Resolution of the Nuclear Localization Mechanism of Glycogen Synthase Kinase-3

FUNCTIONAL EFFECTS IN APOPTOSIS*

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Mechanisms regulating the nuclear localization of glycogen synthase kinase-3β (GSK3β) remained enigmatic despite the crucial regulation by nuclear GSK3β of important cellular functions. These include regulation of gene expression, cell cycle progression, and apoptosis, achieved by the phosphorylation by GSK3 of nuclear substrates (e.g. numerous transcription factors). We resolved this mechanism by identifying a bipartite nuclear localization sequence (NLS) that is necessary for the nuclear accumulation of GSK3β and is sufficient to drive yellow fluorescent protein into the nucleus. Despite the NLS, most GSK3β is cytosolic, sequestered in protein complexes that, although still mobile in the cytosol, block the NLS. Conditions promoting nuclear translocation of GSK3β release it from cytosolic complexes, allowing the NLS to direct nuclear import. Using this information to prepare a nucleus-excluded active GSK3 construct, we found that the antiapoptotic effect of GSK3β in tumor necrosis factor-induced apoptosis is mediated by cytosolic, not nuclear, GSK3β. Identification of a GSK3β NLS allows new strategies to decipher and manipulate its subcellular actions regulating gene expression and apoptosis and its involvement in diseases.

Cells have the inherent ability to rapidly and concurrently modulate the actions of many proteins in response to environmental and biological cues. A large part of these coordinated cellular responses are due to posttranslational modifications of key regulatory proteins. This requires the appropriate localization of the modifying enzymes, particularly protein kinases, since phosphorylation is the predominant mechanism for protein posttranslational modification. Dynamic fluxes in the nuclear level of the serine/threonine kinase glycogen synthase kinase-3β (GSK3β)² occur during key cellular events. Nuclear GSK3β levels markedly increase when cells enter the S-phase of the cell cycle, followed by a loss of nuclear GSK3β as the cell cycle progresses (1). Nuclear GSK3β levels decrease in response to stimulation by proliferative growth factors, whereas insults that induce apoptosis can cause accumulation of GSK3β in the nucleus prior to the activation of the caspase cascade (2). Additionally, GSK3β in the nucleus is highly active relative to cytosolic GSK3β (3). Fluxes in GSK3β in the nucleus at critical periods may be related to the well documented capacity of nuclear GSK3β to regulate many transcription factors, such as nuclear factor-κB (NF-κB), cyclic AMP response element-binding protein (CREB), Snail, p53, AP-1, Myc, and others, that exert widespread effects on cellular functions by regulating the expression of many genes (4–7). Thus, cellular phenotype and survival can be influenced by the level of GSK3β in the nucleus. Despite these crucial cellular events involving nuclear GSK3β, a longstanding problem remains unresolved, deciphering the mechanism of its nuclear import.

There are two isoforms of GSK3, GSK3β and GSK3α, and these are involved in modulating a large and diverse number of cellular functions, including metabolism, development, cellular architecture, gene expression, and survival (7–9). GSK3β is constitutively active, but several mechanisms contribute to controlling its actions. Inhibitory phosphorylation by Akt and other kinases occurs on serine 9 of GSK3β. Conversely, phosphorylation of GSK3β on tyrosine 216 increases its activity (8, 10). GSK3β is also regulated by protein complex formation, especially well known in the cytosolic Wnt signaling pathway, where GSK3β associated with a large protein complex phosphorylates β-catenin to promote its degradation (11). Phosphorylation of substrates by GSK3β is also usually controlled by the phosphorylation state of the substrate, because most substrates of GSK3β must be “primed,” prephosphorylated at a residue four amino acids C-terminal to the GSK3β target site. A mutation in the primed substrate binding pocket of GSK3β, R96A, that blocks its binding to primed substrates is widely used to identify primed substrates of GSK3β (12). In addition to these three regulatory mechanisms, the subcellular distribution of GSK3β regulates its actions by controlling its accessibility to substrates, such as those in the nucleus (7).

Nuclear levels of proteins are balanced by export and import mechanisms. The nuclear export of GSK3β is inhibited by lep-

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² The abbreviations used are: GSK, glycogen synthase kinase; CREB, cyclic AMP-response element-binding protein; EGS, ethylene glycolbis(succinimidylsuccinate); FRAP, fluorescence recovery after photobleaching; MEF, mouse embryonic fibroblast; NES, nuclear export sequence; NLS, nuclear localization sequence; PBS, phosphate-buffered saline; TNF, tumor necro-
sis factor; YFP, yellow fluorescent protein; HA, hemagglutinin; BSA, bovine serum albumin.
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tomycin B, indicating that at least a portion of GSK3β nuclear export is CRM1-dependent (2). Nuclear export of GSK3β is partially mediated by FRAT-1, which binds GSK3β in the nucleus followed by export of the complex via the nuclear export sequence (NES) in FRAT-1 (13). To counter export, we hypothesized that GSK3β may contain an NES; however, examination of the primary amino acid sequence did not reveal a consensus NES, nor was one identified using the CUBIC NES prediction algorithm (14). Therefore, we examined regions in GSK3β rich in basic amino acids (lysine and arginine), a general requirement for an NES. This enabled us to identify an NES motif in GSK3β that is necessary for nuclear import of GSK3β and is sufficient to drive the nuclear import of yellow fluorescent protein (YFP). Further experiments revealed that the subcellular distribution of GSK3β is regulated by the NES, by the N-terminal tail of GSK3β, and by cytosolic protein complexes that are capable of blocking the NES function by sequestering GSK3β in the cytosol. Finally, the controversial intracellular site of action of GSK3β that inhibits tumor necrosis factor (TNF)-induced cytotoxicity was resolved by showing that this was mediated by NES-deficient GSK3β in the cytosol.

MATERIALS AND METHODS

Cell Culture—HEK293, mouse embryonic fibroblasts (MEFs), and HeLa cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen), 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 15 mM HEPES (Cellgro, Herndon, VA) in humidified, 37 °C chambers with 5% CO₂.

Immunoblotting—Cells were washed twice with phosphate-buffered saline (PBS) and were lysed with lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.5% Nonidet P-40, 1 mM sodium orthovanadate, 100 μM phenylmethanesulfonyl fluoride, 0.1 μM okadaic acid, 50 mM sodium fluoride, and 10 μg/ml each of leupeptin, aprotinin, and pepstatin) and incubating on ice for 30 min. After extraction, the nuclear samples were centrifuged at 20,800 × g for 15 min at 4 °C, and the supernatant was centrifuged at 2700 × g for 10 min at 4 °C. The supernatant was centrifuged at 20,000 × g for 15 min at 4 °C to obtain the cytosolic fraction. The pellet containing nuclei was washed twice in 200 μl of wash buffer (5 mM HEPES, pH 7.4, 3 mM MgCl₂, 1 mM EGTA, 250 mM sucrose, 0.1% BSA, with protease and phosphatase inhibitors). The pellet was then resuspended in wash buffer and layered on top of 1 ml of 1 M sucrose (with protease and phosphatase inhibitors), and centrifuged at 2700 × g for 10 min at 4 °C. The nuclear pellet was washed in lysis buffer containing 0.05% Nonidet P-40. The nuclear proteins were extracted by resuspending the pellet in nuclear extraction buffer (20 mM HEPES, pH 7.9, 300 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 100 μM phenylmethanesulfonyl fluoride, 0.1 μM okadaic acid, 50 mM sodium fluoride, and 10 μg/ml each of leupeptin, aprotinin, and pepstatin) and incubating on ice for 30 min. After extraction, the nuclear samples were centrifuged at 20,000 × g for 15 min at 4 °C, and the supernatant was retained as the nuclear extract.

Cloning, Site-directed Mutagenesis, and Transfection—Site-directed mutagenesis was performed on GSK3β-HA in pcDNA3.1+ (generously provided by Dr. J. M. Woodgett, University of Toronto) using the GeneEditor site-directed mutagenesis system (Promega, Madison, WI) following the manufacturer’s protocol. Primers used for mutagenesis were as follows: for lysines 85 and 86 to alanine, GAGAACTGGTCC-CCATCGGCGAGTGATTTGCGGACGAG; for arginine 102 to glycine and lysine 103 to alanine, GAGGTCCAGATC-ATGGGAGGCCTGATCATCACTGTAAC; for lysines 122 and 123 to alanine, CTACTCCAGGATGGAGGCGCACGATGAGGTCTAC. The arginine 96 to alanine and serine 9 to alanine mutants were a kind gift from Dr. G. V. W. Johnson (University of Alabama at Birmingham). To generate Δ9-GSK3β-HA (deletion of the nine N-terminal amino acids), wild-type GSK3β-HA was used as a PCR template with the following primers: 5′-TCTAGTACGATTTTGCGAGAGCAGC and 5′-TAAACTATGCAGGCGCTCGTCTGACTAG. The truncated product was inserted into pcDNA3.1+ using NotI and Nhel restriction enzymes. DNA encoding the GSK3β NLS was synthesized to contain a 5′ EcoRV compatible end and a 3′ overhang compatible with Xhol, a TAG stop codon, and a 5′ phosphorylation, in addition to the coding region for the NLS (sequence available upon request). The eYFP vector was digested with EcoRV and Xhol, and the NLS was ligated into the YFP-NLS fusion. All DNA was synthesized and PAGE-purified by Integrated DNA Technologies (Coralville, IA). The coding regions of all constructs were verified by sequencing at the University of Alabama at Birmingham center for AIDS research DNA sequencing core facility. Cells were transfected 24 h after plating using Fugene 6 (Roche Applied Science) at a ratio of 4 μl of Fugene to 1 μg of DNA following the manufacturer’s protocol. To achieve equal expression levels, all constructs were used at equal amounts except K85A, K86A-GSK3β-HA, which required twice as much DNA to reach an equal expression level.

Immunoprecipitation—Immunoprecipitations were performed using sheep anti-mouse IgG Dynabeads (Dynal Biotech, Oslo, Norway). The Dynabeads, 10 μl of slurry per sample, were washed twice in PBS plus 0.1% BSA; all washes were performed
using a magnetic particle separator. The beads were incubated with 1 μg of anti-GSK3β antibody or 1 μl of anti-HA antibody in PBS/BSA at 4°C with end-over-end mixing overnight. The beads were then washed twice in PBS/BSA followed by the addition of cell lysate (200 μg of protein). The beads plus lysate were incubated at 4°C for 2 h with end-over-end mixing. The beads were then washed three times with PBS/BSA.

**GSK3β Activity Measurement**—GSK3β was immunoprecipitated, and the beads were washed a final time in kinase buffer (20 mM Tris, pH 7.5, 5 mM MgCl₂, 1 mM dithiothreitol). The beads were then resuspended in 30 μl of kinase buffer containing 250 μM ATP, 1.4 μCi of [γ-32P]ATP, and 50 μM phosphoglyceron synthase peptide (Upstate Biotechnology). The samples were incubated at 30°C for 30 min, the reaction tubes were centrifuged for 1 min, and triplicate 9-μl aliquots of each sample were spotted on P81 filter paper circles. The filter paper was then air-dried and counted in a scintillation counter. Activity was normalized to the amount of GSK3β immunoprecipitated, which was determined by immunoblotting.

**Immunofluorescence**—Cells were plated on poly-D-lysine-coated coverslips in 35-mm dishes. After transfection (24 h), cells were fixed in 2% paraformaldehyde at 37°C for 20 min. Cells were then washed twice in PBS followed by permeabilization with 0.1% Triton X-100 in PBS for 5 min at room temperature. The cells were then blocked in 3% BSA, PBS for 1 h at room temperature. Cells were stained with Alexa-488-HA antibody (1:2500 in 3% BSA/PBS; Covance) and incubated with rocking at 4°C overnight. The coverslips were washed with PBS and then incubated with 100 ng/ml Hoechst 33342 for 15 min at 37°C. The cells were then blocked in 3% BSA, PBS for 1 h at room temperature. Cells were washed twice in PBS followed by permeabilization with 0.1% Triton X-100 in PBS for 5 min at room temperature. Cells were then incubated with 100 ng/ml Hoechst 33342 for 15 min at room temperature. The coverslips were washed for several hours in PBS, with frequent changes of the PBS. The coverslips were mounted on slides and examined by fluorescence microscopy (Nikon) with a ×40 oil immersion objective. Images were captured using Image Pro-plus software.

**Fluorescence Recovery after Photobleaching (FRAP) and Confocal Microscopy**—FRAP analysis was performed in live cells expressing GSK3β-eYFP using the FRAP module on a Leica SP2 laser-scanning confocal microscope with a Leica DMXRE upright microscope using a ×100 oil immersion objective. Pre-bleach and postbleach images were captured using a 514-nm laser at 20% power with image acquisition at 800 MHz. For bleaching, an area of 13 μm² was illuminated with the 514-nm laser at 100% power for 6 frames (~0.7 s/frame). The mobile fraction Mₑ was calculated using the equation \( Mₑ = (Fₑ - F₀)/(Fᵢ - F₀) \) (15, 16), where \( Fᵢ \) is the fluorescence intensity in the bleached region immediately after the bleach, and \( F₀ \) is the intensity before the bleach. All values were corrected for loss of fluorescence during acquisition using a region outside of the bleached region. Standard confocal microscopy was performed on live cells using a Leica SP1 laser-scanning confocal with an inverted microscope and ×100 oil immersion objective. All confocal analyses were performed at the University of Alabama at Birmingham high resolution imaging core facility.

**Flow Cytometry**—Annexin-V-PE staining was performed according to the manufacturer’s protocol (BD PharMingen). Stained cells were analyzed using a BD LSRII flow cytometer and BD FACSDiva software. Flow cytometry was performed at the University of Alabama at Birmingham center for AIDS research flow cytometry core facility.

**Cell Death Assay**—Cell death in MEFs was measured as described previously with minor modifications (4). Cells in 60-mm dishes were co-transfected with the indicated construct and LacZ at a ratio of 2:1. After transfection (24 h), cells were trypsinized and counted, and 20,000 cells/well were plated in a 48-well plate. Cells were grown an additional 24 h, followed by treatment with TNF. Following treatment, β-galactosidase activity was measured and compared with the untreated control for each construct as a measure of cell viability.

**In Situ Cross-linking**—Following the indicated treatments, cells were washed twice in 37°C PBS, followed by the addition of the cross-linker, 2 mM ethylene glycolbis(succinimidylsuccinate) (EGS) (Pierce) in PBS, pH 7.4. Cells were incubated at 37°C for 30 min, the reaction was then quenched by the addition of 50 mM Tris, pH 8.0, and samples were incubated at 37°C for 10 min. Cells were washed in PBS and harvested, and subcellular fractions were prepared. Nuclei were lysed using lysis buffer containing 0.5% Nonidet P-40.

**Statistical Analysis**—Statistical analysis was performed using one-way analysis of variance with Dunnett’s post hoc test or Student’s t test using In Stat software.

**RESULTS**

**Identification and Mutagenesis of the Putative NLS in GSK3β**—GSK3β contains a region of 38 amino acids consisting of residues 85–123 that is rich in lysine and arginine (32%), which we hypothesized may encompass an NLS (Fig. 1A). An important feature of an NLS is that it must be externally exposed so that it can bind to nuclear import proteins. The crystal structure of GSK3β (17) shows that this putative NLS domain forms an external loop, indicating that it is easily accessible to proteins involved in the intracellular transport of GSK3β (Fig. 1B). To begin to test if this region contains an NLS, several of the basic amino acids were mutated to alanine or glycine. These mutated constructs of GSK3β (Fig. 1) do not alter the level of endogenous GSK3β in Western blots. Each GSK3β construct was expressed in HEK293 cells, and Western blot analysis demonstrated that expression levels were equivalent to expressed wild-type GSK3β-HA and that the expression of GSK3β constructs did not alter the level of endogenous GSK3β (Fig. 1C). Additionally, the phosphorylation status at serine 9 and tyrosine 216 of each GSK3β construct was examined using site-specific phosphorylation-dependent antibodies. The serine 9 phosphorylation level of wild-type GSK3β-HA and each of the mutants paralleled expression levels of each construct with the exception of K85A,K86A-GSK3β, which exhibited relatively less serine 9 phosphorylation (Fig.
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FIGURE 1. Identification and mutagenesis of an NLS in GSK3β. A, the sequence of GSK3β shows that the region labeled as the basic domain (BD), consisting of amino acids 85–123, contains 12 basic amino acids and is located within the kinase domain. B, an accessible external loop is contained in the basic domain based on the crystal structure of GSK3β. The molecular rendering of a monomer of GSK3β was generated using the Cn3D software from the NCBI based on the protein data bank file 1H8F. C, the lysines and arginines (indicated by the asterisks in A) in the basic domain of GSK3β were mutated to alanine or glycine. HEK293 cells were transiently transfected with wild-type GSK3β-HA or mutants of GSK3β-HA, and 24 h after transfection the cells were harvested, and expression levels of endogenous and expressed GSK3β constructs were examined by Western blot. The expression level for each mutant is similar to wild-type GSK3β-HA, and no detectable phosphorylation at tyrosine 216 (Fig. 1D). Similarly, R96A-GSK3β-HA and K122A,K123A-GSK3β-HA were excluded from both the nucleus and the cytosol (Fig. 2B). In marked contrast, K85A,K86A-GSK3β-HA was completely excluded from the nucleus. Similarly, R96A-GSK3β-HA and R102G,K103A-GSK3β-HA also were excluded from the nucleus. However, K122A,K123A-GSK3β-HA was distributed in both the nucleus and the cytosol to the same extent as wild-type GSK3β-HA, indicating that Lys85 and Lys123 are not required for the nuclear import of GSK3β. These results indicated that a sequence extending from Lys85 to Lys103, but not to Lys123, may constitute an NLS for GSK3β.

To test if the subcellular localization of GSK3β constructs was independent of cell type, immunofluorescent microscopy and immunoblotting were used to examine the localization of each mutant in HeLa cells. Consistent with the results from HEK293 cells, K85A,K86A-GSK3β-HA, R96A-GSK3β-HA, and R102G,K103A-GSK3β-HA were largely excluded from
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The previous findings demonstrated that the region of GSK3β consisting of amino acids 85–103 may be an NLS. In order to test if this domain meets the criteria for an NLS of being sufficient to induce nuclear localization, a YFP fusion protein was constructed containing residues 85–103 of GSK3β on the C terminus (YFP-NLS).Expressed wild-type YFP visualized in live cells was present diffusely throughout the cells (Fig. 3A). In contrast, YFP-NLS preferentially accumulated in the nucleus. Measurements of the nuclear and cytoplasmic distributions of YFP and YFP-NLS demonstrated that the NLS was sufficient to drive the majority of the YFP-NLS into the nucleus in virtually all cells (Fig. 3B). Taken together with the data shown in Fig. 2, these results confirm that the region of GSK3β consisting of amino acids 85–103 is a bona fide bipartite NLS.

GSK3 or its homologue is found in most eukaryotes. To determine if the NLS of human GSK3β is conserved, the sequences of several GSK3 homologues were compared. Alignment of the GSK3 sequences from several species demonstrated that the region containing the NLS, and particularly the identified critical basic residues, is highly conserved (Fig. 3C).

The N Terminus of GSK3 Contributes to Regulating Nuclear Localization—The crystal structure and biochemical evidence indicated that the N terminus of GSK3β interacts with the primed substrate binding site through an intramolecular interaction (12, 17). The primed binding site and the NLS overlap, indicating that the N terminus of GSK3β can interact with the NLS. Therefore, we considered the possibility that the N terminus of GSK3β might contribute to modulating the function of the

...treated with leptomycin B for 5 h (Fig. 2F). However, little nuclear accumulation of expressed wild-type GSK3β or K122A,K123A-GSK3β-HA occurred following treatment with leptomycin B, suggesting that overexpression may circumvent the CRM-1 export mechanism. Nevertheless, even with leptomycin B treatment, little, if any, K85A,K86A-GSK3β-HA, R96A-GSK3β-HA, or R102G,K103A-GSK3β-HA was detected in the nucleus (Fig. 2G), indicating that these mutations impede nuclear import.

Amino Acids 85–103 Are Sufficient for Nuclear Localization—The previous findings demonstrated that the region of GSK3β consisting of amino acids 85–103 may be an NLS. In order to test if this domain meets the criteria for an NLS of being sufficient to induce nuclear localization, a YFP fusion protein was constructed containing residues 85–103 of GSK3β on the C terminus (YFP-NLS).Expressed wild-type YFP visualized in live cells was present diffusely throughout the cells (Fig. 3A). In contrast, YFP-NLS preferentially accumulated in the nucleus. Measurements of the nuclear and cytoplasmic distributions of YFP and YFP-NLS demonstrated that the NLS was sufficient to drive the majority of the YFP-NLS into the nucleus in virtually all cells (Fig. 3B). Taken together with the data shown in Fig. 2, these results confirm that the region of GSK3β consisting of amino acids 85–103 is a bona fide bipartite NLS.

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A. The NLS consisting of amino acids 85–103 from GSK3β is sufficient for nuclear localization and is highly conserved. A, amino acids 85–103 from GSK3β were cloned onto the C terminus of YFP (YFP-NLS). The distributions in HeLa cells of expressed YFP and YFP-NLS were examined by confocal microscopy. Scale bar, 25 μm. β, quantitative values of the nuclear/cytoplasmic ratio of YFP and YFP-NLS expressed in HeLa cells and the percentage of cells with a nuclear/cytoplasmic ratio of >2. *, p < 0.05 compared with YFP. C, sequence alignment of the GSK3 NLS from human, mouse, Caenorhabditis elegans, and Schizosaccharomyces. Basic residues are shown in boldface type, and residues identified as required for nuclear import are marked with boxes.

NLS. To test this, two mutants of GSK3β were used: conversion of serine 9 to alanine (S9A-GSK3β-HA) to test if serine 9 phosphorylation is regulatory and truncation of the first 9 amino acids (Δ9-GSK3β-HA) to test if the N-terminal tail contributes to the function of the NLS. Wild-type GSK3β and each mutant were expressed in HeLa cells and immunoblotted using anti-HA demonstrated equivalent expression levels of all three, and phospho-Ser9-GSK3β immunoblots verified the absence of serine 9 in the two mutant constructs (Fig. 4A).

HeLa (Fig. 4B) or HEK293 (Fig. 4C) cells expressing wild-type GSK3β-HA, S9A-GSK3β-HA, or Δ9-GSK3β-HA were separated into cytoplasmic and nuclear fractions, and these were immunoblotted for GSK3β-HA. The levels of wild-type GSK3β-HA and S9A-GSK3β-HA were equivalent in the nucleus, but the level of Δ9-GSK3β-HA in the nucleus was 40% lower than wild-type GSK3β-HA. Localization in HeLa cells was also examined by immunocytochemistry, and this corroborated the biochemical finding of impaired nuclear localization of Δ9-GSK3β-HA (Fig. 4D). This finding indicates that the nine N-terminal amino acids are not absolutely required for nuclear localization, but they contribute to the nuclear localization via a serine 9 phosphorylation-independent interaction with the NLS.

Nuclear GSK3β Is Not Required for GSK3β-mediated Protection from TNF-induced Apoptosis—An antiapoptotic role for GSK3β in the context of TNF-mediated toxicity was first identified in a study of GSK3β knock-out mice (4) and subsequently was confirmed in a number of reports and expanded to include apoptosis induced by other members of the death receptor family that induce extrinsic apoptosis (21–25). Despite these extensive studies, the important question of whether this action of GSK3β occurs in the nucleus or the cytosol remains unresolved. This is because nuclear GSK3β promotes the cell-protective transcriptional activity of NF-κB, but other data implicate a cytosolic protective effect of GSK3β, because it inhibits activation of the initiator caspase, caspase-8, which occurs directly after receptor activation in the cytosol (reviewed in Ref. 26).

In order to clarify whether cytosolic GSK3β or nuclear GSK3β promotes survival following exposure to TNF, we used wild-type GSK3β and an active, but NLS-deficient, mutant of GSK3β to test if cytosolic GSK3β was capable of protecting GSK3β-null MEFs (GSK3β−/−) from TNF-mediated cell death. Hoeffich et al. (4) designed an elegant strategy to identify the antiapoptotic effect of GSK3β, demonstrating that TNF-induced cytotoxicity was enhanced in GSK3β−/−-MEFs from GSK3β knock-out mice and that this was reversed by expression of wild-type GSK3β. Additionally, they demonstrated that endogenous GSK3α was unable to compensate for the loss of GSK3β in TNF-induced apoptosis in GSK3β−/−-MEFs (4). Consistent with their findings, treating GSK3β−/−-MEFs with TNF for 16 h resulted in nearly half of the cells undergoing apoptosis, as indicated by positive staining for annexin-V, whereas no increase in apoptosis was detected in wild-type GSK3β−/−-MEFs treated with TNF (Fig. 5A). Examination of the subcellular distribution of endogenous GSK3α and GSK3β demonstrated that their nuclear and cytosolic levels did not change after TNF treatment in wild-type MEFs (Fig. 5B). In order to clarify the site of action of the protective effect of GSK3β in TNF-induced apoptosis, we compared the protective capacities of wild-type GSK3β-HA and R102G,K103A-GSK3β-HA expressed in GSK3β−/−-MEFs treated with TNF. First, we confirmed the nuclear and cytosolic localization of wild-type GSK3β-HA and cytosolic localization of R102G,K103A-GSK3β-HA expressed in GSK3β−/−-MEFs (Fig. 5C). Furthermore, TNF treatment did not alter the subcellular distribution of the expressed GSK3β constructs (Fig. 5D). Transfection with vector alone followed by treatment with TNF (5 ng/ml; 16 h) resulted in 44 ± 4% cell death of GSK3β−/−-MEFs (Fig. 5E). As expected, GSK3β−/−-MEFs transfected with wild-type GSK3β-HA and treated with TNF showed a reduction to only 25 ± 4% cell death. Similarly, GSK3β−/−-MEFs expressing R102G,K103A-GSK3β-HA and treated with TNF also displayed reduced cell death of only 19 ± 3%. As an additional control, GSK3β−/−-MEFs expressing the kinase-dead K85A,K86A-GSK3β-HA mutant were treated with TNF and, similar to cells transfected with vector alone, this resulted in 51 ± 3% cell death, demonstrating that cytosolic GSK3β
must be active to provide protection from TNF-induced toxicity. These findings demonstrate that GSK3β attenuates TNF-induced toxicity through a cytoplasmic target.

**Mechanisms Controlling Nuclear Localization of GSK3β**

The predominantly cytosolic distribution of wild-type GSK3β was distinctly different from the predominant nuclear accumulation of YFP-NLS, although both proteins contained the same NLS. This indicated that additional mechanisms must contribute to regulating the nuclear localization of GSK3β. Therefore, we assessed several potential mechanisms that might control the function of the NLS in GSK3β.

Many proteins, including kinases, such as mitogen-activated protein kinases, are known to enter the nucleus by a large transport as dimers (27), and the crystal structure of GSK3β raised the possibility that it also may exist as a dimer (17). Therefore, GSK3β specifically, since GSK3β is known to bind many cytosolic proteins (7), we hypothesized that these may anchor GSK3β in the cytoplasm. If GSK3β is sequestered in the cytosol by protein interactions, it should be possible to saturate the interactions by overexpression of GSK3β-HA, which would lead to an increase in the percentage of GSK3β-HA in the nucleus. Consistent with this, overexpression of GSK3β-HA resulted in a 50% greater nuclear/cytosol distribution of GSK3β-HA (Fig. 6A). However, no endogenous GSK3β was detected in the YFP-immunoprecipitants. To test for the existence of heterodimers of the two isoforms of GSK3, GSK3α was immunoprecipitated, followed by immunoblotting for GSK3β and vice versa (Fig. 6B). No detectable co-immunoprecipitation occurred between GSK3α and GSK3β. Furthermore, expression of K85A,K86A-GSK3β-HA did not alter the nuclear level of endogenous GSK3β, although K85A,K86A-GSK3β-HA was completely absent from the nucleus, indicating that K85A,K86A-GSK3β-HA did not act as a dominant negative regulator of the nuclear import of endogenous GSK3β to prevent its nuclear localization (Fig. 6C). Taken together, these findings suggest that dimerization of GSK3β is unlikely to regulate its distribution to the nucleus.

We next considered the possibility that factors external to GSK3β itself may be important for regulating the nuclear localization of homodimerization of GSK3β or its heterodimerization with GSK3α may represent an additional level of regulation for the subcellular localization of GSK3. However, GSK3 has not been shown to exist as a dimer in situ. To test if wild-type GSK3 forms dimers, GSK3β-YFP was expressed and immunoprecipitated with an antibody recognizing YFP, followed by immunoblotting for GSK3β to test if endogenous GSK3β was associated with the expressed GSK3β-YFP (Fig. 6A). However, no endogenous GSK3β was detected in the YFP-immunoprecipitants. To test for the existence of heterodimers of the two isoforms of GSK3, GSK3α was immunoprecipitated, followed by immunoblotting for GSK3β and vice versa (Fig. 6B). No detectable co-immunoprecipitation occurred between GSK3α and GSK3β. Furthermore, expression of K85A,K86A-GSK3β-HA did not alter the nuclear level of endogenous GSK3β, although K85A,K86A-GSK3β-HA was completely absent from the nucleus, indicating that K85A,K86A-GSK3β-HA did not act as a dominant negative regulator of the nuclear import of endogenous GSK3β to prevent its nuclear localization (Fig. 6C). Taken together, these findings suggest that dimerization of GSK3β is unlikely to regulate its distribution to the nucleus.

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ing strategy was used to verify that cytosolic binding partners are saturated by overexpression of GSK3β and to estimate the proportion of cytosolic GSK3β that was sequestered in protein complexes. Treatment of cells with the membrane-permeable cross-linker EGS to stabilize GSK3β bound in protein complexes showed that very little of the endogenous GSK3β in the cytosol was unbound (not cross-linked to other proteins by EGS). This is indicated by the greatly reduced amount of endog-
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Having found that GSK3 is largely protein-bound in the cytosol raised the possibility that it may be in immobile structures. To test if cytosolic GSK3β is mobile, FRAP analysis was used. GSK3β−/− MEFs were used to reduce the possibility of saturating the GSK3β-binding proteins when exogenous GSK3β was expressed. GSK3β−/− MEFs were transfected with GSK3β-YFP, and FRAP analysis was performed in live cells (Fig. 7F). From this analysis, it was determined that nearly all of the cytosolic GSK3β, 84 ± 3%, was freely mobile. Overall, these data demonstrate that cytosolic GSK3β is predominantly bound in freely mobile protein complexes, which upon stimulation can disassociate and allow nuclear localization of GSK3β.

DISCUSSION

Identifying the central mechanism controlling the nuclear localization of GSK3β has long been a crucial objective needed to clarify the regulatory effects of GSK3β on apoptosis, gene expression, and other nuclear events. We report here the previously unexpected existence of a bipartite NLS in GSK3β consisting of residues 85–103 that was identified by assessing the subcellular localization of GSK3β mutants created by site-directed mutagenesis. The data also provide insight into mechanisms controlling the function of the NLS, including maintenance of a predominantly cytosolic localization of GSK3β while allowing nuclear accumulation in response to demand.

The NLS of GSK3β was found to be both necessary and sufficient for nuclear localization. These properties were shown by the cytosolic localization of GSK3β containing mutations of key residues in the NLS and by the nuclear localization of YFP fused with the NLS of GSK3β. Furthermore, the key residues in the GSK3β NLS are highly conserved in GSK3 homologues throughout the eukaryotic kingdom. The identified NLS in GSK3β is similar to the canonical bipartite NLS in which the amino acids are organized such that there are 2 basic amino acids plus any 10 amino acids plus 3 basic of 5 amino acids. The prototypical bipartite NLS from nucleoplasm has the sequence KRPAATKKAGQAKKKK, where both the N-terminal and C-terminal basic clusters (underlined) are required for nuclear localization (28). The NLS from GSK3β has the sequence KKVLQDKRIFKNRELQ1MRK, which is similar in overall organization except that the C-terminal basic cluster is expanded to 8 amino acids. Thus, the NLS from GSK3β has an enous cytosolic GSK3β that migrated at the native molecular weight in SDS-polyacrylamide gels, indicating that it exits predominantly in protein complexes (Fig. 7B, first two lanes). In contrast, in cells overexpressing wild-type GSK3β-HA, R96A-GSK3β-HA, or R102G,K103A-GSK3β-HA, cross-linking with EGS caused only a minor decrease of the expressed constructs in the cytosol, indicating that they are mostly unbound (not cross-linked to other proteins), consistent with saturation of binding partners (Fig. 7B). Interestingly, K85A,K86A-GSK3β-HA was highly cross-linked following treatment with EGS. The reason for this anomalous behavior is not known, but it may be because this kinase-dead mutant is associated with substrates of GSK3 that it is unable to phosphorylate and release, thereby causing the high level of protein interactions stabilized by the cross-linker. The same cross-linking strategy was used to test if endogenous GSK3β was released from cytosolic protein complexes by conditions that increase nuclear GSK3. As reported previously (2), serum withdrawal or staurosporine treatment increased the nuclear level of endogenous GSK3β (Fig. 7C). In parallel, cells were treated, followed by incubation with or without EGS to stabilize endogenous GSK3β bound in protein complexes. The cytosolic and nuclear proteins were isolated and immunoblotted for the remaining unbound GSK3β or GSK3α.

FIGURE 6. GSK3 dimers are not detectable and do not contribute to nuclear localization. A, GSK3β-YFP was expressed and then immunoprecipitated (IP), and the immunoprecipitate was immunoblotted for GSK3β. No endogenous GSK3β was co-immunoprecipitated with GSK3β-YFP. B, GSK3α was immunoprecipitated from HEK293 cells and immunoblotted for GSK3β, and the reciprocal immunoprecipitation is also shown. No co-immunoprecipitation of the two GSK3 isoforms was detected. Input, 5 µg of protein of cell lysate; negative (Neg) control is isotype-matched nonimmune IgG. C, nuclear extract from HEK293 cells expressing wild-type GSK3β-HA or K85A,K86A-GSK3β was immunoblotted for GSK3β. Shown is a long exposure to demonstrate that the nuclear localization of endogenous GSK3β was not affected by expression of an NLS-deficient GSK3β mutant.

Thus, most of the cytosolic endogenous GSK3β was mobile, FRAP analysis was used. GSK3β−/− MEFs were used to reduce the possibility of saturating the GSK3β-binding proteins when exogenous GSK3β was expressed. GSK3β−/− MEFs were transfected with GSK3β-YFP, and FRAP analysis was performed in live cells (Fig. 7F). From this analysis, it was determined that nearly all of the cytosolic GSK3β, 84 ± 3%, was freely mobile. Overall, these data demonstrate that cytosolic GSK3β is predominantly bound in freely mobile protein complexes, which upon stimulation can disassociate and allow nuclear localization of GSK3β.
Nuclear Localization of GSK3β

A

Endogenous
GSK3β
α-Tubulin
CREB

Expressed

C N

C N

B

Expressed GSK3β-HA

WT K85A,K86A R96A R102G,K103A

Cytosolic

GSK3β

EGS
Free fraction

0.03 0.73 .05 0.74 0.62

C

Nuclear

GSK3α

Cytosolic

Nuclear

S S SF STS S S SF STS

EGS

E

Free cytosolic GSK3β (Percent of Total)

serum SF STS

Free nuclear GSK3β (Percent of Total)

serum SF STS

F

Pre-bleach Bleach Post-bleach

Relative intensity

Time (s)

0 20 40 60 80 100 120 140

1.2

1

0.8

0.6

0.4

0.2

0
Nuclear Localization of GSK3β

organization of 2 basic amino acids plus 9 amino acids plus 3 basic of 8 amino acids. Notably, this NLS of GSK3β overlaps with residues required for its catalytic activity, as well as for substrate binding. Kinase-dead mutations of Lys<sup>96</sup> in GSK3β are often used as a dominant negative construct of GSK3β, but this mutation probably impedes entry to the nucleus, suggesting that its use is restricted to studying functional effects in the cytosol. The mutant R96A-GSK3β-HA blocks binding to primed substrates and is commonly used to identify substrates of GSK3β that must be primed for subsequent phosphorylation by GSK3β. The nuclear exclusion of R96A-GSK3β-HA restricts to the cytosol its use to distinguish primed from unprimed substrates of GSK3β. Thus, the identification of the NLS in GSK3β raises important limitations in the interpretation and design of studies of the functional effects of expressed mutants of GSK3β.

Although GSK3β contains an NLS sufficient to drive YFP into the nucleus, cells maintain only a small portion of GSK3β within the nucleus. Thus, GSK3β is predominantly located in the cytosol without evidence of an NES, indicating that normally the NLS function is largely masked. Since crystallographic studies had suggested that GSK3β may exist as a dimer (17, 29), although this had not been tested <i>in situ</i>, we hypothesized that dimerization might play a role in the nuclear localization of GSK3β. However, we demonstrated that dimerization of GSK3β was unlikely to regulate nuclear localization, because expression of NLS-deficient mutants did not impede the nuclear localization of endogenous GSK3β, and dimers were not detected in co-immunoprecipitation experiments.

Although dimerization of GSK3β was ruled out as a regulatory factor, our data and the data of others indicate that three mechanisms conspire to regulate the NLS: cytosolic protein complexes, the N-terminal tail of GSK3β, and the phosphorylation state of tyrosine 216. Cross-linking analysis demonstrated that cytosolic GSK3β was predominantly bound in protein complexes, and we speculate that these sequester the majority of GSK3β in the cytosol. However, these protein complexes did not immobilize GSK3β in the cytosol, since FRAP analysis demonstrated that ~85% of cytosolic GSK3β was mobile. Perhaps most importantly for nuclear transport, a significant amount of GSK3β was freed from cytosolic protein complexes under conditions that promote nuclear accumulation of GSK3β, suggesting that sequestration in cytoplasmic protein complexes masks the NLS to limit the capability of GSK3β to traverse the nuclear membrane. This conclusion is further supported by the recent demonstration that expression of the GSK3-binding protein Axin2 sequestered GSK3β in the cytosol, thereby blocking its access to its nuclear substrate Snail (30). Thus, despite the NLS, GSK3β is maintained localized predominantly in the cytosol by GSK3β-binding proteins.

In addition to the NLS, the N-terminal tail of GSK3β was found to be capable of modulating nuclear localization of GSK3β, because truncation of the first 9 amino acids, to construct Δ9-GSK3β-HA, reduced its level in the nucleus. This result is in contrast to the previous finding that Δ9-GSK3β-HA is predominantly nuclear in prostate cancer cell lines (31, 32). This discrepancy may be due to cell type-specific effects, but we consistently found that Δ9-GSK3β-HA was not predominantly nuclear compared with wild-type GSK3β-HA, as did another recent report (30). It is also possible that expression of a high level of unregulated, active Δ9-GSK3β-HA could cause toxicity, since overexpressed GSK3β can cause apoptosis with cell shrinkage and nuclear condensation (33), which may contribute to an apparent nuclear accumulation of Δ9-GSK3β-HA in some cell types. We speculate that reduced nuclear localization caused by deletion of the N-terminal tail indicates that the N-terminal tail interacts with the NLS and that the basic amino acids in the N-terminal tail may contribute to the affinity of GSK3β for import molecules. It is already well established that when serine 9 of GSK3β is phosphorylated, the N-terminal tail of GSK3β folds over the region encompassing part of the NLS with proline 5 occupying the phospho-accepting site (12, 17). Furthermore, analysis of peptides corresponding to the sequence of the N-terminal amino acids of GSK3β demonstrated that binding of the peptides to GSK3β was not simply mediated by a phosphate group but was dependent on the sequence context of the phosphoserine, indicating that other residues within this sequence contribute to the intramolecular interaction between the N-terminal tail and its binding site in GSK3β (12, 17). Moreover, merely dephosphorylating serine 9 of GSK3β is not sufficient to influence nuclear localization, because the distribution of S9A-GSK3β is predominantly cytosolic, similar to wild-type GSK3β. Therefore, we suggest that the N-terminal tail interacts with the NLS of GSK3β via an intramolecular interaction to promote nuclear transport, so its elimination reduces the effectiveness of the NLS.

FIGURE 7. Cytosolic GSK3 is bound in protein complexes and released by stimuli. A, the proportions of endogenous and overexpressed GSK3β-HA in the nucleus were calculated by measuring the nuclear to cytosolic ratio by Western blotting for GSK3β. Values are means ± S.E. (n = 3); *, p < 0.05 compared with endogenous GSK3β. B, HeLa cells were either untransfected or transfected with the indicated GSK3β plasmid and treated with the membrane-permeable cross-linker EGS, where indicated, prior to lysis of the cells. Cells were fractionated into nuclear and cytosolic fractions and the level of unbound (noncross-linked) GSK3β was determined by dividing the densitometry value of the lane 5 containing an NLS sufficient to drive YFP under the immunoblot and was determined by dividing the densitometry value of the lane 5 containing an NLS sufficient to drive YFP under the immunoblot and was determined by dividing the densitometry value of the lane 5 containing an NLS sufficient to drive YFP under the immunoblot and was determined by dividing the densitometry value of the lane 5 containing an NLS sufficient to drive YFP under the immunoblot and was determined by dividing the densitometry value of the lane 5 containing an NLS sufficient to drive YFP under the immunoblot and was determined by dividing the densitometry value of the lane 5 containing an NLS sufficient to drive YFP under the immunoblot and was determined by dividing the densitometry value of the lane 5 containing an NLS sufficient to drive YFP under the immunoblot and was determined by dividing the densitometry value of the lane 5 containing an NLS sufficient to drive YFP under the immunoblot and was determined by dividing the densitometry value of the lane 5 containing an NLS sufficient to drive YFP under the immunoblot and was determined by dividing the densitometry value of the lane 5 containing an NLS sufficient to drive YFP under the immunoblot and was determined by dividing the densitometry value of the lane 5 containing an NLS sufficient to drive YFP under the immunoblot and was determined by dividing the densitometry value of the lane 5 containing an NLS sufficient to drive YFP under the immunoblot and was determined by dividing the densitometry value of the lane 5 containing an NLS sufficient to drive YFP under the immunoblot and was determined by dividing the densitometry value of the lane 5 containing an NLS sufficient to drive YFP under the immunoblot and was determined by dividing the densitometry value of the lane 5 containing an NLS sufficient to drive YFP under the immunoblot and was determined by dividing the densitometry value of the lane 5 containing an NLS sufficient to drive YFP under the immunoblot and was determined by dividing the densitometry value of the lane 5 containing an NLS sufficient to drive YFP under the immunoblot and was determined by dividing the densitometry value of the lane 5 containing an NLS sufficient to drive YFP under the immunoblot and was determined by dividing the densitometry value of the lane 5 containing an NLS sufficient to drive YFP under the immunoblot and was determined by dividing the densitometry value of the lane 5 containing an NLS sufficient to drive YFP under the immunoblot and was determined by dividing the densitometry value of the lane 5 containing an NLS sufficient to drive YFP under the imm
Recent findings suggest that phosphorylation of tyrosine 216 of GSK3β may contribute to regulating the nuclear distribution of GSK3β. Upon mutation of tyrosine 216 to phenylalanine, which cannot be phosphorylated, less GSK3β is found in the nucleus (30, 31), whereas there is evidence that GSK3β phosphorylated on tyrosine 216 is the preferred conformation for nuclear accumulation (34). The crystal structure of GSK3β predicts that when tyrosine 216 is not phosphorylated, the activation loop of GSK3β containing tyrosine 216 obstructs the primed substrate binding site in GSK3β (17). Our data show that this binding site is part of the NLS, and therefore the NLS is probably masked by the activation loop containing unphosphorylated tyrosine 216. However, whether or not this mechanism comes into play under physiological conditions is complicated by the controversy about how GSK3β is tyrosine-phosphorylated. Some evidence indicates that GSK3β is nearly completely phosphorylated on tyrosine 216 (19), whereas other evidence shows changes in tyrosine 216 phosphorylation under a variety of experimental conditions, especially notably within the nucleus (34).

GSK3β is well established to be an antiapoptotic regulator of death receptor-induced extrinsic apoptosis (26). This was first identified with TNF-induced apoptosis (4, 21) and subsequently was expanded to include all other apoptosis-inducing members of the death receptor family (22–24). Despite this important regulatory effect, the site of action of GSK3β responsible for attenuating death receptor-induced apoptotic signaling was not clear. Several studies reported that GSK3β inhibits apoptotic signaling proximal to activation of the initiating caspase, caspase-8, at the death receptor (21, 22, 35), indicating a cytosolic action of GSK3β. However, much evidence has shown that GSK3β in the nucleus promotes the transcriptional activity of NF-κB (4, 25), which is antiapoptotic in this context, providing circumstantial support for the idea that this action of GSK3β in the nucleus mediates its inhibitory effect on death receptor-induced apoptosis. We were able to address this issue by utilizing a nucleus-excluded, but active, GSK3β construct (R102G,K103A-GSK3β-HA) with GSK3β−/− MEFs, which can be rescued from TNF-induced cell death by expression of GSK3β (4). These experiments demonstrated that GSK3β restricted to the cytosol was as efficient as wild-type GSK3β in rescuing GSK3β−/− MEFs from TNF-induced cytotoxicity. These results do not negate the clearly established regulatory effect of GSK3β on NF-κB, but they show that the protective action of GSK3β is cytosolic, in agreement with findings that GSK3β inhibits an early step in the death receptor-induced apoptotic process (21, 22, 35).

In summary, the identification of an NLS in GSK3β, in conjunction with previous reports, provides considerable insight into the mechanisms controlling the dynamic flux of nuclear GSK3β. However, the identification of an NLS in GSK3β, but no NES, raised the question of why GSK3β is nearly completely nuclear despite the NLS. Under basal conditions, GSK3β is mostly cytosolic where it is anchored to cytoplasmic binding proteins, since GSK3β is well known to be associated in multiprotein complexes that serve as a mechanism to direct the actions of GSK3β toward specific substrates, an important task, since over 50 substrates of GSK3β have been identified (7). We speculate that these GSK3β-binding proteins limit the pool of GSK3β that is free to translocate into the nucleus. Following stimulation, GSK3β is released from the cytoplasmic anchors, and GSK3β phosphorylated on tyrosine 216 is then available to be imported into the nucleus via the NLS in conjunction with support by the N-terminal tail. Inside the nucleus, GSK3β is highly active (3) and phosphorylates numerous substrates (7). Within the nucleus, GSK3β can bind FRAT and be exported from the nucleus in a CRM1-dependent fashion (2, 13). Taken together, these mechanisms may conspire to largely block the NLS of GSK3β to maintain it predominantly in the cytosol but allow nuclear localization in the appropriate cellular conditions. Keeping in mind that the NLS encompasses residues required for kinase activity and substrate binding, these interactions provide an elegant mechanism in which GSK3β activity and nuclear localization can be tightly coupled and regulated.

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