol 3-kinase/AKT-mediated phosphorylation of its NH2 terminus (18–20). Furthermore, it is suggested that in NF-κB activation the role of GSK-3β is non-redundant as the closely related enzyme GSK-3α cannot compensate for the loss of GSK-3β (8, 18). In contrast with the results derived from knock-out mice, in neuronal cells expression of an active form of GSK-3β suppresses NF-κB activity by inhibiting IκB kinase (IKK)1 and stabilizing IκB (21, 22). GSK-3β has also recently been shown to phosphorylate a GST-p65 fusion protein in vitro, but the relevant site(s) have not been determined and it has remained unclear if p65 is a physiological substrate for GSK-3β in vivo (23). Using a peptide array-based approach (24) we detected a protein kinase activity that specifically phosphorylated Ser468 of p65 NF-κB. Experiments presented here strongly suggest that GSK-3β is a physiological Ser468 protein kinase and we imply this phosphorylation site in negative control of NF-κB. In the light of the opposing findings regarding the role of GSK-3β in NF-κB signaling our results close an important gap in the understanding of the role of GSK-3β in regulation of p65 activity.

EXPERIMENTAL PROCEDURES

Cells and Materials—HeLa cells stably expressing the tet transactivator protein were a kind gift of H. Bujard, Heidelberg, Germany. p65−/− cells were a kind gift of H. Nakano, Tokyo, Japan. All cells were cultured in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal calf serum, 2 mM l-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin. Antibodies against the following proteins or peptides were used in this study: IκBα (9242), phospho-(Ser32/36)IκBα (9241), phospho-Ser329 NF-κB (3069), phospho-Ser311 NF-κB (3069), all from Cell Signaling Technology and p65 NF-κB (C-20) from Santa Cruz and GSK-3β (610201) from BD Biosciences. Recombinant GSK-3β (P6040S) was from New England Biosciences. Horseradish peroxidase-coupled secondary antibodies were from Sigma. Human recombinant IL-1α was a kind gift of J. Saklatvala,

1 The abbreviations used are: IKK, IκB kinase; IL, interleukin; TNF, tumor necrosis factor; GST, glutathione S-transferase; PP1 and PP2A, protein phosphatase 1 and 2A, respectively.

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Peptide Arrays—The peptide array containing p65 NF-κB peptide spots was generated following SPOT synthesis (28). 180 peptide fragments of 15 amino acid residues in length and overlapping by 12 residues were generated such that the entire p65 NF-κB protein sequence was covered. These peptides were chemically synthesized as an array of spots on an amino-polyethylene glycol-modified cellulose membrane (AC-S01, AIMS Scientific Products GmbH, Braunschweig, Germany) as described previously (26). All peptides are NH₂-terminal acetylated and remain covalently attached to the membrane via their carbamyl termini.

Plasmids and Transfections—The expression plasmid for the p65 TAD, pGEX-p65-(354–551) was a kind gift of H. Sakurai, Toyama, Japan. GST fusion proteins were expressed in bacteria and purified on GSH-Sepharose using standard procedures. pMT7-p65 NF-κB has been published (27) and NF-κB (3)/lac contained three NF-κB-binding sites upstream of a luciferase cDNA. pBS-β-gal coding for SV40 promoter driven β-galactosidase was from plasmids containing p65 cDNA seeded in 6-well plates and transfected at 70–80% confluence using Rotifect (Roth) according to the manufacturer’s instructions.

Preparation of Cell Extracts—For the preparation of whole cell extracts cells were lysed directly in SDS-PAGE sample buffer. DNA was sheared by brief sonification, and soluble proteins were recovered after centrifugation of lysates at 15,000 × g for 15 min at 4 °C. For in vitro kinase assays cells were lysed in 10 mM Tris, pH 7.65, 30 mM NaF, 1% Triton X-100, 2 mM Na3VO4, 50 mM NaF, 20 mM β-glycerophosphate, and freshly added 0.5 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin, 400 mM octylid acid. After 10 min on ice, lysates were clarified by centrifugation at 10,000 × g for 15 min at 4 °C.

Nuclear and cytosolic extracts were prepared as described previously (27). The protein concentration of cell extracts was determined by the method of Bradford, and samples were stored at −80 °C.

In Vitro Kinase Assays—For the kinase assay shown in Fig. 1A, 10 μl of cell lysate (50 μg of protein) was added to 1 μl of recombinant protein substrates (GST-p65-(354–551) or mutants thereof) in 10 μl of H2O and 10 μl of kinase buffer (150 mM Tris, pH 7.4, 30 mM MgCl2, 60 μM ATP, 4 μCi of [γ-32P]ATP). After 15 min at 30 °C in vitro phosphorylated GST-p65 fusion proteins were purified on GSH-Sepharose prior to SDS-PAGE as described by Holtmann et al. (28). Then, SDS-PAGE sample buffer was added, and proteins were eluted from the beads by boiling for 5 min. After centrifugation at 10,000 × g for 5 min, supernatants were separated on 10% SDS-PAGE. Phosphorylated proteins were visualized by autoradiography. For in vitro phosphorylation of immobilized peptides the peptide arrays were incubated with 2 ml of cell extract (4.7 mg of protein), 2 ml of cell lysis buffer, and 2 ml of kinase buffer (150 mM Tris, pH 7.4, 30 mM MgCl2, 60 μM ATP, 200 μCi of [γ-32P]ATP). After 30 min at 30 °C membranes were washed twice in phosphate-buffered saline, once in 8 M urea, 1% SDS, 0.5% β-mercaptoethanol for 30 min at 40 °C, twice in H2O, and three times in EtOH. Air-dried membranes were autoradiographed at 4 °C or at room temperature. For the experiments shown in Fig. 2A 100 units of recombinant GSK-3β was incubated with 1 μg of GST-p65 fusions proteins in 1× GSK-3β reaction buffer (New England Biolabs) supplemented with 20 μM ATP and 2.5 μCi of [γ-32P]ATP in a total volume of 30 μl for 30 min at 30 °C. Reactions were stopped by the addition of SDS-PAGE sample buffer and phosphorylation of proteins visualized as described above. For detection of phosphorylated proteins by immunoblotting as shown in Fig. 2B 500 units of GSK-3β, 50 ng of GST-p65 fusion proteins, and 135 μM ATP were used in the kinase reaction, and radioactive ATP was omitted. Western blotting and site-directed mutagenesis were performed as described by Buse et al. (14).

RESULTS AND DISCUSSION

To investigate the occurrence of IL-1-inducible phosphorylation sites within the transactivating COOH terminus of p65, whole cell extracts isolated from unstimulated and IL-1-treated HeLa cells were incubated with a recombinant GST-p65-(354–551) fusion protein in the presence of [γ-32P]ATP. These experiments revealed constitutive and IL-1-inducible protein kinase activities. Mutations in Ser529 and Ser536, the sites that have been found as targets for the hitherto identified p65 TAD kinases IKKα and IKKβ (7), casein kinase II (6), TRKB (14, 29), RSK1 (16), and IKKe (14), did not completely abolish in vitro phosphorylation of GST-p65, suggesting the existence of further other p65 phosphorylation site(s) and kinases (Fig. 1A). To profile phosphorylation sites in p65 the complete p65 coding sequence arrayed as overlapping 15-mer peptides was subjected to in vitro kinase assays with cell extracts from IL-1 stimulated HeLa cells. This approach revealed more than 20 peptides that were phosphorylated in vitro (Fig. 1B), including some that contained already identified phosphorylation sites such Ser529 (Fig. 1B, peptides H1–H3). 19 peptides that are indicated by white circles in Fig. 1B contained amino acids 463–477 of p65 and systematic alanine mutations in all possible phosphorylation sites (underlined).
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Results

In the reaction mixtures were analyzed by reprobing the membrane with Ser468 specific antibodies. The amounts of GST-p65 fusion proteins in the reaction mixtures were confirmed by Coomassie Brilliant Blue staining (CBB) of gels. Black arrows indicate the position of GST-p65, and white arrows indicate autophosphorylation of GSK-3β. B, kinase assays essentially performed as described for A were analyzed for phosphorylation of Ser468 by immunoblotting using anti-phospho-Ser468 specific antibodies. The amounts of GST-p65 fusion proteins in the reaction mixtures were analyzed by reprobing the membrane with anti-p65 antibodies. For details see “Experimental Procedures.”

To directly address the question whether this consensus sequence is phosphorylated by recombinant GSK-3β in vitro, the purified kinase was incubated with a GST-p65-(354–551) substrate protein and a GST-p65-(354–551-Ser468-Ala) control protein where the phosphorylation site was point mutated. GSK-3β efficiently phosphorylated the GST-p65-(354–551) protein, while the GST-p65-(354–551-Ser468-Ala) mutant was phosphorylated to a minor extent (Fig. 2A), revealing GSK-3β as a serine 468 kinase. To obtain evidence for GSK-3β-mediated p65 serine 468 phosphorylation by an independent experimental approach, the products of the in vitro kinase assays were analyzed using an antibody specifically recognizing the phosphorylated form of serine 468. These experiments clearly confirmed that GSK-3β phosphorylated specifically Ser468, as no signal with the antibody was obtained using the S468A mutant protein as substrate or by omitting the substrate (Fig. 2B). Thus the experiments shown in Fig. 2 identify p65 Ser468 as a specific GSK-3β phosphorylation site in vitro. The residual phosphorylation observed in the radioactive kinase assay (Fig. 2A) and the detection of three protein bands of different mobility on SDS-PAGE by immunoblotting with the phospho-Ser468-specific antibody (Fig. 2B) leave the possibility that the p65 TAD might contain additional GSK-3β sites as previously suggested by Schwab and Brenner (23). To investigate whether GSK-3β phosphorylates Ser468 in vitro, HeLa cells were stimulated with IL-1, TNF, or phorbol ester plus ionomycin, but phosphorylation of endogenous Ser468 was only faintly activated (data not shown). Intriguingly, treatment of cells with the PP1/PP2A Ser/Thr phosphatase inhibitor calyculin A (32) for 30 min strongly induced Ser468 phosphorylation. These results can be reconciled with a recently suggested model whereby PP1 dephosphorylates GSK-3β at the NH2-terminal serine 9, thereby activating the kinase (33). In this model, mutual control is ensured by GSK-3β-mediated inhibition of the protein I-2, a negative regulator of PP1, thus maintaining GSK-3β in an active state in unstimulated cells. This mechanism also implies that inhibition of GSK-3β accelerates inactivation of PP1 by I-2 (33). We therefore analyzed if inhibition of PP1 affects phosphorylation of GSK-3β in the cells employed in this study. Incubation of HeLa cells with calyculin A triggered the phosphorylation of GSK-3α at serine 21 and of GSK-3β at serine 9, as revealed by immunoblotting with a phospho-spe-

FIG. 2. GSK-3β phosphorylates Ser468 of p65 in vitro. A, recombinant GSK-3β and GST-p65-(354–551) or a version in which Ser468 was mutated to alanine (GST-p65S468A) were subjected to in vitro kinase assays (ka). Reaction mixtures were separated by SDS-PAGE and phosphorylated proteins detected by autoradiography. Equal amounts of GST-p65 fusion proteins were confirmed by Coomassie Brilliant Blue staining (CBB) of gels. Black arrows indicate the position of GST-p65, and white arrows indicate autophosphorylation of GSK-3β. B, kinase assays essentially performed as described for A were analyzed for phosphorylation of Ser468 by immunoblotting using anti-phospho-Ser468 specific antibodies. The amounts of GST-p65 fusion proteins in the reaction mixtures were analyzed by reprobing the membrane with anti-p65 antibodies. For details see “Experimental Procedures.”

FIG. 3. GSK-3β phosphorylates Ser468 in vitro. HeLa cells were treated for 30 min with calyculin A (100 nM) or left untreated. Then cells were treated with increasing concentrations of LiCl for further 30 min as indicated. Cells were lysed in SDS sample buffer, and proteins were separated by SDS-PAGE and analyzed by Western blotting using antibodies against phospho-Ser468 of p65 (P-S468p65), p65, phospho-Ser21 of GSK-3α (P-GSK-3α), and phospho-Ser9 of GSK-3β (p-GSK-3β), respectively, GSK-3β, phospho-Ser32/36 of IκBα (P-IκBa) and IκBa.

FIG. 4. Mutation of Ser468 to alanine enhances activity of p65 in p65-deficient cells. A, 1 μg of pMT7 expression plasmids for wild type p65 (wt), the Ser468 mutant (S468A), or empty vector (−/−) were transiently transfected in p65-deficient fibroblasts. After 24 h cytosolic and nuclear extracts were prepared as described, and expression of p65 proteins analyzed by Western blotting (black arrows). White arrows indicate a protein that is detected unspecifically by the p65 antibodies. B, 0.3 μg of expression plasmids for wild type p65 (wt) or the Ser468 mutant (S468A) were cotransfected in p65-deficient fibroblasts together with 0.5 μg of NF-κB (3)luc and 1 μg of SV40-β-gal. After 24 h the cells were lysed, and luciferase activities were determined and normalized for β-gal activity. Shown are the mean values ± S.E. from four independent experiments performed in duplicates comparing the activity of the p65 S468A mutant relative to the p65 wild type protein.
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![Diagram showing the phosphorylation of p65 NF-κB at Serine 468](image)

Here, we suggest a function for GSK-3β-mediated phosphorylation of Ser468 in negative regulation of p65. Collectively, these results predict that the balance of active GSK-3β and PP1 determines the phosphorylation status of Ser468 in unstimulated cells (see Fig. 5). In conjunction with other potential GSK-3β phosphorylation sites Ser468 may contribute to the altered constitutive activity of NF-κB that has been observed in chronic inflammatory disease (36) and in different tumors (37). Thus Ser468 phosphorylation may be another crucial determinant responsible for the outcome of NF-κB activation in inflammation and cancer.

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