

Interaction of checkpoint kinase 1 and the X-linked inhibitor of apoptosis during mitosis

V. Galvan, A.V. Kurakin, D.E. Bredesen*

Buck Institute for Age Research, 8001 Redwood Blvd., Novato, CA 94945, USA

Received 11 September 2003; revised 3 December 2003; accepted 3 December 2003

First published online 14 January 2004

Edited by Jesus Avila

Abstract We report here that the checkpoint kinase Chk1 and the inhibitor of apoptosis protein (IAP) family member XIAP can be found in a complex in association with condensed chromosomes aligned at the metaphase plate during mitosis. The interaction between Chk1 and XIAP was transient and followed the breakdown of the nuclear envelope. Chk1 and XIAP also formed a complex in vitro and in coimmunoprecipitation experiments. The interaction between Chk1 and the BIR3 domain of XIAP in vitro required an N-terminal sequence in Chk1 that is identical to the BIR-binding motif at the N-terminus of HID. An interaction of Chk1 and XIAP may imply a mechanism of coupling between the regulatory networks that control cell cycle progression and apoptosis during mitosis.

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Key words: Chk1; XIAP; Mitosis; Apoptosis; Baculoviral IAP repeat domain

1. Introduction

The existence of an interdependence between cell cycle control and the regulation of apoptotic cell death has been suggested. However, the way in which cell cycle regulation and apoptosis intersect is still for the most part unknown.

Chk1, an evolutionarily conserved dual-specificity protein kinase, has a role in preventing progression into mitosis in the presence of DNA damage [1,2]. In mammalian cells, however, Chk1 is expressed and active as a kinase during the transition from S phase to M phase [3] and regulates Cdc25A function [4] in the absence of DNA damage. Disruption of both alleles of Chk1 in mice results in embryonic lethality associated with massive, p53-independent apoptosis at the blastocyst stage [5,6].

The role of the inhibitor of apoptosis [7] family of proteins (IAP), which contain one or more zinc-binding motifs named baculoviral IAP repeat (BIR) [8] domains, has long been established in the control of apoptosis through direct inhibition of caspases [9,7]. Recently, a member of the IAP family, named survivin/Tiap in mammals, was also implicated in the control of chromosome segregation and cytokinesis, hinting at the possibility that members of the IAP family may have a role both in the regulation of apoptosis and in the control of cell division [10–12]. The three best characterized IAP family

members, XIAP, cIAP1 and cIAP2, interact with and inhibit caspase-3 and caspase-7, while XIAP binds and inhibits caspase-9, as well. The BIR3 domain of XIAP interacts with processed caspase-9 while caspase-3 and -7 contact XIAP through the linker region upstream of its BIR2 domain [13]. The N-terminal four residues of the mammalian pro-apoptotic molecule Smac/DIABLO and those of processed caspase-9 compete for the same binding groove on the BIR3 domain of XIAP. Thus, Smac/DIABLO is thought to function by relieving XIAP inhibition of caspase-9 activity [14]. The N-terminal motif of Smac/DIABLO is present in the pro-apoptotic *Drosophila* proteins Grim, Reaper and Hid as well, suggesting that this mode of activation of apoptosis is evolutionarily conserved.

We report in the present study that a motif homologous to the sequences that target processed caspase-9 and Smac/DIABLO to the BIR3 domain of XIAP is present at the N-terminus of Chk1. Chk1 bound the BIR3 domain of XIAP and cIAP1 in vitro, and XIAP was present in complexes immunoprecipitated with Chk1 from cultured cells. Mutation of the Chk1 N-terminal AVP motif was sufficient to disrupt its interaction with the BIR3 domain of XIAP but did not affect Chk1 binding to the BIR3 domain of cIAP1 in vitro. The interaction of Chk1 and XIAP appeared to be restricted to mitosis and occurred after the disruption of the nuclear envelope at prometaphase. During mitosis, Chk1 associated with XIAP in juxtaposition with condensed chromosomes aligned at the metaphase plate.

Chk1 may be the first BIR-interacting protein for which a function in the regulation of apoptosis has not been described. An interaction between Chk1 and IAPs may have a role in the cross-talk between the mechanisms that drive apoptosis and those that control the cell cycle.

2. Materials and methods

2.1. Cell culture and transient transfections

L929, HeLa and HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Wild-type and catalytically inactive (D130A) human Chk1 (hChk1) expression vectors were described previously [1] and were kindly provided by S. Elledge. HEK293 cells were transfected with 3–5 or 6–8 µg of control (pcDNA3.1) or hChk1 plasmid constructs respectively using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The amount of plasmid DNA transfected was equalized by the addition of pcDNA3.1 vector DNA. Thirteen to 15 h after transfection, cells were lysed in 1 mM EDTA, 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EGTA, 1% Triton X-100, 0.1% NP-40, 0.5% sodium deoxycholate and Mini protease inhibitors (Roche) (lysis buffer).

*Corresponding author. Fax: (1)-415-209 2230.

E-mail address: dbredesen@buckinstitute.org (D.E. Bredesen).

2.2. Western blotting

Synchronized-released HeLa cells were collected at the times indicated in the figures and resuspended in lysis buffer. Lysates were spun at $16000\times g$. Equivalent amounts of protein from supernatants were resolved in denaturing gels (Invitrogen) and immunoblotted with the antibodies indicated, or used in immunoprecipitation assays.

2.3. Immunofluorescence assays

L929 cells were grown on 8-well slides and either left untreated or incubated in 0.2 $\mu\text{g/ml}$ nocodazole for 16 h in the absence of serum, fixed by immersion in -20°C methanol and blocked in 10% normal donkey serum (Jackson ImmunoResearch Laboratories) and 1% bovine serum albumin (BSA) in $1\times$ phosphate-buffered saline (PBS) for 1 h at room temperature (RT). L929 cells were incubated with polyclonal anti-Chk1(FL-476) (Santa Cruz Biotechnology) and mouse anti-XIAP (StressGen). Synchronized HeLa cells were incubated in a mixture of rabbit anti-Chk1 (PEP, kindly provided by S. Elledge) and rabbit anti-Chk1(FL-476) and mouse anti-XIAP. In all cases, incubation was done in 1% BSA for 1 h at RT, followed by three PBS washes and incubation with fluorescein isothiocyanate (FITC)-conjugated, donkey anti-mouse and Texas Red-conjugated, donkey anti-rabbit antibodies (Jackson Laboratories) for 1 h at RT. Cells were then washed extensively (8–10 times, 15 min) with PBS at room temperature, mounted in DAPI-Vectashield (Vector Laboratories) and visualized by confocal microscopy using a Nikon PCM-2000 Laser Confocal Scanning Microscope. Alternatively, asynchronous HEK293 cells were fixed in 4% paraformaldehyde in PBS for 40 min at RT and processed for immunofluorescence as described above. Wide-field images were acquired for unsynchronized HeLa cell cultures using a Nikon Eclipse-800 microscope and appropriate filters and collected using Compix Simple PCI software.

2.4. Cell synchronization

HeLa cells were synchronized by a double-thymidine block. Briefly, cells were incubated in 2 mM thymidine for 19 h, washed twice with pre-warmed PBS and released in 5% FBS DMEM for 10 h. Thymidine was re-added to the cultures to a final concentration of 2 mM and incubated for an additional 17 h. Cells were then washed twice with pre-warmed PBS and placed into normal medium. This point, at which 85% of cells are at the G1/S boundary (not shown), was designated t_0 . Cultures were either collected and subjected to lysis or fixed at the times indicated, processed for immunolocalization of Chk1 and XIAP as described and visualized by confocal microscopy.

2.5. Glutathione S-transferase (GST) pulldown assays

GST fusion proteins of the BIR3 domains of XIAP and cIAP1 were expressed and purified from bacteria as described [15]. The GST-cIAP1(BIR3) expression construct was kindly provided by Dr. Catherine L. Day [16]. The GST-XIAP(BIR3) expression construct was generated as described [15]. Lysates from untreated or transiently transfected HEK293 cells were spun at $16000\times g$ for 5 min at 4°C . Supernatants were precleared with GST and then incubated with 20 μg of the indicated GST fusion proteins or GST alone bound to Sepharose 4B beads. Complexes were washed, resolved in gels and probed with antibodies as described in the figure legends. Ten-well, 4 mm wide-well polyacrylamide gels were used in all experiments to maximize the sharpness and resolution of closely migrating bands. In cases in which single experiment sample number exceeded what could be run on a single gel, all procedures (sample handling, electrophoretic runs and transfers, antibody incubations, membrane washes and development) were done simultaneously to avoid variability due to differences in processing.

2.6. Immunoprecipitation

An agarose-conjugated anti-Chk1(FL-476) antibody was purchased from Santa Cruz Biotechnology. To remove unbound IgGs, 500 μg of antibody was washed twice in 15 ml of buffered 0.1% Triton X-100, 0.5% Na deoxycholate, 0.1% NP-40 and 3 mM dithiothreitol (DTT) followed by five washes in 15 ml of PBS. The concentration of the washed agarose-conjugated antibody was estimated by Coomassie blue staining of the starting material and the agarose pellet after the final wash. 650 μg of total protein from supernatants of synchronized HeLa cells was precleared by incubation with Protein A/G-PLUS agarose (Santa Cruz Biotechnology) for 30 min at 4°C followed by incubation with 3 μg of washed, agarose-conjugated anti-Chk1 antibody overnight at 4°C . Agarose pellets were washed twice with lysis

buffer, followed by three additional washes with 25 mM HEPES pH 7.6, 20 mM MgCl₂, 2 mM DTT, 1 mM sodium orthovanadate and 20 mM β -glycerophosphate. Disruption of the immunoprecipitated complexes was done in Laemmli buffer (Invitrogen) at 55°C to minimize dissociation of the IgGs from the agarose matrix, and resolved in 7% or 3–8% Tris-acetate gels (Invitrogen). The separated polypeptides were immunoblotted with anti-XIAP monoclonal antibody and detected with horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (Vector Laboratories).

3. Results

3.1. Chk1 contains a BIR3-targeting motif at its N-terminus

A domain-based search for proteins that contain an N-terminal motif homologous to the sequences that target processed caspase-9 and Smac/DIABLO to the BIR3 domain of XIAP revealed a motif in Chk1 (Fig. 1). The Chk1 N-terminal motif is identical to the one present in Hid and homologous to the N-terminus of processed caspase-9, Smac/DIABLO, Grim and Reaper. The N-terminal motif present in Chk1 is conserved in the human, mouse and *Xenopus laevis* homologs.

3.2. Chk1 and XIAP are found in perinuclear structures in nocodazole-treated L929 cells

Chk1 has been localized to the nucleus [1,3], while XIAP is thought to be strictly cytoplasmic [7]. Thinking that an interaction between Chk1 and XIAP would possibly require the disruption of the nuclear envelope, we performed coimmunolocalization studies in untreated and nocodazole-treated L929 cell cultures. Nocodazole is an inhibitor of microtubule polymerization/depolymerization that disrupts mitotic spindle function. In the presence of nocodazole, dividing cells arrest as they attempt to enter mitosis. In agreement with previous reports, we found that Chk1 immunoreactivity was predominantly nuclear and XIAP immunoreactivity was restricted to the cytoplasm of untreated L929 cells. In contrast, Chk1 and XIAP immunoreactivity colocalized in structures juxtaposed to a DNA-containing compartment in 46% of L929 cells after 24 h of exposure to nocodazole (Fig. 2A). These results suggested that an interaction between Chk1 and XIAP could occur in intact cells and that this interaction might be dependent on the breakdown of the nuclear envelope and be restricted to mitosis.

3.3. Chk1 and XIAP are present in complexes associated with condensed chromosomes aligned at the metaphase plate

To determine whether an interaction between Chk1 and XIAP may be regulated by the cell cycle, we assayed HeLa cells in double-thymidine block and release experiments followed by coimmunolocalization (Fig. 2B). Synchronization

Smac/DIABLO	-AVPIAQKSEPHSL
Caspase-9 human	-ATPFQEGRLRTFDQ
Caspase-9 rat	-AVPYQEGPRTLDDQ
Reaper	MAVAFYIPDQATLL
Grim	MAIAYFIPDQAQLL
Hid	MAVPFYLPEGGADD
Chk1 human	MAVPFVEDWDLVQT
Chk1 mouse	MAVPFVEDWDLVQT
Chk1 Xenopus	MAVPFVEDWDLVQT

Fig. 1. Alignment of the N-terminal sequences of the indicated proteins. The BIR3 domain-binding motif is highlighted.

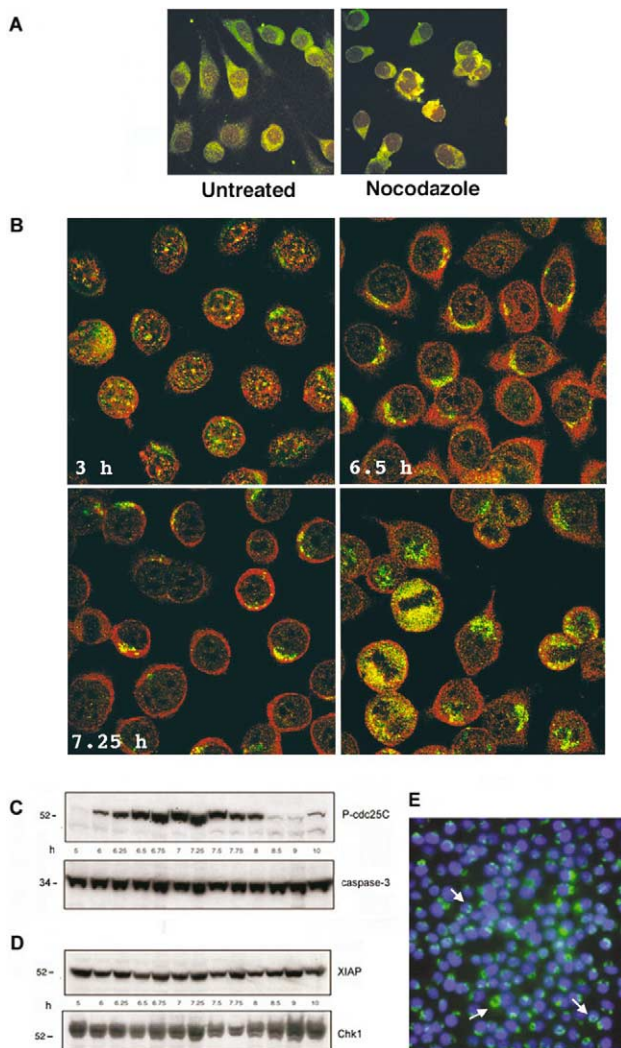


Fig. 2. A: L929 cells were left untreated or treated with nocodazole for 16 h, fixed and incubated with rabbit anti-Chk1(FL-476) and mouse anti-XIAP antibodies followed by Texas Red-conjugated anti-rabbit and FITC-conjugated anti-mouse antibodies. B: HeLa cells were synchronized by a double-thymidine block, fixed at the times indicated and incubated with rabbit anti-Chk1(FL-476) and mouse anti-XIAP antibodies followed by Texas Red-conjugated anti-rabbit and FITC-conjugated anti-mouse antibodies. C: Lysates from synchronized-released HeLa cells were collected at the times indicated, resolved in denaturing gels (Invitrogen) and immunoblotted with the indicated antibodies. D: Synchronized-released HeLa cell lysates were processed as in C. E: Asynchronous HEK293 cells fixed in 4% paraformaldehyde were processed as described in B. Wide-field images were acquired using Compix Simple PCI software.

experiments were monitored by immunoblotting lysates with phospho-CDC25C(Ser216)-specific antibodies and anti-caspase-3 antibodies as a loading control (Fig. 2C). To increase the sensitivity of the Chk1 immunostain in synchronized HeLa cells, we used a mixture of rabbit anti-Chk1(FL-476) and rabbit anti-Chk1(PEP) in immunohistochemistry assays. The use of two antibodies directed to different epitopes of the same protein in a single immunoassay to extend the dynamic range of staining has been described previously [17]. A comparison of the staining pattern of the mixture of anti-Chk1(FL-476) and anti-Chk1(PEP) vs. anti-Chk1(FL-476) alone in immunohistochemistry assays was determined in pre-

liminary experiments using HEK293 cells transiently transfected with constructs encoding hChk1 and XIAP. Non-specific binding was negligible, and not significantly different for the mixture of anti-Chk1(FL-476) and anti-Chk1(PEP) vs. anti-Chk1(FL-476) alone (not shown).

In agreement with our observations in unsynchronized L929 cells and previous reports, Chk1 immunoreactivity was found predominantly in punctate structures in the nuclear compartment in HeLa cells during S phase (0–4 h after release). As released cells progressed through G2 (5–7.5 h), Chk1 immunoreactivity was found also in the cytoplasm of cells approaching mitosis (7.25–9 h) that had not yet undergone nuclear envelope breakdown. In these rounded cells, Chk1 immunoreactivity in the cytoplasm appeared to be increased (Fig. 2B, 7.25 h), while nuclear punctate staining decreased in some cells. The apparent increase in staining intensity may be a consequence of rounded morphology of the cells approaching mitosis. As cells progressed through G2 and entered mitosis, XIAP immunoreactivity was redistributed to discrete cytoplasmic structures juxtaposed to the nuclear compartment. In some cells, XIAP immunoreactivity formed ring-like structures that were stable as long as the nucleus remained intact, disappeared during mitosis, and seemed to re-form in daughter cells immediately after cytokinesis (Fig. 2B, 6.5, 7.25 and 9.5 h). No association between Chk1- and XIAP-immunoreactive signals could be detected through early and late anaphase in synchronized-released HeLa cells (Fig. 2B, 3, 6.5 and 7.5 h). In cells undergoing mitosis (8–10 h after release), however, Chk1 and XIAP were detected as complexes associated with condensed chromosomes aligned at the metaphase plate (Fig. 2B, 9.5 h). We found no evidence of interaction between Chk1 and XIAP at other points in the cell cycle in synchronization-release experiments performed in HeLa cells (data not shown). To determine whether the apparent increase in Chk1 immunoreactivity in cells entering mitosis was due to an increase in total Chk1 protein, we performed immunoblotting on synchronized-released HeLa cell lysates. No significant differences in the levels of Chk1 protein were found between lysates of HeLa cells at 6.5–7 h and 7–7.5 h after release, although a small decrease in Chk1 levels was observed at 7.75–8 h. Total XIAP expression levels were unchanged during S to M progression in HeLa cells (Fig. 2D). We concluded from these experiments that an interaction between Chk1 and XIAP may be restricted to mitosis, and may occur in association with condensed chromosomes aligned at the metaphase plate. Cytoplasmic Chk1 localization and function in phosphorylation of Cdc25 has been documented in *Xenopus* oocytes during normal cell cycle progression [18]. Diffuse cytoplasmic staining, accompanied by punctate nuclear staining, was observed in human fibroblasts stained with anti-Chk1(PEP) antibody when imaged at low magnifications [1]. Also, Kaneko and collaborators reported the cell cycle-dependent regulation of Chk1 subcellular localization and protein levels in HeLa cells [3].

To exclude the possibility that the XIAP-immunoreactive ring-like structures detected in HeLa cells traversing the G2–M boundary represented artifacts of methanol fixation, and to determine whether these structures would be present in asynchronous cultures, we fixed HeLa cell cultures with 4% paraformaldehyde, then performed immunostaining with anti-XIAP antibodies and counterstained with DAPI as in the previous experiments. XIAP immunoreactivity formed ring-

like structures closely juxtaposed to the nucleus in cells fixed with paraformaldehyde, ruling out the possibility that these structures are an artifact of methanol fixation (Fig. 2E). Our results suggest that XIAP-immunoreactive ring-like structures are present in HeLa cells throughout the cell cycle, become undetectable during S phase, and re-form after cytokinesis. The significance of these XIAP-immunoreactive structures remains to be determined.

3.4. Chk1 binds the BIR3 domain of XIAP in vitro

Chk1 shares an N-terminal motif with XIAP-binding proteins that targets these proteins to the XIAP BIR3 domain (Fig. 1). To determine whether Chk1 may interact with the BIR3 domain of XIAP as well, we performed in vitro binding experiments using lysates of HEK293 cells transiently transfected with hChk1 or with enhanced green fluorescent protein. These lysates were incubated with a GST fusion of the XIAP BIR3 domain (GST-XIAP(BIR3)) or with GST alone as a control. Chk1-immunoreactive material was detected in complexes pulled down from lysates of HEK293 cells when GST-XIAP(BIR3), but not when GST alone, was included in the reaction (Fig. 3A). An additional band of slightly slower apparent mobility was present in complexes pulled down by GST-XIAP(BIR3); this may correspond to endogenous Chk1. Thus hChk1, and possibly endogenous Chk1, bound the BIR3 domain of XIAP in vitro.

3.5. Chk1 binds the BIR3 domain of cIAP1 in vitro

BIR3 domains found in IAP family members share a significant degree of homology and are thought to display generally similar binding preferences. To determine whether

hChk1 may interact with other BIR3 domains in the IAP family, we performed GST pulldown experiments using a GST fusion of the BIR3 domain of cIAP1 (GST-cIAP1(BIR3)) as a bait. We found that GST-cIAP1(BIR3) pulled down Chk1-immunoreactive material both from untreated HEK293 cells and from cells expressing hChk1 (Fig. 3B, lanes 3 and 6). These results indicate that GST-cIAP1(BIR3) can bind both endogenous and transiently expressed Chk1 in vitro. Even though we observed higher amounts of Chk1-immunoreactive material pulled down with GST-cIAP1(BIR3) than with GST-XIAP(BIR3) (Fig. 3B, compare lanes 2 and 5 to lanes 3 and 6), no conclusion about the relative affinities of binding of GST-XIAP(BIR3) and GST-cIAP1(BIR3) to Chk1 can be made given the non-quantitative nature of the GST pulldown assays performed. The difference in mobility observed for Chk1-immunoreactive bands present in lysates from untreated cells in panels A and B may be due to differences in the resolving ranges of the polyacrylamide gels used in these experiments (7% Tris-acetate as compared to 10% Bis-Tris polyacrylamide gels).

3.6. The interaction of Chk1 with the BIR3 domain of XIAP, but not cIAP1, depends on an N-terminal AVP motif in Chk1

To determine whether the N-terminal four residues homologous to a BIR3 domain-targeting motif in hChk1 may function as a BIR3-targeting sequence, we generated full-length hChk1 mutants in which the motif AVPF was mutated to GVPF (hChk1-GV) or GGPF (hChk1-GG). Lysates from HEK293 cells expressing wild-type hChk1, hChk1-GV and hChk1-GG were tested in GST pulldown experiments using

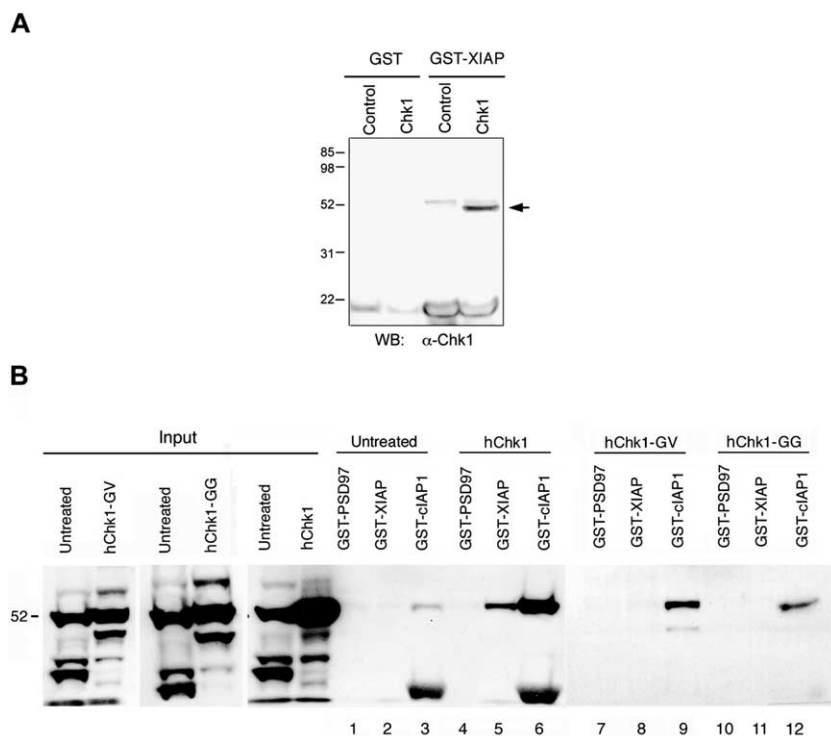


Fig. 3. A: Lysates of HEK293 cells treated as indicated were used in GST pulldown assays as described in Section 2. Pulled-down complexes were washed, resolved in gels and probed with monoclonal anti-Chk1(G4) antibody (Santa Cruz Biotechnology). B: HEK293 cells were left untreated or transfected with the indicated expression vectors, lysed and precleared and then incubated with the indicated GST fusion proteins. Lysates and pulled-down complexes were resolved in gels and probed with anti-Chk1(G4) antibody.

GST-XIAP(BIR3) or GST-SAP97 as a control. No Chk1 immunoreactivity was detected in complexes pulled down by GST-XIAP(BIR3) from cells transiently expressing the N-terminal mutants hChk1-GV or hChk1-GG (Fig. 3B, lanes 8 and 11), even when the immunoblots were exposed to film for extended periods of time (up to 30 min; not shown). These results indicate that mutation of the N-terminal motif AVPF to GVPF or GGPF in hChk1 is sufficient to abolish its interaction with the BIR3 domain of XIAP *in vitro*. A more rapidly migrating Chk1-immunoreactive band was found in lysates of cells expressing hChk1 N-terminal mutants but not in cells expressing wild-type hChk1 (Fig. 3B, compare input lanes hChk1-GV, hChk1-GG and hChk1), which suggests that endogenous and/or transiently expressed Chk1 is proteolytically cleaved or degraded in the presence of hChk1 N-terminal mutants. However, in a manner similar to what has been described for kinase-dead and also for Chk1 phosphorylation mutants [19], we did not observe any dominant-negative effect of N-terminal mutants with respect to endogenous wild-type Chk1 in DNA damage-induced cell cycle arrest or in the control of apoptosis in transient transfection experiments. It was also conceivable that wild-type Chk1 might have a 'dominant-negative', pro-apoptotic effect through the displacement of processed caspase-9 and Smac/DIABLO from binding to the BIR3 domain of XIAP. However, in a manner similar to what we observed for the N-terminal Chk1 mutants, wild-type Chk1 did not have an effect on cell death in transient transfection assays (data not shown).

We conclude that the association between Chk1 and the BIR3 domain of XIAP *in vitro* requires the presence of an intact N-terminal AVP motif in hChk1.

In contrast to what we observed for GST-XIAP(BIR3), there was no difference in the ability of GST-cIAP1(BIR3) to bind hChk1-GV and hChk1-GG mutants vs. wild-type hChk1 (Fig. 3B, lanes 6, 9 and 12). The relatively higher amounts of wild-type hChk1 found in complexes pulled down with GST-cIAP1(BIR3) with respect to hChk1-GV and hChk1-GG may be due to differences in the levels of expression of wild-type hChk1 and hChk1-GV and -GG mutants. These results suggest that the interaction between hChk1 and the BIR3 domain of cIAP1 does not depend on the hChk1 N-terminal AVP motif. More rapidly migrating Chk1-immunoreactive bands were also present in the complexes pulled down by GST-cIAP1(BIR3), supporting the notion that they represent cleavage products of hChk1 N-terminal mutants, or of endogenous Chk1. The use of untagged hChk1 constructs in the present studies precluded the distinction between these two possibilities.

3.7. Chk1 and XIAP interact in lysates from synchronized HeLa cells

To determine whether Chk1 and XIAP may interact in cell lysates, we performed immunoprecipitation experiments using lysates of synchronized HeLa cells progressing through G2 and undergoing mitosis (6.5 h and 8.5 h after release, respectively) (Figs. 4 and 2B,C). Detection of XIAP and Chk1 in immunoprecipitated complexes was complicated by the fact that both proteins and the immunoglobulin G heavy chain (IgGHC) are of similar size and migrate in denaturing gels at almost identical apparent relative molecular masses. To reduce IgGHC cross-reactivity on the membranes, we used an agarose conjugate of the rabbit polyclonal anti-Chk1(FL-

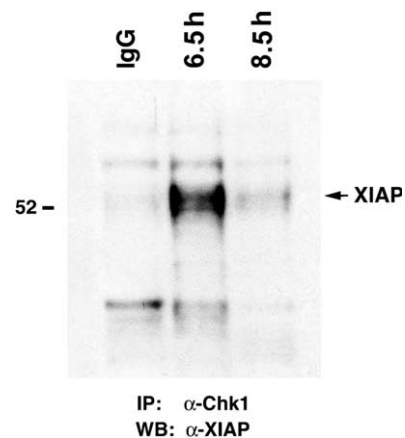


Fig. 4. Synchronized HeLa cells were lysed at the indicated times after release and subjected to immunoprecipitation with an agarose-conjugated anti-Chk1(FL-476) polyclonal antibody or with an agarose conjugate of non-specific rabbit IgG. Immunoprecipitated complexes were resolved in Tris-acetate gels and incubated with anti-XIAP monoclonal antibodies.

476) antibody that was extensively washed to reduce the amount of unbound IgGs present. Also, we used pre-adsorbed secondary antibodies at high dilutions (to minimize cross-species reactivity of the IgGHCs) and resolved the immunoprecipitated complexes in 6% Tris-acetate gels. A prominent XIAP-immunoreactive band was found in complexes immunoprecipitated with agarose-conjugated anti-Chk1 antibody from lysates of synchronized HeLa cells traversing G2 (6.5 h, Fig. 4). Unexpectedly, the amount of XIAP coimmunoprecipitated with anti-Chk1 antibody from HeLa cells in mitosis (8.5 h) was lower than that present in lysates from cells in G2 (6.5 h). As compartmentalization is not preserved when cells are lysed, it is possible that the total amount of Chk1 protein in cells traversing G2 is higher than in those undergoing mitosis. Indeed, we observed slightly lower amounts of total Chk1 protein (but not of XIAP) in lysates from HeLa cells 8.5 h after release from a double-thymidine block (Fig. 2D). Low levels of immunoreactivity were also observed in complexes immunoprecipitated from HeLa lysates with an agarose conjugate of rabbit IgG, which may correspond to small amounts of IgGHC released from the agarose beads at the time of heating of the samples in preparation for the electrophoretic run. These results indicate that XIAP is present in a complex with Chk1 in lysates of synchronized HeLa cells.

4. Discussion

The results presented here suggest that a four-residue N-terminal motif homologous to a BIR3-targeting sequence may be present in Chk1. This motif was required for the interaction of hChk1 with the BIR3 domain of XIAP *in vitro*. It is possible that the N-terminus of Chk1 interacts with the same binding groove on the BIR3 domain of XIAP that mediates the interaction of XIAP with processed caspase-9 and Smac/DIABLO. Even though it is conceivable that Chk1 binding to XIAP might displace activated caspase-9 and thus be pro-apoptotic, we did not find any effect of the expression of wild-type Chk1 on cell death in transient transfection assays (data not shown). Furthermore, the activation of apoptosis in blastocysts in the absence of Chk1 [5] suggests that Chk1 might in fact have a

function in the suppression, not in the activation, of apoptosis during cell division. To test this hypothesis, we transiently expressed kinase-active Chk1 mutants in which the N-terminal BIR3 binding motif was obliterated by point mutations (hChk1-GV and hChk1-GG) in HEK293 cells, but did not detect an effect on cell survival or on cell cycle progression in the presence or absence of DNA damage, possibly owing to the presence of endogenous Chk1 in HEK293 cells (data not shown). That the overexpression of Chk1 mutants, including those that abolish its kinase function, does not interfere with the function of endogenous Chk1 has been documented by others [19]. Thus, further studies will be required to assess the potential contribution of the N-terminal sequences of Chk1 to the modulation of apoptosis through the interaction with XIAP, or to its function at the G2/M checkpoint. Elucidation of the significance of the interaction between Chk1 and XIAP may require the construction of N-terminal mutant hChk1 knock-in embryonic stem cell lines, an experiment that we have attempted so far without success.

Our results suggest that interaction of Chk1 and XIAP is transient and restricted to metaphase, probably immediately after nuclear envelope breakdown. In cells undergoing mitosis, Chk1 and XIAP colocalized in juxtaposition with condensed chromosomes aligned at the metaphase plate.

The level of ectopic expression of N-terminally mutated Chk1 cDNA constructs was lower than that of wild-type Chk1. This observation suggests that the levels of mutant hChk1 expression in cells might be regulated at the post-transcriptional level. Inhibition of proteasome activity, however, did not have any effect on the levels of either endogenous or transiently expressed mutant or wild-type hChk1 in 293 cells.

Chk1 may be the only IAP-interacting protein described to date that has not been directly implicated in the control of apoptosis. However, indirect evidence has been presented: removal of Chk1 function results in embryonic lethality associated with massive, p53-independent apoptosis in blastocysts, suggesting that Chk1 is essential for the survival of mammalian cells even in the absence of DNA damage. A role for Chk1 in the control of unperturbed cell cycle progression was recently confirmed by experiments using Chk1 siRNA [4]. The study of the interaction between Chk1 and XIAP may help to shed light on mechanisms that modulate apopto-

sis during the DNA damage-activated G2/M checkpoint, and possibly at the time of entry into mitosis during normal cell cycle progression, as well.

Acknowledgements: We thank Dr. Stephen Elledge (Baylor College of Medicine, Houston, TX, USA) for providing us with Chk1 constructs and antibodies and Dr. Catherine L. Day (University of Otago, New Zealand) for her kind gift of the GST-cIAP1(BIR3) expression construct. We also thank Elaine Carlson for valuable discussions and Molly A. Susag for administrative assistance. The present work was supported by a grant from the Eppley Foundation for Research to V.G., and NIH AG12282, the Joseph Drown Foundation and the Institute for the Study of Aging to D.E.B.

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