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XIAP associates with GSK3 and inhibits the promotion of intrinsic apoptotic signaling by GSK3

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Abstract

This study examined if there are interactions between two key proteins that oppositely regulate intrinsic apoptosis, X-linked inhibitor of apoptosis protein (XIAP), a key suppressor of apoptosis that binds to inhibit active caspases, and glycogen synthase kinase-3 (GSK3), which promotes intrinsic apoptosis. Immunoprecipitation of GSK3 β revealed that XIAP associates with GSK3 β , as do two other members of the IAP family, cIAP-1, and cIAP-2. Cell fractionation revealed that XIAP is predominantly cytosolic, cIAP-1 is predominantly nuclear, and cIAP-2 is present in both compartments, and nearly all of the nuclear cIAPs are associated with GSK3. Expression of individual domains of XIAP demonstrated that the Ring domain of XIAP associates GSK3. Inhibition of GSK3 did not alter the binding of XIAP to active caspase-9 or caspase-3 after stimulation of apoptosis with staurosporine. However, inhibition of GSK3 reduced apoptosis and apoptosome formation, including the recruitments of caspase-9 and XIAP to Apaf-1, in response to staurosporine treatment. Cell free measurements of apoptosome-induced caspase-3 activation demonstrated that GSK3 acts upstream of the apoptosome to facilitate intrinsic apoptotic signaling. This facilitation was blocked by overexpression of XIAP. These findings indicate that the Ring domain of XIAP (and probably cIAP-1 and cIAP-2) associates with GSK3, GSK3 acts upstream of the apoptosome to promote intrinsic apoptosis, and the association between XIAP and GSK3 may block the pro-apoptotic function of GSK3.

Keywords

XIAP; GSK3; apoptosis

1. Introduction

Apoptosis is the major mechanism used to eliminate cells for replenishment in most organisms, thus it is a vital process, but one that must be very tightly regulated since it controls cell death [1]. Dysregulated apoptosis is associated with many prevalent diseases, such as cancers that are associated with impaired apoptosis, and neurodegenerative diseases that are associated with aberrantly activated apoptosis. Two predominant apoptotic signaling pathways have been identified, the extrinsic pathway activated by death receptors [2] and the intrinsic pathway

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mediated by mitochondrial disruption [3]. Although there is much overlap between these two pathways and they converge on the same executioner caspases, caspase-3 and caspase-7, to cause cell death [4].

Both apoptotic signaling pathways are regulated by a number of vital proteins, including the inhibitors of apoptosis (IAP) family of proteins [5]. Of the eight members of the IAP family, the most well-characterized is X-linked IAP (XIAP), the most potent suppressor of apoptosis [6,7]. XIAP, and possibly other IAPs, can inhibit apoptosis by directly binding and inhibiting several caspases, such as caspase-3, -7 and -9 [5-7]. XIAP also is a component of the apoptosome, a complex of Apaf-1, Cytochrome c and caspase-9 that facilitates caspase-9 autoactivation and activation of the executioner caspase-3. The function of XIAP in the apoptosome has not been fully clarified, it apparently both directly inhibits activated caspase-9 but also recruits caspase-3 to the complex [8]. IAPs also have been reported to inhibit apoptosis by other mechanisms, particularly by binding to death receptors that induce apoptosis [9-11]. For example, cIAP-1 is part of an anti-apoptotic complex containing DDX3 and glycogen synthase kinase-3 (GSK3) that inhibits death receptor-induced apoptosis [12].

GSK3 is also a regulator of apoptosis, promoting intrinsic and inhibiting extrinsic apoptotic signaling [13]. GSK3 is comprised of two highly homologous isoforms, GSK3 α and GSK3 β [14]. GSK3 phosphorylates over 50 substrates, thus it influences many cellular functions [15]. GSK3 differs from most kinases in that it is constitutively partially active, but regulated through inhibitory phosphorylation on serine21-GSK3 α and serine9-GSK3 β . A precise mechanism by which GSK3 promotes intrinsic apoptotic signaling has not been identified, but this action has been demonstrated with a wide variety of insults. GSK3 appears to promote intrinsic apoptotic signaling both by interacting with proteins directly involved in the cascade, and by regulating a number of transcription factors that modulate the expression of proteins that inhibit or promote apoptotic signaling [13].

Due to the prevalent apoptosis-inhibiting actions of IAPs and the widespread promotion of intrinsic apoptotic signaling by GSK3, in this study we examined if GSK3 interacts with IAP proteins, particularly XIAP, and if the functions of these proteins are affected by their association. The results demonstrate that GSK3 β associates with XIAP, cIAP-1, and cIAP-2, and specifically binds to the C-terminal RING domain of XIAP. Inhibition of GSK3 did not affect XIAP binding to caspases, but interfered with the intrinsic apoptotic signaling upstream of the apoptosome. Overexpression of XIAP disabled the pro-apoptotic action of GSK3, suggesting that the association of XIAP with GSK3 might inhibit the promotion of intrinsic apoptotic signaling by GSK3.

2. Materials and Methods

2.1 Cell culture and materials

HeLa and MDA-MB-231 cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) with 15 mM HEPES, 100 units/ml penicillin and 100 μ g/ml streptomycin, in humidified, 37°C chambers with 5% CO₂. Human neuroblastoma SH-SY5Y cells were grown in 50% Minimum Essential Medium (MEM) (Cellgro) and 50% Kaighn's Modification of Ham's F-12 (ATCC) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Jurkat cells were grown in RPMI 1640 medium (Cellgro) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium-pyruvate, 2.5 g/L glucose, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Sources of chemicals were: lithium chloride, staurosporine, cytochrome c, dATP (Sigma, St. Louis, MO), 6-bromoindirubin-3'-oxime (BIO; CalBiochem), CHIR99021 (Dr. Hilary Mclauchlan, University of Dundee), insulin-like growth factor-1 (IGF-1; Intergen, Purchase, NY), LY294002, (Alexis Biochemicals, San Diego, CA),

recombinant GSK3 β (Biolab Inc, Ipswich, MA), horseradish peroxidase (HRP)-conjugated secondary reagents (Southern Biotechnology Associates, Birmingham, AL), and Smac cDNA (Origene, Rockville, MD). The following sources provided antibodies: caspase-8, caspase-9 (BD Pharmingen, San Diego, CA), caspase-3, cleaved 85 kDa fragment of poly(ADP-ribose) polymerase (PARP), phospho-Ser9-GSK3 β , total GSK3 α/β , cleaved, active caspase-3 (Cell Signaling Technology, Beverly, MA), β -actin (Sigma), Apaf-1 (R&D systems, Minneapolis, MN). Monoclonal antibodies to caspase 9, cIAP-1, cIAP-2, and XIAP were provided by Dr. T. Zhou (University of Alabama at Birmingham). Full length XIAP was a gift from Dr. John Reed (Burham Institute).

2.2 Subcellular fractionation

Nuclei were isolated by washing adherent cells twice with phosphate-buffered saline (PBS) and adding 200 μ l of lysis buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 3 mM MgCl₂, 0.05% NP-40, 1 mM EGTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 μ M phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 5 μ g/ml pepstatin A, and 1 nM okadaic acid). Lysed cells were collected in microcentrifuge tubes and centrifuged at $2,700 \times g$ for 10 min at 4 $^{\circ}$ C. The supernatant containing the cytosol was further centrifuged at $20,800 \times g$ for 15 min at 4 $^{\circ}$ C to obtain the cytosolic fraction. The nuclei in the pellet were washed three times by gently resuspending the nuclei in 200 μ l of wash buffer (5 mM HEPES, pH 7.4, 250 mM sucrose, 3 mM MgCl₂, 1 mM EGTA, 25 mM NaCl, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 μ M phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, and 5 μ g/ml pepstatin A) and centrifuging at $2,700 \times g$ for 5 min at 4 $^{\circ}$ C. For a final wash, the nuclei were resuspended in 100 μ l of wash buffer, layered over a cushion of 1 ml of sucrose buffer (1 M sucrose, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 μ M phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, and 5 μ g/ml pepstatin A), and centrifuged at $2,700 \times g$ for 10 min. The pellet containing nuclei was washed in 100 μ l of lysis buffer and centrifuged at $2,700 \times g$ for 5 min at 4 $^{\circ}$ C to remove residual sucrose buffer.

2.3 Immunoprecipitation

After washing with PBS twice, cells were harvested in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM sodium orthovanadate, 100 μ M phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 5 μ g/ml pepstatin, 50 mM NaF, 1 nM okadaic acid, 1% Triton X-100, and 10% glycerol) and centrifuged at $20,800 \times g$ for 15 min. For immunoprecipitations, cell lysates were incubated with anti-GSK3 α or anti-GSK3 β antibody conjugated to sepharose beads for 2 hr, or with anti-XIAP or anti-caspase-9 antibody for overnight, followed by incubation with protein G or protein A sepharose beads for 2 hr. Beads were washed 3 times with 400 μ l lysis buffer, resuspended in 40 μ l of Laemmli buffer, and the denatured samples were run on 8-15% Tris/Glycine gels to detect the indicated proteins.

2.4 Plasmids

Full length FLAG tagged (N-terminal) XIAP was subcloned by PCR into pcDNA3.1+ using the following primers (GAGGTACCCATGGACTACAAAGACGATCA) and (CGTCTAGAGCCCTACTATAGAGTTAGATTAAGA). The restriction enzymes KPN1 and XbaI were used to digest the vector and insert followed by ligation. To generate GFP-FLAG-BIR1/2, BIR1/2 was cloned into the CeGFP vector (Clontech, Mountain View, CA) using the following primers (CCGCTCGAGTCATGGACTACAAAGACGATCA) and (GCTCTAGATGGATTCTTGAAGATTG) and restriction enzymes XhoI and XbaI. GFP-FLAG-BIR3 was generated by replacing BIR1/2 with BIR3 in the GFP-FLAG-BIR1/2. BIR3 was subcloned by PCR using the following primers (CGAATTCTCACCATCCATGGCAGATTATGAAG) and

(GCTCTAGACTATTGTCCCTTCTGTTCTAACAG) and restriction enzymes EcoRI and XbaI. GFP-FLAG-RING was generated by replacing BIR1/2 with RING in the GFP-FLAG-BIR1/2. RING was subcloned by PCR with following primers (GCGAATTCGCGGGACAAGAATATATAACAATATTC) and (CGTCTAGAGCCCTACTATAGAGTTAGATTAAGA) and restriction enzymes EcoRI and XbaI. All restriction enzymes were purchased from New England Biolabs (Ipswich, MA). (Plasmids were amplified in *e. coli* (JM109) and purified by MaxiPrep (Qiagen, Germantown, MD). All inserts were sequenced at the UAB Center for AIDS research DNA core facility.

2.5 shRNA and expression

Lentiviral mediated shRNA was performed using shRNA lentiviral (pLKO.1-puro) plasmids (Sigma). The oligonucleotides containing the XIAP target sequence that were used are: sequence #3, 5'-

CCGGGACATGGATATACTCAGTTAACTCGAGTTAACTGAGTATATCCATGTCT
TTTTT One 100 mm dish of 293FT cells was co-transfected with 3 µg of the pLKO.1-puro plasmids plus 3 µg each of the packaging vectors pLP1, pLP2, and pLP/VSVG (Invitrogen) using Lipofectamine 2000. The media was changed approximately 16 hr after transfection, and the cells were cultured an additional 48–72 hr. The media was then collected, centrifuged at 3000rpm for 5 min, and filtered through a 0.45 µm filter. Experimental cells were incubated with the virus-containing medium overnight in 6-well plates, the media was changed, and cells incubated for 24 hr. Cells were transferred to 100 mm dishes and infected cells were selected by incubation in puromycin (1 µg/ml). To overexpress XIAP, cells were transiently transfected with full length and deleted XIAP plasmids for 24 hr by using fuge 6 reagent (Roche Indianapolis, IN), followed by experiment assays.

2.6 Immunoblotting

Cells were washed twice with PBS, and lysed in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM sodium orthovanadate, 100 µM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 5 µg/ml pepstatin, 50 mM NaF, 1 mM okadaic acid, 1% Triton X-100, and 10% glycerol). The lysates were incubated for 30 min on ice, centrifuged at 20,800×g for 15 min, and supernatants were collected. Protein concentrations were determined using the bicinchoninic method (Pierce, Rockford, IL). Cell lysates were mixed with Laemmli sample buffer and placed in a boiling water bath for 5 min. Proteins (10–20 µg) were resolved in 8–12% SDS-polyacrylamide gels, and transferred to nitrocellulose. Blots were probed with the indicated antibodies, and were developed using horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG, followed by detection with enhanced chemiluminescence.

2.7 Cytochrome c-induced apoptosis in a cell free system

Cytosolic fractions for in vitro assays were isolated from cells by washing with PBS and adding 1 ml of cavitation buffer (5 mM HEPES, pH 7.4, 3 mM MgCl₂, 1 mM EGTA, 250 mM sucrose) containing protease and phosphatase inhibitors (1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 µM phenylmethylsulfonyl fluoride, 10 µg/mL leupeptin, 10 µg/mL aprotinin, 5 µg/mL pepstatin A, and 1 nM okadaic acid). The adherent cells were harvested and homogenized by nitrogen cavitation (200 p.s.i., for 5 min) in a cell disruption bomb (Parr Instrument Co., Moline, IL). Cell homogenates were centrifuged twice at 500×g for 5 min to remove unbroken cells and nuclei. The crude cytosolic supernatant was centrifuged at 14000 rpm for 20 min to obtain cytosolic fractions. The cell free assay of apoptosis was modified from [16]. The cytosolic fraction (70 µg) was mixed with 1 mM dATP, 1 mM DTT and cytochrome c (0.03 µg/µl), and incubated at 37°C for 1 hr, followed by immunoblotting or caspase activity assay.

2.8 Caspase activities

Fluorometric assays of caspase-3 activity were conducted as described previously [17] in 96-well clear-bottom plates, and all measurements were carried out in triplicate wells. To each well, 200 μ l of assay buffer (20 mM HEPES, pH 7.5, 10 % glycerol, 2 mM dithiothreitol) was added. The peptide substrate for caspase-3 DEVD-AMC (Alexis Biochemicals, San Diego, CA) was added to each well to a final concentration of 25 ng/ μ l. Cell lysates (20 μ g protein) were added to start the reaction. Fluorescence was measured on a fluorescence plate reader (Bio-Tek, Winooski, VT) set at 360 nm excitation and 460 nm emission. Caspase activity was calculated as [(mean AMC fluorescence from triplicate wells) – [background fluorescence]]/ μ g protein.

2.9 Immunocytochemistry

Cells were seeded on coverslips and fixed with 2% paraformaldehyde in PBS for 20 min at 37°C and then permeabilized with 0.1% Triton X-100 for 5 min at room temperature. Non-specific sites were blocked with 3% BSA for 1 h at 37°C. Cells were incubated with primary antibody at 4°C for overnight, washed five times with PBS, and incubated with the corresponding secondary antibodies for 1 h at room temperature. After washing three times with PBS, the cells were incubated with 4', 6'-diamidino-2-phenylindole dihydrochloride (DAPI) to counterstain cell nuclei for 5 min at room temperature. The coverslips were then washed three times in PBS for 10 min each, the cells were mounted with mounting media, and examined with confocal fluorescence microscopy.

3. Results

3.1 IAP proteins associate with GSK3

To test if members of the IAP family of proteins associate with GSK3 β , immunoprecipitates of GSK3 β were probed with antibodies to XIAP, cIAP-1, or cIAP-2. All three IAP proteins co-immunoprecipitated with GSK3 β from four different types of cells, including Jurkat, SH-SY5Y, HeLa, and MDA-MB-231 cells (Figure 1A). Confocal images also show the co-localization of GSK3 β with XIAP and c-IAP1 (Supplementary Figure 1). This extends our recent finding that cIAP-1 co-immunoprecipitates with GSK3 β [12] to other IAP proteins. Since the primary mechanism that regulates the activity of GSK3 β is inhibition by phosphorylation on serine-9 [18], we tested if changes in serine-9 phosphorylation altered the association of GSK3 β with IAPs. The phosphorylation of ser9-GSK3 β was reduced by incubating SH-SY5Y or HeLa cells overnight in serum-free media, and further reduced by incubation with the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 (10 μ M), and phospho-ser9-GSK3 β was increased by incubating cells overnight in serum-free media followed by treatment with IGF-1 (50 ng/ml) for 30 min (Figure 1B, top panel). These treatments did not alter the cellular levels of GSK3 β or XIAP, and although large changes in the serine phosphorylation of GSK3 β were attained, co-immunoprecipitation experiments showed that these alterations in serine-9 phosphorylation of GSK3 β did not modulate its association with XIAP in either cell type (Figure 1B, lower panel). Furthermore, treatment with three selective GSK3 inhibitors, 20 mM lithium [19], 10 μ M CHIR99021 [20], or 5 μ M BIO [21] did not alter the association of XIAP with GSK3 β , indicating that GSK3 activity is not necessary for its association with XIAP (Figure 1C).

3.2 Subcellular localization of IAPs and GSK3

The IAP proteins and GSK3 isoforms displayed distinct subcellular distributions that were similar among Jurkat, SH-SY5Y, and HeLa cells. XIAP, GSK3 α , and GSK3 β are predominantly cytosolic proteins, whereas cIAP-1 and cIAP-2 are predominantly localized in the nucleus (Figure 2A). These subcellular distributions are also evident in confocal images

(Supplementary Figure 1), confirming previous findings that XIAP is largely cytosolic, cIAP-1 is mostly in the nucleus, and cIAP-2 is in both compartments [22-26].

We next estimated the portion of each IAP protein that was associated with GSK3 β . GSK3 β was immunoprecipitated from cytosolic and nuclear fractions and the co-immunoprecipitated and non-precipitated amount of each IAP protein was measured by immunoblotting the entire fraction. Less than 10% of the total cytosolic XIAP co-immunoprecipitated with GSK3 β (data not shown). In contrast, nearly all of the nuclear cIAP-1 and cIAP-2 co-immunoprecipitated with GSK3 β (Figure 2B).

3.3 GSK3 β binds the RING domain of XIAP

The IAP proteins contain three tandem copies of the BIR (baculovirus IAP repeat) domain and a C-terminal RING domain [27,28]. Death receptor-induced apoptosis causes cleavage of XIAP between the BIR2 and BIR3 domains to produce fragments containing the BIR1-2 and the BIR3-RING domains [29]. We examined the effect of activation of the extrinsic apoptotic signaling pathway on the association of GSK3 with IAP proteins in MDA-MB-231 cells by stimulating the TRAIL receptor (TRAIL-R2) using the agonistic antibody TRA-8 [30]. Treatment with TRA-8 caused a time-dependent activation of caspase-3 between 1 and 5 hr after stimulation (Figure 3A, left panel). Simultaneously with caspase-3 activation, there was loss of intact XIAP, cIAP-1, and cIAP-2 and production of proteolytic products of each IAP protein. Each of the IAP proteins co-immunoprecipitated with GSK3 β in untreated cells, and after TRA-8 stimulation cleavage products of each of the IAPs remained associated with GSK3 β (Figure 3A, right panel). Thus, extrinsic apoptotic signaling causes cleavage of IAPs but proteolytic fragments remain associated with GSK3. This cleavage likely is mediated by caspase-8 because IAP proteins were not cleaved during intrinsic apoptotic signaling induced by staurosporine (Figure 3B).

To determine if the N-terminal BIR1-2 or the C-terminal BIR3-RING domains of XIAP bind GSK3 β , constructs of these fragments were expressed in HeLa cells (Figure 4A). Immunoprecipitation of GSK3 β showed that the BIR3-RING fragment of XIAP co-immunoprecipitated with GSK3 β and replaced a portion of the endogenous full length XIAP bound to GSK3 β (Figure 4B). However the BIR1-2 fragment of XIAP did not bind to GSK3 β even though a greater amount of BIR1-2 than BIR3-RING was expressed (Figure 4C). To further identify the domain of XIAP bound to GSK3 β , full length, BIR1-2, BIR3, and RING fragments of XIAP were expressed. Immunoprecipitation of GSK3 β demonstrated that only full length XIAP and the RING domain of XIAP associated with GSK3 β (Figure 4D). This data indicates that GSK3 interacts with XIAP through the RING domain.

3.4 GSK3 activity does not affect the binding of XIAP with caspases

One possibility is that GSK3 could impede the anti-apoptotic function of XIAP by interfering with the binding of XIAP to active caspase-3, caspase-7, or caspase-9. No caspase-3 was detected associated with immunoprecipitated GSK3 β from cells treated with staurosporine with or without lithium treatment (data not shown), indicating they do not directly associate. To test if active GSK3 affects the binding of XIAP to caspase-3, caspase-7 or caspase-9, HeLa cells were pretreated with the selective GSK3 inhibitor lithium for 30 min, followed by treatment with 1 μ M staurosporine to induce apoptosis. XIAP was immunoprecipitated after 120 and 150 min, and immunoblotting detected active caspase-3, caspase-7 and caspase-9 in the immunoprecipitants, confirming previous reports that these active caspases associate with XIAP during apoptosis [5-7]. However, the inhibition of GSK3 by lithium decreased the amount of active caspases that co-immunoprecipitated with XIAP in proportion to decreases in the active caspase levels caused by lithium's protection from apoptotic signaling (Figure 5). This indicates that GSK3 did not regulate the binding of XIAP to active caspases.

3.5 GSK3 facilitates apoptotic signaling upstream of the apoptosome

XIAP is recruited to Apaf-1 as part of the apoptosome where XIAP is thought to bind both caspase-9 and caspase-3 during apoptotic signaling [8]. XIAP binding to caspase-3 in the apoptosome has been proposed both to participate in recruiting caspase-3 to the apoptosome and to inhibit caspase-3 activity [31]. Therefore, we tested if inhibition of GSK3 with lithium influenced the association of XIAP with Apaf-1. Treatment with 1 μ M staurosporine caused a time-dependent increase in the activation of caspase-3 as determined by cleavage of PARP (Figure 6A) and this matched the time-dependent association of XIAP with Apaf-1 (Figure 6B). Staurosporine treatment also induced the association of Apaf-1 with caspase-9, but Apaf-1 did not co-immunoprecipitate with GSK3 β (data not shown). Lithium treatment reduced both caspase-3 activation and the apoptosome formation indicated by the recruitment of XIAP and caspase-9 to Apaf-1, as well as of XIAP. This indicates that GSK3 promotes signaling that leads to apoptosome formation.

To determine if GSK3 facilitates apoptosome formation directly, or indirectly by regulating upstream apoptotic signaling that induces the apoptosome, a cell-free system was used. Apoptosome formation was induced by adding recombinant cytochrome c to cytosolic extracts [16], and this was followed by measurements of caspase-3 activation. Addition of GSK3 inhibitors lithium, SB216763, or BIO to cytosolic extracts did not change caspase-3 activation induced by addition of cytochrome c (Figure 7A). Conversely, addition of recombinant GSK3 β with cytochrome c also did not alter caspase-3 activation as measured by PARP cleavage (Figure 7B). Thus, neither decreasing nor increasing GSK3 β activity altered apoptosome-induced activation of caspase-3, indicating that GSK3 stimulates intrinsic apoptotic signaling upstream of apoptosome formation and does not directly act on the apoptosome. However, comparing cytosolic extracts from cells in which the level of GSK3 β was knocked down with cytosol from wild-type cells demonstrated that knocking down GSK3 β reduced cytochrome c-induced caspase-9 and caspase-3 activation (Figure 7C) and the association of XIAP with Apaf-1, active caspase-9, and active caspase-3 (Figure 7D). Conversely, there was increased caspase-3 activation, as indicated by PARP cleavage, induced by cytochrome c addition to cytosolic extracts from cells in which GSK3 β was overexpressed compared with cytosol from wild-type cells (Figure 7E). As positive controls, cytosol from cells overexpressing XIAP reduced cytochrome c-induced caspase-3 activation (Figures 7E and 7F), and cytosol from cells overexpressing Smac increased cytochrome c-induced caspase-3 activation (Figure 7F). These results indicate that GSK3 regulates the function or expression levels of cytosolic proteins that regulate apoptosome formation induced by cytochrome c.

3.6 Overexpression of XIAP disables the anti-apoptotic effect of lithium in the intrinsic apoptotic pathway

We tested if XIAP affects the promotion by GSK3 of intrinsic apoptotic signaling. Wild-type MDA-MB-231 cells and MDA-MB-231 cells with XIAP levels knocked down were pretreated with lithium to inhibit GSK3, followed by treatment with staurosporine to activate apoptosis. Inhibition of GSK3 with lithium reduced staurosporine-induced caspase-3 activity in both wild-type and XIAP knockdown cells to a similar extent (Figure 8A). However, lithium treatment did not reduce staurosporine-induced caspase-3 activity in cells with XIAP overexpressed (Figure 8B). These results indicated that XIAP overexpression inhibits the promotion of intrinsic apoptotic signaling by GSK3, which might occur through their association.

4. Discussion

GSK3 promotes mitochondrial-mediated intrinsic apoptotic signaling induced by many insults, including growth factor withdrawal, endoplasmic reticulum stress, DNA damage, and others

[13]. However, the precise underlying mechanisms of this action are not clear. Part of this effect may be due to regulation by GSK3 of proteins directly involved in intrinsic apoptotic signaling, such as phosphorylating Ser-163 of pro-apoptotic Bax [32]. GSK3 also may reduce the threshold for apoptosis by regulating transcription factors that control expression of anti-apoptotic or pro-apoptotic proteins, such as CREB [33,34] and p53 [35]. However, one of the main inhibitors of apoptosis is XIAP, and no information is available concerning possible interactions between GSK3 and XIAP. Since cIAP-1 recently was shown to associate with GSK3 [8], we considered the possibility that GSK3 also may associate with other members of the IAP family. Therefore, this study focused on possible interactions between GSK3 and XIAP, because XIAP is the most well-established inhibitor of caspases in the IAP family of proteins. The results showed that GSK3 β associated with XIAP, as well as cIAP-1 and cIAP-2, in several cell types. Rather than finding that GSK3 regulated XIAP, the results indicate that the association of XIAP with GSK3 may mitigate the pro-apoptotic action of GSK3 during intrinsic apoptotic signaling.

XIAP and cIAPs share three tandem Baculovirus IAP repeat BIR domains and one RING domain [36-38]. The BIR1-BIR2 domain was reported to bind caspase-3/7, while the BIR3 domain specifically binds caspase-9. However, compared with BIR domains, the functions of the C-terminal RING domain of IAPs remain unresolved. It appears most likely that the RING domain of IAPs confers ubiquitin protease ligase (E3) activity to IAPs and is responsible for their auto-ubiquitination [39,40] and for promoting the degradation of other proteins, such as active caspase-3 [41]. Therefore, determining the domain of XIAP that binds to GSK3 β could provide leads for understanding the functional interactions of these two proteins. We found that the XIAP C-terminal BIR3-RING fragment, which was produced by cleavage of XIAP during extrinsic apoptotic signaling, binds to GSK3. Further co-immunoprecipitation evidence showed that the RING domain of XIAP was the predominant domain capable of individually associating with GSK3. Since GSK3 also associates with cIAP-1 and cIAP-2, which contain a RING domain similar to that of XIAP, it is very likely the cIAP Ring domains also account for their interaction with GSK3. This finding raises the possibility that GSK3 may regulate the E3 ligase function of IAPs.

During intrinsic apoptotic signaling, the apoptosome is a critical platform for extending the signal from mitochondrial disruption to activation of executioner caspases. Apoptotic signaling causes the release of cytochrome c from mitochondria which binds to cytosolic Apaf-1, forming an oligomerized signaling complex called the apoptosome [42]. Caspase-9 is recruited to the apoptosome, which facilitates the autocatalytic cleavage and activation of caspase-9, a crucial step in intrinsic apoptotic signaling. XIAP also has been shown to bind the apoptosome where it appears to contribute to the regulation and recruitment of caspase-9 and caspase-3 [8,31]. Our results confirmed that XIAP is recruited to Apaf-1 during intrinsic apoptotic signaling in a time-dependent manner. In confirmation of previous results that GSK3 promotes intrinsic apoptotic signaling, inhibition of GSK3 with lithium greatly reduced apoptosome formation. This raised the question of whether the action of GSK3 was upstream of the apoptosome or if it directly regulated the promotion of caspase activation by the apoptosome. This was addressed using the cell-free assay of apoptosome formation and function, and these demonstrated that neither recombinant GSK3 nor GSK3 inhibitors directly regulate cytochrome c-induced apoptosis signaling, indicating that GSK3 does not directly regulate the apoptosome. However, cytochrome c-induced apoptotic signaling was promoted with cytosol from cells in which GSK3 was overexpressed, and was reduced in cytosol from cells with GSK3 knocked down, indicating that GSK3 regulates cytosolic proteins that regulate apoptosome formation induced by cytochrome c release.

Conversely, we tested if XIAP activity could regulate the pro-apoptotic activity of GSK3 in intrinsic apoptotic signaling. staurosporine-induced caspase-3 activity was potentiated by

knocking down XIAP, confirming the anti-apoptotic activity of XIAP [6]. Inhibition of GSK3 with lithium blocked staurosporine-induced caspase-3 activity in both wild-type cells and in cells with XIAP knocked down to a similar extent. However, intrinsic apoptotic signaling was reduced by overexpression of XIAP, and inhibition of GSK3 no longer impeded apoptotic signaling as it did in wild-type cells. In wild-type cells only very small amount of XIAP associated with GSK3, but more association occurs upon overexpression of XIAP. These results raise the possibility that part of the anti-apoptotic action of XIAP may be attributable to binding GSK3 to block the promotion of apoptotic signaling by GSK3.

In summary, we reported that RING domain of XIAP binds GSK3 and GSK3 facilitates intrinsic apoptosome formation and following apoptotic signaling could be through cytosolic proteins expression level which regulate apoptosome formation. XIAP could inhibit the promotion of intrinsic apoptosis signaling by GSK3 through binding to GSK3.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. GSK3 β associates with IAP proteins. (A) Endogenous GSK3 β was immunoprecipitated (IP) from Jurkat, SH-SY5Y, HeLa and MDA-MB-231 cells, and the immunoprecipitates (left panel) or cell lysates (right panel) were immunoblotted for XIAP, cIAP-1, cIAP-2 and GSK3 β . (B) SH-SY5Y and HeLa cells were incubated overnight in serum-free medium (SF) followed by treatment with 10 μ M LY294002 for 1 hr or 50 ng/ml IGF-1 for 30 min. Cell lysates (top panel) were immunoblotted for phospho-serine9-GSK3 β , total GSK3 β , and XIAP. GSK3 β immunoprecipitates (bottom panel) were immunoblotted for XIAP and GSK3 β . (C) HeLa cells were treated with 20 mM lithium, 10 μ M CHIR99021, or 5 μ M BIO for 1 hr,

GSK3 β was immunoprecipitated, and the immunoprecipitates (left panel) or cell lysates (right panel) were immunoblotted for XIAP and GSK3 β .

Fig. 2. Nuclear and cytosolic distribution and association of IAP proteins with GSK3 β . (A) Jurkat, SH-SY5Y and HeLa cells were fractionated to cytosolic (C) and nuclear (N) fractions, followed by immunoblotting XIAP, cIAP-1, cIAP-2, GSK3 α and GSK3 β . (B) GSK3 β was immunoprecipitated (IP) from cytosolic and nuclear fractions of SH-SY5Y and HeLa cells, cIAP-1, cIAP-2 proteins were detected by immunoblotting both immunoprecipitants (P) and supernatants (S).

Fig. 3.

IAP protein levels and association with GSK3β after stimulation of TRAIL-R2. (A) MDA-MB-231 cells were treated with the TRAIL-R2 agonistic antibody TRA-8 for 0 to 5 hr. Cell lysates (left panels) were immunoblotted for caspase-3, XIAP, cIAP-1, and cIAP-2 (FL, full length protein; CL, cleaved fragment). MDA-MB-231 cells were treated with TRA-8 followed by immunoprecipitation (IP) of GSK3β and immunoblotting for XIAP, cIAP-1, cIAP-2, and GSK3β (right panels). (B) HeLa cells were treated with staurosporine (1 μM) for 0 to 150 min. Cell lysates were immunoblotted for cleaved caspase-3, cleaved PARP, XIAP, cIAP-1, cIAP-2, and GSK3β.

Fig. 4.

GSK3 β binds the RING domain of XIAP. (A) Diagram of XIAP domains and constructs. HeLa cells were transiently transfected with (B) vector (V) or FLAG-BIR3-RING, or (C) vector (V) or GFP-FLAG-BIR1-BIR2 (GFP-BIR1-2) fragments of XIAP, followed by immunoprecipitation (IP) of GSK3 β . Immunoprecipitants (left panels) and cell lysates (right panels) were immunoblotted for XIAP (B) or XIAP, GFP (C) and GSK3 β . (D) HEK293 cells were transiently transfected with vector (V), full length GFP-FLAG-XIAP (FL), GFP-FLAG-BIR1-2 (B1-2), GFP-FLAG-BIR3, and GFP-FLAG-RING domains of XIAP, followed by immunoprecipitation of GSK3 β and immunoblotting with anti-GFP.

Fig. 5.

Inhibition of GSK3 does not affect the binding of XIAP to active caspases. HeLa cells were pretreated with 20 mM lithium for 30 min, followed by treatment with 1 μ M staurosporine (STS). After 120 and 150 min GSK3 β was immunoprecipitated (IP), and cell lysates (top panels) and immunoprecipitants (bottom panels) were immunoblotted for active caspase-3, active-caspase-7 and active caspase-9.

Fig. 6.

Lithium blocks apoptosome formation. HeLa cells were pretreated with 20 mM lithium for 30 min, followed by 1 μ M staurosporine treatment for 90, 120, and 150 min. (A) Cell lysates were immunoblotted for cleaved PARP as an indicator of caspase-3 activity. (B) XIAP or caspase-9 was immunoprecipitated (IP), followed by immunoblotting for Apaf-1 to examine apoptosome formation and the association of XIAP.

Fig. 7.

GSK3 facilitates apoptosis upstream of apoptosome. (A) Cytosolic extracts were isolated from SH-SY5Y cells, followed by incubation with 3 different GSK3 inhibitors, lithium (20 mM), SB216763 (5 μ M), or BIO (5 μ M) and dATP (1mM) for 30 min. Recombinant cytochrome c (2 μ g) and DTT (1mM) was added and after 60 min caspase-3 activity was measured. (B) Cytosolic extracts were incubated with or without recombinant GSK3 β and dATP followed by addition of recombinant cytochrome c and DTT, and after incubation for 60 min cleaved PARP was immunoblotted. Cytosolic extracts were isolated from wild-type and GSK3 β knockdown MDA-MB-231 cells, followed by incubation with recombinant cytochrome c, dATP and DTT for 60 min, and (C) procaspase-9, active cleaved caspase-9, active cleaved caspase-3, and cleaved PARP were immunoblotted, or (D) XIAP was immunoprecipitated (IP), followed by immunoblotting immunoprecipitants for Apaf-1, active cleaved caspase-9, and active cleaved caspase-3, as indicators of apoptosome formation. (E) GSK3 β , XIAP or (F) XIAP, Smac were expressed in HeLa cells, and cytosolic fractions were isolated. After

incubation with cytochrome c, dATP and DTT for 60 min, caspase-3 activity was measured by immunoblotting cleaved PARP.

Fig. 8.

Effect of XIAP on promotion of apoptosis by GSK3. (A) Wild-type and XIAP knockdown MDA-MB-231 cells were pretreated with 20 mM lithium for 30 min and then treated with 1 μ M staurosporine for 120 min, followed by measurement of caspase-3 activity. Results are expressed relative to caspase-3 activity in wild-type cells not treated with lithium. Means $\pm$ SEM, $n=3$, * $p<0.05$ STS + Li compared with STS, ** $p<0.05$ XIAP knockdown cells compared with wild-type cells. (B) Vector and stably overexpressing XIAP SH-SY5Y cells were pretreated with 20 mM lithium for 30 min and then treated with 1 μ M staurosporine for 120 min, followed by measurement of caspase-3 activity. Results are expressed relative to caspase-3 activity in wild-type cells not treated with lithium. Means $\pm$ SEM, $n=3$, * $p<0.05$ STS + Li compared with STS, ** $p<0.05$ XIAP overexpression cells compared with wild type cells.