A number of different kinases have been implicated in NF-κB regulation and survival function. Here we investigated the molecular cross-talk between glycogen synthase kinase-3β (GSK-3β) and the p105 precursor of the NF-κB p50 subunit. GSK-3β forms an in vitro complex with and specifically phosphorylates NF-κB1/p105 at Ser-903 and Ser-907 in vitro. In addition, the p105 phosphorylation level is reduced in fibroblasts lacking GSK-3β as compared with wild-type cells. GSK-3β has a dual effect on p105: it stabilizes p105 under resting conditions and primes p105 for degradation upon tumor necrosis factor (TNF-α) treatment. Indeed, constitutive processing of p105 to p50 occurs at a higher rate in cells lacking GSK-3β with respect to wild-type cells and can be reduced upon reintroduction of GSK-3β by transfection. Moreover, p105 degradation in response to TNF-α is prevented in GSK-3β−/− fibroblasts and by a Ser to Ala point mutation on p105 at positions 903 or 907. Interestingly, the increased sensitiveness to TNF-α-induced death occurring in GSK-3β−/− fibroblasts, which is coupled to a perturbation of p50/p105 ratio, can be reproduced by p105 silencing in wild-type fibroblasts.

Nuclear factor κB (NF-κB) is a group of closely related protein dimers that bind a common DNA sequence motif and are known to regulate basic processes, such as immune and proinflammatory responses, development and differentiation, cell proliferation, and apoptosis (1). Regulation of NF-κB activity is achieved at three main levels. 1) Control of p105 and p100 processing; 2) interaction of the active heterodimers with IκB inhibitory molecules (IκBa, β, γ, δ, ε, p105, and p100); and 3) phosphorylation-dependent interaction either with histone deacetylases or the CMAP-response element-binding protein/p300 (CBP/p300) (reviewed in Ref. 1 and references therein).

Homeostasis of p105 and p50 appears to be physiologically relevant since transgenic mice expressing p50, but not p105, exhibit severe inflammation and an increased susceptibility to opportunistic infections (2). In contrast NF-κB1 knockout mice lacking expression of both p50 and p105 do not display such inflammatory changes and only manifest minor defects in B cell function (3). These data indicate that p105 likely plays an important role in regulating p50 function and that this property of p105 is not compensated for by other IκBs.

Processing/degradation of p105 through the ubiquitin pathway occurs under both basal and activated conditions (4). It was reported that p50/p105 heterodimers are generated cotranslationally from a single mRNA, post-translational steps regulate p50 homodimer formation, and the intracellular ratio of p50 and p105 (5). Three structural motifs are known to be involved in the processing of the NF-κB precursor p105. Namely a ubiquitination domain between amino acids 441 and 450, an upstream glycine-rich region (amino acids 376–404) required to stop further degradation of p50 and to stabilize the molecule (6), and a death domain located at the carboxyl terminus of the protein (7). Stimulation of cells with tumor necrosis factor-α (TNF-α) triggers phosphorylation of serine 927 within the p105 PEST region by IκB kinase (IKK) (8, 9). The phosphorylation triggers rapid ubiquitination by the SCFβ-TRCP ubiquitin ligase and subsequent NF-κB1 p105 proteolysis, releasing associated Rel subunits to translocate into the nucleus and modulate target gene expression (10–12).

The two members of the protein serine/threonine kinase GSK-3 family, GSK-3α and -β, are involved in regulating cell fate and differentiation in a variety of organisms (13). Unlike most kinases, GSK-3β is active in resting cells. Stimulation with mitogens or growth factors leads to the inactivation of GSK-3β by phosphorylation of the regulatory serine residue at position 9 (14). There is evidence that GSK-3β is downstream of the wingless pathway as well as the ERK-MAPK pathway. GSK-3β acts on a wide variety of substrates including glycogen synthase, c-Jun, c-Myc, eIF-2B, NFAT1, β-catenin, and cyclin D1 (15).

Interestingly, disruption of the murine GSK-3β gene results in embryonic lethality caused by severe liver degeneration during mid-gestation (16). This phenotype is consistent with excessive tumor necrosis factor toxicity, as observed in mice lacking genes involved in the activation of NF-κB (17). In addition GSK-3β−/− fibroblasts are impaired in NF-κB activation in response to TNF-α (16). A possible explanation of the cross-talk between GSK-3β and NF-κB emerges from a recent study indicating that GSK-3β can phosphorylate RelA/p65 in vitro (18). On the other hand we have previously shown that GSK-3β and p105/NF-κB1 form a complex in vivo and that GSK-3β can phosphorylate p105 in vitro (19). In this article we further analyze the molecular connection between GSK-3β and p105/NF-κB1 and propose a model to explain the lack of NF-κB induction in GSK-3β−/− cells.
EXPERIMENTAL PROCEDURES

Chemicals and Reagents—The proteasome inhibitors ALLN and MG132 and TNF-α were purchased from Sigma. LipofectAMINE reagent and OligofectAMINE were bought from Invitrogen. GSK-3β was purchased from New England BioLabs Inc. Protein kinase A (PKA) and protein kinase inhibitor (PKI) were a kind gift of Dr. Christian Kühne.

Plasmids and Constructs—The eukaryotic expression vector p105-pcDNA3/FLAG was a kind gift of Dr. Claus Scheidereit (Max Delbrück, Center for Molecular Medicine, MDC, Berlin) and has been described before (8).

p105 cDNA was PCR amplified and subcloned into vectors pEGFP-N1 and pEGFP-C3. Deletion mutants were obtained by means of PCR amplification and subcloning into pEGFP-N1. The GeneTailor site-directed mutagenesis system (Invitrogen) was used for site-directed mutagenesis according to the manufacturer’s instructions. p50-GFP was obtained by subcloning NF-κB1 (p50) cDNA obtained from pBSV NF-κB1 (p50) kindly donated by Dr. G. J. Nabel and described in Ref. 20 into pEGFP-N1. All constructs were checked by sequence analysis.

Cell Culture—Wild-type and GSK-3β−/− mouse fibroblasts were kindly provided by Dr. Jim Woodgett. p50−/− mouse fibroblasts were a kind gift of Dr. D. Baltimore. HeLa, 293, Phoenix cells, and the mouse fibroblasts described above were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum.

Immunological Procedures—Standard protocols for immunoblotting and immunoprecipitation were used. Monoclonal antibodies against GSK-3 were purchased from BioSource Int., CA. Polyclonal antibody against GSK-3β, NF-κB p50 (C-19) and (D-17), and NF-κB p65 (C-20) were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Polyclonal antibody anti-human NF-κB NH2-terminal peptide was purchased from BIOMOL Inc. Monoclonal anti-β-galactosidase antibody was purchased from Promega Corp. Monoclonal anti-phospho-Ser/Thr-Pro and MPM-2 was purchased from Upstate, Lake Placid, NY.

Transfection—Transfections of HeLa, 293, and Phoenix cells were performed by the calcium phosphate method using standard procedures. GSK-3β−/−, p50−/−, and control mouse fibroblasts at 60–80% confluence were transiently transfected or oligofected using LipofectAMINE Plus reagent (Invitrogen) or OligofectAMINE according to the manufacturer’s instructions.

Pulse-Chase Experiments—For pulse-chase experiments to study endogenous p105, cells were grown in 10-cm plates and serum starved for 16 h. After a labeling period of 4 h with [35S]methionine/cysteine (150 Ci/mmol), cells were chased for 90 min in the absence or presence of TNF-α (20 ng/ml). At each time point cells were lysed in immunoprecipitation buffer containing 0.5% Nonidet P-40 and subjected to immunoprecipitation experiments. For the analysis of overexpressed p105, labeling time was 1 h.

Vitro Kinase Assay—293 cells grown on 10-cm plates were lysed in a buffer containing 20 mM Tris, pH 8, 100 mM KCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μM/ml pepstatin, 1 μM/ml leupeptin, 20 mM β-glycerophosphate, and 10 mM sodium orthovanadate on ice. Cells were disrupted by repeated aspiration through a 26-gauge needle, and cellular debris was removed by centrifugation. The lysates were incubated with the appropriate antibodies overnight at 4 °C, and subsequently protein A-Sepharose beads (Amersham Biosciences) were added and incubated for 2 h.

Afterward the immunoprecipitation products were centrifuged and washed twice with kinase buffer (20 mM Tris, pH 7.5, 10 mM MgCl2, 5 mM dithiothreitol, 20 μM ATP, 20 mM β-glycerophosphate, and 10 mM sodium orthovanadate) and resuspended in the same buffer. Kinase assay was performed by adding to the immunoprecipitation product 5 μCi of [32P]ATP and 150 units of recombinant GSK-3β (Cell Signaling) and incubating 30 min at 30 °C. Reactions were terminated by adding SDS-PAGE loading buffer and analyzed on a 7.5% SDS-PAGE after boiling for 2 min.

RESULTS

GSK-3β Interacts with p105 and p65 in HeLa Cells—We have previously shown that p105/NF-κB1 and GSK-3β can be co-immunoprecipitated from 293 cell extracts with an antibody specific for GSK-3β (19). Here we investigated the interaction between endogenous p105, GSK-3β, and p65/RelA in HeLa cells. We confirmed that an antibody raised against GSK-3β is able to pull down p105 and p65 from HeLa cell extracts (Fig. 1, A, lane 3, and D, lane 3). Conversely, a NF-κB1-specific antibody can co-immunoprecipitate GSK-3β (Fig. 1C, lane 2), as well as p65/RelA (Fig. 1D, lane 2). The specificity of the NF-κB1 immunoprecipitation was assessed using an antibody raised against a specific p50 peptide and by adding the same peptide as competitor in the reaction. The NF-κB1-specific antibody fails to immunoprecipitate both p105 and GSK-3β when the competitor is present (Fig. 1B). Interestingly, as shown in the first lane of Fig. 1C, p65 immunoprecipitation products contain also GSK-3β, besides the expected p105 (Fig. 1A, lane 1). The interaction between GSK-3β and p65 is in line with a recent report showing that GSK-3β phosphorylates p65 in vitro (18). Moreover it might potentially suggest the existence of a complex containing p105, p65, and GSK-3β in living cells, although
further studies are required to formally prove the existence of a ternary complex in the cell.

Expression plasmids containing full-length p105 and COOH-terminal deletion mutants: Δ920, Δ906, Δ898, fused to the NH₂-terminus of green fluorescent protein (GFP) were constructed as tools to dissect the molecular link between p105 and GSK-3β. A schematic drawing of p105 domains indicating the location of the deletions is reported in Fig. 1E. The first deletion mutant (Δ920) lacks serine 927, which has been shown to be the target for IKK kinase and to be required for inducible p105 degradation (9). The other two deletions remove candidate targets for GSK-3β kinase, namely: serine 907 (mutant Δ906), serine 903, and serine 899 (mutant Δ898) that we previously identified by sequence inspection. The ectopically expressed GFP fusion proteins migrate in an SDS-PAGE with the expected molecular mass as shown in the blot reported in the middle panel of Fig. 1F decorated with a GFP-specific antibody, and act as inhibitors of NF-κB driven translocation in transient transfection experiments (data not shown).

The p105-GFP constructs described above were assayed in immunoprecipitation experiments to map the site of interaction of GSK-3β on p105. Full-length p105 and mutants were transfected in 293 cells, 24 hours after transfection cell lysates were collected and immunoprecipitated with a GSK-3β-specific antibody. The immunoprecipitation products were challenged for the presence of ectopically expressed p105 proteins. The deletion mutants still retain the capability to bind GSK-3β, albeit with a significant reduction with respect to the wild-type protein (Fig. 1F, compare lane 1 with lanes 2–4).

GSK-3β Phosphorylates p105 in Vitro and in Vivo—As a first approach to map GSK-3β phosphorylation sites on the p105 protein we employed the p105 COOH-terminal deletion mutants in a kinase assay. Full-length p105 and deletion mutants were transfected in Phoenix cells, 24 h after transfection cell lysates were prepared and subjected to immunoprecipitation with a GFP-specific antibody. An aliquot of each immunoprecipitation product was used as a substrate in a kinase assay (Fig. 2A, lower and middle panel), another aliquot was used for a control blot decorated with anti-GFP antibody to check immunoprecipitation efficiency (Fig. 2A, upper panel).

Ectopically expressed full-length p105 can be efficiently phosphorylated by GSK-3β in vitro (lane 1). Interestingly, Δ920 mutant, lacking the last 48 amino acids, is still a substrate of GSK-3β, albeit much poorer compared with full-length p105 (lane 2). On the contrary, the level of GSK-3β-mediated phosphorylation of the other two mutant proteins appears similar to the one observed in the mock kinase assay. Altogether these data suggest that one or more GSK-3β phosphorylation sites are located between amino acids 896 and 920 and that the downstream region located between amino acid 920 and the COOH terminus of the protein might contain target sites for other kinases with a priming function.

It has recently been shown that the hedgehog signaling effector Cubitus interruptus is phosphorylated at three specific sites by PKA and this event primes further phosphorylation at adjacent serines by GSK-3β (21). It is well established that PKA is involved in NF-κB-mediated transcription regulation (22, 23). In addition we predicted a candidate PKA phosphorylation site by sequence inspection at the COOH-terminal end of p105. Therefore full-length p105 and deletion mutants, obtained by immunoprecipitation, were used to investigate whether p105 could be phosphorylated by PKA in vitro, and to define the relevant cis-acting sequences. As shown in Fig. 2B, p105 can be efficiently phosphorylated by PKA (lane 1) and the specific PKA inhibitor PKI can prevent this event (lane 2). On the contrary neither one of the deletion mutants is a substrate for PKA in vitro, indicating an absolute requirement of the last 48 amino acids for PKA phosphorylation.

To investigate whether phosphorylation of p105 by GSK-3β could be enhanced by a PKA-dependent priming event, p105 immunoprecipitation products were phosphorylated in vitro by PKA in the presence of cold ATP and then challenged with GSK-3β in the presence of 32P-labeled ATP (lane 5). PKI was included to prevent further PKA activity. A control reaction was preincubated in the presence of cold ATP and PKI (lane 4), or PKA (lane 5) and then utilized for in vitro kinase assays with GSK-3β in the presence of [32P]ATP. PKA and GSK-3β function were individually tested in standard kinase assays (lanes 2 and 3, respectively). Background levels of phosphorylation were assessed by a mock kinase assay (lane 1). D, Phoenix cells were transfected with COOH-terminal GFP-tagged p105 full-length and point mutants as indicated. Twenty-four hours after transfection cells were collected and lysed. The lysates were immunoprecipitated with anti-GFP antibody and utilized for an in vitro GSK-3β kinase assay (lower panel), mock kinase assays (central panel, or control blot decorated with an anti-GFP-specific antibody (upper panel). E, wild-type and GSK-3β−/− fibroblasts were grown in serum-free medium for 16 h and then lysed in immunoprecipitation buffer. Equal amounts of lysates were subjected to immunoprecipitation with an anti-NF-κB1 antibody (upper panel) or with an anti-phospho-Ser-903/907 antibody (lower panel). The immunoprecipitation products were resolved on a SDS-PAGE, transferred to nitrocellulose, and decorated with an NF-κB1-specific antibody.

**Fig. 2.** p105 is phosphorylated at Ser-903 and Ser-907 by GSK-3β in vitro. A, Phoenix cells were transfected with GFP-tagged full-length and COOH-terminal deleted p105 as indicated. Twenty-four hours after transfection cells were collected and lysed. The lysates were immunoprecipitated with anti-GFP-specific antibody and used for an in vitro GSK-3β kinase assay (lower panel), mock kinase assays (central panel, or control blot decorated with a GFP-specific antibody (upper panel). B, an aliquot of each immunoprecipitation product described in the legend to panel A was utilized as substrate for a PKA kinase assay. PKI was included into one reaction containing full-length 105-GFP immunoprecipitation product (second lane). C, p105 GFP protein obtained as described above was preincubated in the presence of cold ATP and PKI (lane 4), or PKA (lane 5) and then utilized for in vitro kinase assays with GSK-3β in the presence of [32P]ATP. PKA and GSK-3β function were individually tested in standard kinase assays (lanes 2 and 3, respectively). Background levels of phosphorylation were assessed by a mock kinase assay (lane 1). D, Phoenix cells were transfected with COOH-terminal GFP-tagged p105 full-length and point mutants as indicated. Twenty-four hours after transfection cells were collected and lysed. The lysates were immunoprecipitated with anti-GFP antibody and utilized for an in vitro GSK-3β kinase assay (lower panel), mock kinase assays (central panel, or control blot decorated with an anti-GFP-specific antibody (upper panel). E, wild-type and GSK-3β−/− fibroblasts were grown in serum-free medium for 16 h and then lysed in immunoprecipitation buffer. Equal amounts of lysates were subjected to immunoprecipitation with an anti-NF-κB1 antibody (upper panel) or with an anti-phospho-Ser-903/907 antibody (lower panel). The immunoprecipitation products were resolved on a SDS-PAGE, transferred to nitrocellulose, and decorated with an NF-κB1-specific antibody.
5), although further studies are required to formally prove this hypothesis.

For a finer mapping of GSK-3β phosphorylation sites on p105 we produced COOH-terminal tagged p105-GFP constructs with specific point mutations: S903A and S907A. Ser-903 and Ser-907 were previously identified as candidate targets for GSK-3β kinase by sequence inspection (19). These expression plasmids were transfected in Phoenix cells and 24 h later ectopically expressed proteins were recovered by immunoprecipitation and used as substrates in a GSK-3β kinase assay. Fig. 2D shows that the phosphorylation level of p105 point mutants is clearly reduced with respect to the wild-type protein, indicating that both Ser-903 and Ser-907 are specific targets for GSK-3β in vitro. Notably, mutation of either site appears to significantly reduce in vitro phosphorylation of the other. Interestingly, both p105–903A and p105–907A mutant proteins show a similar affinity for GSK-3β with respect to the wild-type p105 in co-immunoprecipitation experiments (data not shown).

To address whether GSK-3β phosphorylates p105 also in vivo we investigated the phosphorylation level of p105 in GSK-3β knockout as compared with wild-type fibroblasts. GSK-3β−/− and wild-type cells were serum starved for 16 h and then cell lysates were prepared and subjected to immunoprecipitation using a commercial anti-phospho-Ser/Thr-Pro antibody, or an anti-NF-κB1 antibody for standardization. As shown in Fig. 2E the amount of p105 that can be immunoprecipitated by the phospho-specific antibody is significantly lower when using cells lacking GSK-3β as compared with wild-type cells, thus strongly suggesting that GSK-3β phosphorylates p105 in vivo. Interestingly, one of the possible targets of the phospho-Ser-Thr-Pro-specific antibody is Ser-907, which is indeed followed by a proline residue.

**GSK-3β Kinase Regulates the Ratio between p50 and p105 in Vivo**—Since we showed that GSK-3β interacts with and phosphorylates p105, we addressed the effect of GSK-3β on p105 stability. NH2- and COOH-terminal GFP-tagged p105 expression constructs were transfected in mouse embryo fibroblasts lacking GSK-3β and in wild-type cells. The COOH-terminal tag allows the detection of the p105 precursor only, whereas the NH2-terminal tag is conserved also in the processed form p50. Twenty-four hours after transfection cells were fixed and analyzed by immunofluorescence. NH2-terminal tagged p105/50 shows cytoplasmic localization in wild-type cells. A typical field is shown in Fig. 3A. The picture is more composite in cells derived from GSK-3β knockout mice: p105/50 proteins are present in the cytoplasm of some cells, in others they prevalently show nuclear localization and in others they are present both in the nucleus and the cytoplasm (Fig. 3B). On the other hand overexpressed COOH-terminal-tagged p105 appears to be localized exclusively in the cytoplasm both in GSK-3β−/− and in wild-type cells (data not shown), indicating that GSK-3β does not affect p105 localization. Since overexpressed p50 localizes exclusively in the nucleus (data not shown) (23) we can suggest that constitutive processing of overexpressed p105 to p50 is taking place at a higher rate in cells lacking GSK-3β. We cannot, however, rule out the possibility that the GFP tag at the COOH terminus affects the stabilization and/or the targeting.

The ratio between the NF-κB1 precursor and its processed form p50 was also evaluated by a biochemical approach with similar outcomes. In particular, the levels of endogenous p50 can be reduced upon transfection of a GSK-3β expression plasmid in cells lacking this gene (Fig. 3C). Although the level of p105 does not seem to be affected, the ratio between p105 and p50 does increase resembling the one observed in wild-type cells.

To evaluate the involvement of cellular proteases in the constitutive processing of p105 to p50 we incubated GSK-3β−/− and wild-type cells with two proteasome inhibitors, ALLN and MG132, for different time intervals, as indicated in Fig. 3D. Both inhibitors appear to be effective and determine an increase of p105/p50 ratio in cells lacking GSK-3β (compare Fig. 3. p105/p50 ratio is influenced by GSK-3β. Wild-type (A) and GSK-3β−/− fibroblasts (B) were transiently transfected with NH2-terminal GFP-tagged p105. 24 h after transfection cells were fixed and analyzed by immunofluorescence. C, GSK-3β−/− cells were transiently transfected with a control empty vector with an expression vector for GSK-3β. After 24 h lysates were collected, separated on SDS-PAGE, and analyzed by immunoblotting with an anti-NF-κB1 antibody (center and lower panels) or anti-GSK-3 (upper panel). The intensity of p105 and p50 bands was quantified by the NIH Image 1.62 program and the ratio is reported below the blot. D, wild-type and GSK-3β−/− fibroblasts were treated with ALLN for 1, 3, and 6 h, with MG132 for 1 and 3 h, or left untreated. Lysates were collected, separated on SDS-PAGE, and analyzed by immunoblotting with an anti-NF-κB1 antibody. Bands intensity was measured with the NIH Image 1.62 program. The ratio between p105 and p50 is reported below each lane. E, wild-type and GSK-3β−/− fibroblasts were transiently transfected with an expression vector encoding for GFP-p105. A β-galactosidase expression vector was cotransfected as control to normalize transfection efficiency. Twenty-four hours later cells were incubated with MG132 for 1 h or left untreated. Lysates were collected, separated on SDS-PAGE, and analyzed by immunoblotting with anti-GFP (upper panel) or anti-β-galactosidase antibody (lower panel).
The effect of MG132 on p105 stability was also investigated by overexpression experiments. The NH\textsubscript{2}-terminal-tagged GFP-p105 expression plasmid was transfected into GSK-3\textbeta\textsuperscript{--/-} and wild-type mouse fibroblasts along with a \beta-galactosidase expression vector for standardization. Twenty-four hours after transfection cells were incubated for 1 h with MG132 and the lysates were analyzed by immunoblot to detect overexpressed p105. MG132 treatment determines a net increase in overexpressed p105 stability in GSK-3\textbeta\textsuperscript{--/-} cells (Fig. 3A), in accordance with the results obtained with endogenous p105 (Fig. 3D). The differential effect of MG132 on p105 in cells with or without GSK-3\textbeta can be considered as indirect evidence to support the stabilization effect of GSK-3\textbeta on p105, as recently reported for Notch (24).

**TNF-\textalpha-induced p105 Degradation Is Prevented in GSK-3\textbeta\textsuperscript{--/-} Cells**—It is well established that TNF-\textalpha can induce p105 proteolysis, however, the final outcome of this process is not unequivocally defined. Indeed, some reports demonstrate that TNF-\textalpha induces complete degradation of the protein (8, 27). Mouse fibroblasts lacking GSK-3\textbeta have been shown to be impaired in NF-\kappaB activation, although I\kappaB degradation occurs normally in response to TNF-\textalpha (16). To investigate whether GSK-3\textbeta plays any role in the regulation of TNF-\textalpha-induced p105 proteolysis we evaluated the effect of TNF-\textalpha on p105 stability in cells lacking GSK-3\textbeta.

Wild type and GSK-3\textbeta\textsuperscript{--/-} fibroblasts were serum starved for 16 h, as previously reported for the dissection of the NF-\kappaB activation pathways by TNF (28), pulsed for 4 h with \textsuperscript{35}S)methionine/cysteine, and chased for 90 min in the presence or absence of TNF-\textalpha. Lysates were collected 0 and 90 min following the addition of TNF-\textalpha to the medium and utilized to perform immunoprecipitation reactions with anti-NF-\kappaB1 antibody, as shown in Fig. 4A. In wild-type mouse fibroblasts p105 remains stable in the control chase and is degraded upon TNF-\textalpha treatment, indicating that endogenous p105 protein is degraded rather than processed in response to TNF-\textalpha treatment in GSK-3\textbeta wild-type fibroblasts, in accordance with other reports (8). On the contrary, in GSK-3\textbeta\textsuperscript{--/-} fibroblasts p50 appears to accumulate significantly in the control chase, while TNF-\textalpha fails to induce p105 degradation. To further study the effect of TNF-\textalpha on p105 stability, a time course experiment of TNF-\textalpha induction (0, 15, 30, 60 min) was performed in GSK-3\textbeta\textsuperscript{--/-} and wild-type cells and the lysates were used to analyze p105 protein levels by immunoblot. The same samples were used to detect P-cadherin and GSK-3 as loading controls. The blot reported in Fig. 4B shows that p105 degradation upon TNF-\textalpha treatment is blocked in cells lacking GSK-3\textbeta, confirming the data obtained by the pulse-chase experiment described above.

To investigate the molecular basis of the differential p105 regulation in wild-type and GSK-3\textbeta\textsuperscript{--/-} cells in response to TNF-\textalpha we analyzed the polyubiquitination pattern of p105. The same approach has been useful to detect polyubiquitinated intermediates of \beta-catenin and demonstrate the requirement of GSK-3\textbeta for \beta-catenin ubiquitination (29). Wild type and GSK-3\textbeta\textsuperscript{--/-} fibroblasts were co-transfected with a plasmid expressing FLAG-tagged p105 and a plasmid expressing HA-tagged \textit{ubiquitin}. A \beta-galactosidase expression plasmid was included to check transfection efficiency.

Twenty-four hours post-transfection cells were preincubated in the presence or absence of the proteasome inhibitor MG132 for 30 min and then stimulated with TNF-\textalpha for 30 min or left untreated. Cell extracts were then prepared and ectopically expressed p105 proteins were isolated by immunoprecipitation with a FLAG-specific antibody. The polyubiquitination intermediates appear as a smear of high molecular weight products when detected with an antibody against HA tag (Fig. 4C, upper panel). None of these products was detected in the absence of HA-ubiquitin indicating the specificity of the ladder. In wild-type cells a significant increase in the amount of polyubiquitination intermediates was detected after treatment with MG132 and TNF-\textalpha. On the other hand in GSK-3\textbeta\textsuperscript{--/-} cells a background level of p105 ubiquitination is detected in \textit{vivo} in all the conditions tested and TNF treatment did not induce any significant increase in ubiquitination. A control blot of the inputs decorated with an anti-\beta-galactosidase-specific antibody is shown in the lower panel of the figure. These results indicate

![Fig. 4. TNF-\textalpha-induced p105 degradation is altered in GSK-3\textbeta\textsuperscript{--/-} cells.](image-url)
that the ubiquitination process is significantly enhanced upon TNF-α treatment in wild-type cells. Ubiquitination products are strongly stabilized by MG132, suggesting that proteolytic degradation by 26 S occur rapidly after polyubiquitination. Polyubiquitination of p105 can occur also in cells lacking GSK-3β, as demonstrated by the constitutive accumulation of polyubiquitinated intermediates, but is unaffected by TNF-α.

**TNF-α-induced Degradation of p105 Requires Ser-903 and Ser-907**—TNF-α has been reported to activate Akt (28), and Akt was shown to phosphorylate GSK-3β on serine 9 with consequent inhibition of its activity (14). To evaluate the effect of TNF-α treatment on GSK-3β phosphorylation, serum-starved Phoenix cells were stimulated with TNF-α for 0, 15, and 30 min or with insulin for 15 min as positive control. Cell lysates were collected and analyzed by an immunoblot probed with a phospho-GSK-3β (Ser-9)-specific antibody (Fig. 5A). Both insulin and TNF-α treatment induce phosphorylation of GSK-3β on serine 9.

In the previous paragraphs we have shown that an active GSK-3β is required for p105 proteolysis in response to TNF-α treatment and that Ser-903 and Ser-907 are targets for GSK-3β phosphorylation *in vitro*. To evaluate the biological significance of these findings we investigated the role of Ser-903 and Ser-907 in the regulation of p105 stability following TNF-α stimulation. We transfected the COOH-terminal GFP-tagged expression plasmids encoding for wild-type p105 and its respective S903A and S907A point mutants in GSK-3β wild-type fibroblasts and analyzed p105 stability in a pulse-chase experiment. A β-galactosidase expression plasmid was co-transfected for normalization. Twenty-four hours after transfection the cells were labeled with [35S]methionine/cysteine for 60 min and then chased with or without TNF-α for an additional 90 min. As shown in Fig. 5B a single Ser to Ala mutation at positions 903 or 907 prevents p105 proteolysis in response to TNF-α, whereas the wild-type p105 protein level decreases in response to TNF-α, as expected.

**p105 silencing sensitizes cells to TNF-α-induced apoptosis**—Since we have shown that GSK-3β regulates p105 stability, a crucial biological question arises. Does the alteration in the p105/p50 ratio that occurs in cells lacking GSK-3β play any role in TNF-α-induced apoptosis? To approach this problem we designed a small interfering RNA oligonucleotide for p105 silencing. As shown in Fig. 6A, p105 silencing by RNA interference was effective in reducing p105/p50 protein levels compared with a nonspecific small interfering RNA. Silencing of NF-κB1 expression by RNA interference determines a clear loss of p105 protein after 48 h (Fig. 6A), while some residual p50 is still present 72 h after silencing (not shown), probably because of its higher stability. We then scored the sensitivity to TNF-α-induced apoptosis after p105 silencing both in wild-type and GSK-3β knockout fibroblasts. Cells were transfected with small interfering RNA targeting p105 mRNA or unspecific small interfering RNA as negative control. Twenty-four hours later cells were stimulated for a further 24 h with TNF-α and then cell viability was evaluated by propidium iodide staining and fluorescence-activated cell sorter analysis for apoptosis quantification.
experiments were repeated three times. Fig. 6B shows the percentage of cells in sub-G1 for each experimental condition. TNF-α treatment induces a net increase in cell death in GSK-3β−/− cells, as previously reported (16). Inhibition of p105 expression renders control wild-type cells sensitive to death induced by TNF-α, as it occurs in cells lacking GSK-3β. Trypan blue negative staining gave similar results (not shown). Altogether these results suggest that p105 silenced wild-type cells respond to TNF-α similarly to GSK-3β−/− cells.

**DISCUSSION**

The characterization of embryos lacking GSK-3β (16) evidenced embryonic lethality due to hepatocyte apoptosis similarly to p65−/− mice (17). Interestingly, NF-κB-dependent induction of transcription in response to TNF-α was reduced in cells lacking this kinase, while nuclear translocation and DNA binding of NF-κB being unaffected. This phenotype was surprising since it has been previously shown that NF-κB activation by tumor necrosis factor requires activation of the Akt serine-threonine kinase. Akt mediates IKK phosphorylation at threonine 23 and subsequent NF-κB activation (28). On the other hand Akt directly phosphorylates GSK-3β at serine 9 and inactivates it (14). In addition in the present work we have shown that TNF-α treatment is associated with the phosphorylation of GSK-3β on serine 9.

Apparently controversial data have been reported on the effect of the GSK-3β inhibitor lithium chloride on NF-κB activity (16, 18, 30, 31), possibly due to the different timing of lithium addition or to the different cellular contexts and might be reconciled by our present findings. We show that GSK-3β increases p105 stability in basal conditions as recently reported for the Notch1 receptor (24). Indeed, we demonstrate that constitutive p105 processing to p50 occurs at an increased rate in cells lacking GSK-3β with respect to wild-type fibroblasts, and that the processing rate can be reduced upon reintroduction of GSK-3β by transfection. In addition we show that GSK-3β activity is required to prime TNF-α-induced p105 degradation and that point mutations at specific GSK-3β phosphorylation sites in p105 prevent its degradation in response to TNF-α. Our working model depicting the dual role of GSK-3β toward p105 in constitutive and TNF-α-induced conditions is presented in Fig. 7. We suggest that p105 phosphorylation by GSK-3β stabilizes p105 in resting conditions and prevents constitutive processing/degradation. On the basis of our observations we propose that phosphorylation of p105 by GSK-3β adds a further level of regulation to the NF-κB/1xB system.

Akt activation induced by TNF-α or other cytokines determines both IKK activation and GSK-3β inhibition (14) (present work). Therefore, whereas the pre-existing p105 molecules, pre-phosphorylated by GSK-3β, follow the degradation route switched on by IKK, the newly synthesized p105 inhibitors cannot be phosphorylated by GSK-3β and are excluded from the ubiquitin/proteasome degradation pathway, ensuring a tight and rapid negative control of NF-κB activity. This hypothesis is in line with the established role of GSK-3β as a general repressor, keeping its targets switched off or inaccessible under resting conditions (15).

The diametrically different effects resulting from the lack of GSK-3β on p105 processing/degradation in basal versus TNF-α-induced conditions are not surprising. A number of observations from different laboratories indicate that constitutive processing and TNF-α-induced degradation may be differentially regulated. p105 undergoes degradation in lipopolysaccharide-stimulated monocytes without enhanced generation of p50 by processing (32). Furthermore, p105 degradation without increased processing occurs in cells stimulated with TNF-α or overexpressing Tpl-2 (27) and deletion of the IKK sites on p105 does not interfere with processing (8).

Many different kinases are involved in NF-κB regulation (1) including Tpl-2 (27), PKA (22), and GSK-3β (16) and possibly some of them are interconnected. It was recently shown that various GSK-3β substrates including Cubitus interruptus (21, 33), NF-AT (34), and heterogeneous nuclear ribonucleoprotein D (35) require priming by PKA. In the present study we identified a PKA phosphorylation site within the last 48 amino acids of p105 and found that PKA priming enhances phosphorylation of p105 by GSK-3β in vitro. Additional studies are certainly required to assess the in vivo relevance of this finding and the cross-talk between PKA and GSK-3β in NF-κB regulation.

A major goal of our work was to investigate why GSK-3β−/− fibroblasts are impaired in NF-κB activation in response to TNF-α. Our study shows the involvement of p105. We suggest that the constitutive higher p50/p105 ratio occurring in GSK-3β−/− cells could slow down the replacement process of the p50 homodimer by the activated p65/p50 heterodimer. Indeed a recent report shows that the p50 homodimer interacting with DNA under resting conditions prevents transcription activation from NF-κB-driven promoters by means of histone deacetylases recruitment (23). Moreover, the failure of TNF-α to induce the degradation of p105 in GSK-3β−/− cells could maintain a fraction of the potential transcription activators in a frozen state, despite the fact that other IkB proteins are normally degraded. Interestingly the unbalance of the p50/p105 ratio occurring in cells lacking GSK-3β can be reproduced in wild-type cells by small interfering RNA-p105 silencing where it is coupled to an increase sensitiveness to TNF-α-induced apoptosis, just like it occurs in GSK-3β−/− fibroblasts. An alternative explanation for the block of NF-κB activation in cells lacking GSK-3β was recently reported on the basis of the evidence that p65 can be phosphorylated by GSK-3β (18). It is likely that the intersection between GSK-3β and NF-κB pathways occurs at multiple levels. Altogether the findings here reported support the idea that control of p105 stability plays an
important role in the delicate equilibrium of NF-κB regulation and survival function.

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