Abstract. Raf-1 serine/threonine protein kinase plays an important role in cell growth, differentiation and cell survival. Recent reports using c-raf-1 gene-knockouts have observed MEK/ERK independent functions of Raf-1 in cell survival and protection from apoptosis. Raf-1 has also been shown to be involved in counteracting specific apoptotic pathways by restraining caspase activation, although the precise mechanism is unknown. XIAP is a potent inhibitor of apoptosis that blocks both the mitochondria and death receptor mediated pathways of apoptosis by directly binding to and inhibiting the initiator and effector caspases. In our efforts to understand the mechanism by which Raf-1 inhibits caspase activation, we discovered a novel interaction between Raf-1 and XIAP. In this study, we describe the physical interaction between Raf-1 and XIAP in vitro and in vivo in mammalian cells. We also demonstrate that Raf-1 phosphorylates XIAP in vitro and in vivo. Additionally, Raf-1 prevents XIAP degradation in response to different apoptotic triggers. Our studies identify XIAP as a new substrate of Raf-1 and provide potentially important insight into mechanisms underlying Raf-1 effects on cell survival.

Introduction

Raf-1 protein kinase, an integral component of the Ras/Raf/MEK/ERK signaling pathway, has been shown to play an important role in cell growth, differentiation and cell survival (1,2). However, reports using genetic knockouts of the mouse c-raf-1 gene have demonstrated a MEK/ERK independent role for Raf-1 in cell survival and protection from apoptosis (3,4). Studies have linked the anti-apoptotic function of Raf-1 to a re-localization of Raf-1 to mitochondria (5,6). Raf-1 is reported to exert prosurvival effects upstream of cytochrome c release by phosphorylating the pro-apoptotic protein Bad, a Bcl-2 family member (7-9). Raf-1 is known to regulate the anti-apoptotic transcription factor NF-κB (10) that has been shown to participate in the transcriptional regulation of IAPs (11,12) as well as c-FLIP, an inhibitor of caspase-8 activation (13). Raf-1 apparently induces NF-κB activation by degradation of IκB, an inhibitor of NF-κB, via MEKK-1, independent of MEK/ERK (14). Raf-1 has also been shown to phosphorylate and inhibit the pro-apoptotic function of the ankyrin-repeat protein Tvl-1 by binding to it (15). Tvl-1 promotes the assembly of pro-apoptotic Apaf-1 complexes, caspase activation and apoptosis (16). Furthermore, the MEK/ERK-independent prosurvival function of Raf-1 may be due to antagonizing the activity of apoptotic signal-regulated kinase (ASK1) (17). Recently, Raf-1 was shown to counteract apoptosis by suppressing the activation of mammalian sterile 20-like kinase (MST2) (18). Intriguingly, in Raf-1 deficient macrophages and fibroblasts with normal activation of MEK/ERK and NF-κB, cytochrome c release is not increased (4,19). However, caspase activation by selected apoptotic stimuli such as growth factor deprivation and Fas, is enhanced (4). Thus, Raf-1 appears to be involved in counteracting specific apoptotic pathways by restraining caspase activation, although the precise mechanism is unknown.

Members of Bcl-2 and IAP gene families are two classes of cellular apoptotic inhibitors that oppose the cellular destruction by caspases. Bcl-2 proteins can block only the release of cytochrome c from mitochondria (20), whereas IAPs block both the mitochondria and death receptor mediated pathways of apoptosis by directly binding to and inhibiting the...
initiator and effector caspases (21-24). X-linked inhibitor of apoptosis protein (XIAP) is the widely studied IAP member that binds to and potently inhibits caspase-3, caspase-7 and caspase-9 activity (23,24).

In this study, we show that Raf-1 protein kinase can physically interact with XIAP both under in vitro and in vivo conditions in mammalian cells. We further demonstrate that Raf-1 phosphorylates XIAP in vitro and in vivo. Additionally, we also show that Raf-1 prevents XIAP degradation in response to different apoptotic triggers.

Materials and methods

Cell culture and transfections. MDA-MB 231 breast cancer cells, PC-3 prostate cancer cells and A549 lung cancer cells were grown as a monolayer in improved minimum essential medium (IMEM) supplemented with 10% FBS, 2 mM glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin. Human embryonic kidney 293T/17 (ATCC, Manassas, VA, USA) cells and Panc-1 pancreatic cancer cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 2 mM glutamine and penicillin/ streptomycin. Cell cultures were maintained at 37°C in an atmosphere containing 5% carbon dioxide. Transient transfections were carried out with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instructions.

Plasmid construction. DNA inserts for plasmid constructs were amplified by the polymerase chain reaction (PCR) using the AccuPrime Taq DNA Polymerase High Fidelity PCR system (Invitrogen) and primers linked to restriction enzyme sites suitable for cloning. Inserts were sequenced to rule out possible mutations introduced by PCR.

Wild-type Raf-1 expression vector (pCMV-Raf-1) and dominant negative Raf-1 expression vector (Raf-1-S621A) were purchased from BD Biosciences (Palo Alto, CA, USA). Kinase inactive Raf-1 mutant (Raf-1-K375M) was generated using the PCR-based QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) following the manufacturer's instructions. Constitutively active Raf-1 mutant (Raf-1-BXB) in pSRα expression vector was kindly provided by Dr Michael White (The University of Texas, Southwestern Medical Center, Dallas, TX, USA). His-Xpress-tagged-Raf-1 was generated by PCR using pCMV-Raf-1 as template and cloned into pcDNA4/HisMAX TOPO vector (Invitrogen).

cDNA fragment encoding the open reading frame of XIAP was amplified by PCR using XIAP cDNA (Image clone #5532247; Open Biosystems, Huntsville, AL, USA) as the template and cloned into the green fluorescent protein (GFP) expression vector pcDNA3.1/NT-GFP-TOPO or pcDNA4/HisMAX TOPO according to the instructions (Invitrogen) to generate GFP-tagged-XIAP and His-Xpress-tagged XIAP, respectively. The mammalian expression plasmid encoding GST-tagged XIAP (pEBG-XIAP) was obtained as a gift from Dr Colin Duckett (University of Michigan, Ann Arbor, MI, USA).

For in vitro studies, bacterial expression plasmid was made by cloning XIAP open reading frame into the pGEX-4T-3 vector (Amersham Biosciences, Piscataway, NJ, USA). XIAP insert construct was generated by PCR and cloned into the BamHI and EcoRI sites in pGEX-4T-3.

For the mammalian two-hybrid assays, pM-XIAP and pVP16-Raf-1 plasmids were constructed. XIAP insert was generated by PCR and cloned into the EcoRI and BamHI sites in pM vector (BD Biosciences). Raf-1 insert was generated by PCR and cloned into the EcoRI and BamHI sites in pVP16 vector (BD Biosciences).

Co-immunoprecipitation assays. Transfected and untransfected cells were lysed in buffer containing 500 mM HEPES, pH 7.2, 1% NP-40, 10% glycerol, 5 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 20 μg/ml leupeptin. In addition, we also performed co-immunoprecipitation using NP-40 lysis buffer (25) (10 mM HEPES, pH 7.2, 142.5 mM KCl, 5 mM MgCl2, 1 mM EGTA, 0.2% NP-40, 1 mM PMSF, 5 μg/ml leupeptin and 5 μg/ml aprotinin) or RIPA lysis buffer (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 20 μg/ml aprotinin, 20 μg/ml leupeptin and 5 mM sodium orthovanadate). The cell extracts were clarified by centrifugation and used for immunoprecipitation with various antibodies and protein A/G-Agarose (Roche, Indianapolis, IN, USA). As a control we used normal mouse or rabbit IgG (Sigma, St. Louis, MO, USA). Immune complexes were washed three times with lysis buffer, once with 0.5 M LiCl, 100 mM Tris-HCl, pH 7.4, and once with 10 mM Tris-HCl, pH 7.4, and resolved on SDS-PAGE. For Western blot analysis, following antibodies were used: monoclonal anti-Raf-1 antibody (BD Biosciences), polyclonal anti-Raf-1 antibody (Santa Cruz, Biotechnology, CA, USA), monoclonal anti-XIAP antibody (BD Biosciences), polyclonal anti-XIAP antibody (Cell Signaling, Beverly, MA, USA), monoclonal and polyclonal anti-GFP antibody (BD Biosciences), monoclonal anti-GST antibody (Novagen, San Diego, CA, USA) and monoclonal anti-Xpress antibody (Invitrogen). The proteins were detected using an enhanced chemiluminescence assay system (Amersham Biosciences).

Production and purification of GST-XIAP. Expression plasmid pGEX-XIAP is described above. pGEX-XIAP and pGEX-4T-3 were transformed into E. coli BL21 (Amersham Biosciences). The protein expression was induced at 25°C with 0.5 mM isopropyl-1-thio-ß-D-galactopyranoside for 2 h. Protein extracts were made from the induced cultures by sonication as described in the product manual (Amersham Biosciences). The GST and GST-XIAP proteins were affinity purified using Glutathione Sepharose 4 FastFlow beads (Amersham Biosciences). Eluted GST-XIAP was dialyzed into 20 mM HEPES, pH 8.0, for 4 h.

In vitro binding assay. In vitro translated [35S]L-methionine-labeled Raf-1 protein was prepared from pcDNA4/HisMAX-Raf-1 template using the TNT Coupled Reticulocyte Lysate Systems (Promega Corporation, Madison, WI, USA) according
to the manufacturer’s instruction. [35S]-L-methionine-labeled Raf-1 protein was incubated with GST or GST-XIAP bound to Glutathione Sepharose 4 FastFlow beads for 3 h at 4°C. The beads were washed three times with PBS containing protease inhibitor cocktail (Roche) and analyzed by SDS-PAGE and autoradiography.

Mammalian two-hybrid assay. For in vivo protein-protein interaction studies, the BD Matchmaker Mammalian Two-Hybrid Assay system (BD Biosciences) and HEK 293T/17 cells were used. XIAP and Raf-1 open reading frames were cloned into mammalian two-hybrid vectors pM and pVP16, respectively, as described above. HEK 293T/17 cells in 6-well tissue culture plates were transfected with 2.0 μg pM-XIAP and pVP16-Raf-1 constructs, with 0.4 μg of the reporter construct pG5SEAP according to the manufacturer’s protocol. Reporter gene assay was performed 36 h after transfection using GreatEscApE SEAP assay kit (BD Biosciences) as per the manufacturer’s protocol.

Immunofluorescence microscopy. MDA-MB 231, PC-3 and HEK 293T/17 cells were seeded in Lab-Tek II chamber slide (Nalge Nunc International) and allowed to attach overnight. HEK 293T/17 cells were transiently transfected with pcDNA4/HisMAX-Raf-1 and pEBG-XIAP for 48 h. Cells were fixed in 4% paraformaldehyde (pH 7.4), and permeabilized with 0.1% Triton X-100 in PBS. Cells were blocked with 2% BSA and incubated with primary antibodies for 1 h. The cells were then washed with PBS and incubated with secondary antibody in 2% BSA/PBS along with 2 μM TO-PRO-3 reagent (Molecular Probes) for nucleic acid staining. The slides were then washed with PBS and mounted with anti-fade medium (Molecular Probes). Cells were visualized using Olympus Fluoview FV300 confocal laser scanning biological microscope.

In vitro kinase assay. Purified GST-XIAP or GST proteins (5 μg) were incubated with 0.1 μg of active Raf-1 (Upstate, Charlottesville, VA, USA) in kinase assay buffer (Upstate) together with 10 mCi [32P]-orthophosphate (0.5 mCi/ml) in phosphate-free DMEM medium for 3 h. GST-XIAP was immunoprecipitated with anti-GST antibody. The immunoprecipitates were separated on SDS-PAGE and the gel dried using gel drying solution (Invitrogen) and analyzed by autoradiography.

In vivo phosphorylation assay. HEK 293T/17 cells were transfected with GST-XIAP together with Raf-1-BXB, Raf-1-S621A or control vector. Cells were serum starved overnight and labeled with [32P]-orthophosphate (0.5 mCi/ml) in phosphate-free DMEM medium for 3 h. GST-XIAP was immunoprecipitated with anti-GST antibody. The immunoprecipitates were separated on SDS-PAGE and the gel dried using gel drying solution (Invitrogen). Phosphorylated XIAP was detected by autoradiography.

Results

Raf-1 interacts with XIAP. Initially, to examine whether endogenous Raf-1 is associated with endogenous XIAP in mammalian cells, we performed co-immunoprecipitation experiments. As can be seen in Fig. 1A, Raf-1 could be detected in XIAP immunoprecipitates of various tumor cell extracts but not in control immunoprecipitates, where normal mouse IgG was used. The experiment was also performed in opposite fashion, i.e. Raf-1 was immunoprecipitated from cell extracts and detected for XIAP by Western blotting. The results were comparable (data not shown). Further, to establish that the co-immunoprecipitation of XIAP with Raf-1 is not due to cross reactivity of the antibody, three different Raf-1 antibodies (one monoclonal and two polyclonal) raised against different regions of Raf-1 were used in Western blotting. The results were comparable (data not shown). To further confirm Raf-1-XIAP interaction, co-immunoprecipitation experiments were performed with transiently transfected epitope-tagged Raf-1 and XIAP plasmid constructs (Fig. 2). For this purpose, we used two constructs of XIAP (GFP-tagged and GST-tagged) and a Raf-1 construct (His-Xpress-tagged). First, we transiently transfected HEK 293T/17

![Figure 1](image)

Figure 1. Raf-1 protein kinase associates with XIAP in mammalian cells. (A) Endogenous XIAP was immunoprecipitated (IP) from various tumor cell extracts using anti-XIAP monoclonal antibody, and the immune complexes were resolved by 8% SDS-PAGE and co-precipitated Raf-1 was detected by immunoblotting (IB) with anti-Raf-1 polyclonal antibody. Normal mouse IgG was used as a negative control. The blot was reprobed for XIAP as a loading control. (B) Endogenous Raf-1 was immunoprecipitated using anti-Raf-1 monoclonal antibody and resolved by 4-12% SDS-PAGE and co-precipitated XIAP was detected by immunoblotting with anti-XIAP monoclonal antibody. Normal rabbit IgG was used as a negative control. The blot was reprobed for Raf-1 as a loading control. (C and D) MDA-MB 231 cells were lysed using RIPA lysis buffer. Endogenous XIAP was immunoprecipitated from cell extracts using anti-XIAP monoclonal antibody and co-precipitated Raf-1 was detected by immunoblotting with anti-XIAP monoclonal antibody (D). The blot was reprobed for Raf-1.
cells with the GFP-XIAP or GST-XIAP constructs and performed co-immunoprecipitation with endogenous Raf-1. Raf-1 could be detected in GFP or GST immunoprecipitates of cell extracts transfected with tagged-XIAP constructs and not in the vector transfected cells (Fig. 2A and C). Similar observations were made with Raf-1 transfected cells (Fig. 2B and D). We then carried out transient double transfections of GFP-XIAP and His-Xpress-Raf-1 in HEK 293T/17 cells and performed co-immunoprecipitation experiments. As seen in Fig. 2E and F, exogenous XIAP was found to be associated with exogenous Raf-1. Furthermore, co-immunoprecipitation experiments with COS-1 cells transfected with Raf-1 gave similar results (data not shown). These data confirmed that Raf-1 is associated with XIAP in mammalian cells.

Raf-1 binds with XIAP in vitro. In order to determine whether Raf-1 can bind to XIAP, we performed in vitro binding assay with GST-XIAP and in vitro translated [35S]-L-methionine-labeled Raf-1 protein. As a control, we incubated GST protein with the in vitro translated Raf-1 protein. Fig. 2G shows that Raf-1 can indeed bind to XIAP, but not to control GST protein.

Raf-1 binds to XIAP in vivo in mammalian cells. As a further confirmation on the Raf-1-XIAP interaction, we performed the in vivo mammalian two-hybrid assay. In this experiment, XIAP fused to the Gal4 DNA binding domain in pM vector and Raf-1 fused to the activation domain in pVP16 vector were co-transfected in HEK 293T/17 cells along with a reporter.
plasmid pG5SEAP encoding secreted alkaline phosphatase (SEAP). Raf-1-XIAP interaction resulted in the production of high levels of SEAP activity (Fig. 2F). Control experiments, which included co-transfecting pM plasmid together with pVP16 plasmid, pM along with pVP16-Raf-1 or pVP16 along with pVP16-plasmid pM-XIAP, showed significantly lower reporter activity (P<0.001). Thus, Raf-1 can interact with XIAP in vivo.

Raf-1 co-localizes with XIAP in mammalian cells. We carried out immunofluorescence experiments in tumor cells (MDA-MB 231 and PC-3) and in HEK 293T/17 cells transfected with epitope-tagged Raf-1 and XIAP constructs. Fig. 3 shows that Raf-1 and XIAP often co-localize in the cell. In tumor cells, co-localization was performed on endogenous Raf-1 and XIAP proteins using antibodies specific for Raf-1 and XIAP. Additionally, we performed co-localization studies with exogenously transfected His-Xpress-tagged Raf-1 and GST-tagged XIAP using antibodies specific for His and GST.

Raf-1 kinase phosphorylates XIAP in vitro and in vivo. To investigate whether the physical association of Raf-1 and XIAP results in phosphorylation of XIAP by Raf-1, in vitro and in vivo phosphorylation assays were performed. In vitro kinase assays were performed using purified GST-tagged XIAP as a substrate of Raf-1. In a direct in vitro kinase assay, followed by SDS-PAGE and autoradiography, active Raf-1 was observed to phosphorylate GST-XIAP, while no phosphorylation was observed with purified GST protein (Fig. 4A).

To further examine whether Raf-1 can phosphorylate XIAP in vivo, HEK 293T/17 cells were co-transfected with GST-XIAP and constitutively active Raf-1 construct (Raf-1-BXB) or dominant negative Raf-1 (Raf-1-S621A) construct. After 36 h of transfection, cells were labeled with [32P]-orthophosphate for 3 h. SDS-PAGE analysis of GST-XIAP immunoprecipitates showed that constitutively active Raf-1 increase phosphorylation of XIAP as compared with cells transfected with dominant negative Raf-1 and control vector (Fig. 4B).

Raf-1 kinase prevents XIAP degradation in response to apoptotic stimuli. Degradation of XIAP has been shown to be an important mechanism for caspase activation in response to different apoptotic stimuli (26,27). To study whether Raf-1 inhibits XIAP degradation following apoptotic stimuli, we transfected HEK 293T/17 cells with His-XIAP along with various Raf-1 constructs and exposed them to staurosporine treatment or serum starved them. In response to apoptotic stimuli, a significant degradation of XIAP protein expression was observed (Fig. 5A and B). In contrast, XIAP levels were stable in constitutively active Raf-1 transfected cells. On the other hand, kinase inactive Raf-1 constructs (Raf-1-K375M or Raf-1-S621A) or vector control could not protect XIAP from degradation (Fig. 5A and B). To exclude the involvement of ERK in constitutively active Raf-1 transfected cells, cells were treated with MEK inhibitors (PD98059 and U0126). XIAP levels were found to be stable in Raf-1-BXB transfected cells treated with MEK inhibitors (Fig. 5A and B). Fig. 5C shows significant inhibition of ERK activity following treatment with MEK inhibitors in constitutively active Raf-1 transfected cells. This suggests a direct role of Raf-1 in XIAP stability. Next we examined whether active Raf-1 stabilized XIAP can suppress caspase-3 activity. In HEK 293T/17 cells treated with staurosporine, caspase-3 activity was markedly decreased by Raf-1-BXB, while kinase inactive Raf-1 mutants or vector control did not inhibit caspase-3 activity (Fig. 5D).
Raf-1 kinase has been shown to play a central role in cell survival (28). Although, Raf-1 activation of the MEK-ERK cascade has been associated with inhibition of apoptosis, growing evidence also suggests MEK-ERK independent role of Raf-1 in apoptosis suppression and cell survival (29,30). Previously, we and others have demonstrated that antisense raf oligonucleotide mediated inhibition of Raf-1 leads to significant antitumor activity and radiosensitization and chemosensitization of various tumor cell types (31-34). Also, inhibition of Raf-1 is associated with apoptotic cell death as indicated by caspase-3 activation and chemosensitization of various tumor cell types (31-34). Also, inhibition of Raf-1 is associated with apoptotic cell death as indicated by caspase-3 activation and chemosensitization of various tumor cell types (31-34). Raf-1 exerts pro-survival effects at the premitochondrial levels with inactivation of proapoptotic protein Bad (7-9). However, recent studies have suggested a pro-survival role for Raf-1 at the post-mitochondrial levels (4,19). In our efforts to understand the role of Raf-1 in inhibiting caspase activation, we discovered a novel interaction between Raf-1 and XIAP in mammalian cells.

Members of the IAP family of proteins, especially XIAP, function as potent inhibitors of caspases (23,24). The IAP families of proteins are characterized by baculovirus IAP repeat (BIR) domains and the RING domain. XIAP contains three BIR domains (24). BIR2 domain of XIAP binds and inhibits caspases-3 and-7, while BIR3 domain inhibits caspase-9. The binding domains between Raf-1 and XIAP remain to be ascertained. The C-terminal RING domain of XIAP has been shown to have ubiquitin protease ligase (E3) activity and is responsible for the autoubiquitination and degradation of XIAP after apoptosis stimuli (22). Here we demonstrate that Raf-1 kinase can phosphorylate XIAP and prevent its degradation in response to apoptotic stimuli. Also, active Raf-1 stabilized XIAP could suppress caspase-3 activity. These results suggest that XIAP is a new physiological substrate of Raf-1. Recent report has also indicated interaction between chicken IAP homologue ITA and Raf kinases (37) and has proposed a role of Raf at the post-mitochondrial event.

Recently, Akt was also shown to phosphorylate and interact with XIAP (38). XIAP protein sequence contains Akt phosphorylation consensus sequence at residue serine 87 of XIAP (38). It remains to be determined which serine/threonine residues are phosphorylated in XIAP by Raf-1 protein kinase. Previously, B-Raf has been shown to confer protection against apoptosis at the level of caspase activation, downstream of cytochrome c release from mitochondria (39). This suggests that XIAP may also be a relevant target of B-Raf. Future studies are aimed at exploring targeted disruption of Raf-XIAP complex for anti-proliferative properties.

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References


