The F-box protein Fbxo7 interacts with human inhibitor of apoptosis protein cIAP1 and promotes cIAP1 ubiquitination

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Abstract

Human cIAP1 protein is a member of the inhibitor of apoptosis proteins (IAPs) that are involved in apoptosis regulation and an increasing number of other functions, including cell cycle and intracellular signal transduction. In order to identify novel proteins involved in cIAP1 regulation, we performed a yeast two-hybrid screen and identified an F-box protein Fbxo7 as a cIAP1 interacting protein. Co-immunoprecipitation assay showed that cIAP1 can interact with Fbxo7 in human cells. When co-expressed in cells, cIAP1 and Fbxo7 co-localized remarkably both in the cytoplasm and nucleus, and considerable amounts of these often co-localized at one or few distinct Golgi-like structures close to the nucleus. Furthermore, we showed that overexpression of Fbxo7 promotes the ubiquitination of cIAP1. Since F-box proteins are specificity determining subunits of SCF ubiquitin protein ligases, our results suggest that Fbxo7 can mediate the ubiquitination of cIAP1 by SCF ubiquitin protein ligase and thus have important implication in the regulation of cIAP1 function.

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The inhibitor of apoptosis protein (IAP) family consists of a number of evolutionarily conserved proteins identified in baculovirus, yeast, nematodes, drosophila, and mammals [1]. In addition to suppressing apoptotic cell death, IAPs are involved in an increasing number of other cellular functions, including cell cycle and intracellular signal transduction [2,3]. IAP family proteins are characterized by baculoviral IAP repeat (BIR) domain [4]. cIAP1 is one of the eight human IAPs. It has three BIR domains and one RING-finger domain. It was shown that cIAP1 inhibits apoptosis by directly binding and inhibiting cell death proteases, caspase-3 and -7 [5–7]. The BIR-domain containing region of cIAP1 is sufficient for inhibition of these caspases. cIAP1 can also prevent the proteolytic processing of

pro-caspases-3, -6, and -7 by blocking the cytochrome *c*-induced activation of pro-caspase-9 [8]. In addition to direct caspase inhibition, cIAP1 modulates death receptor signaling by interacting with the TNF receptor-associated factors 1 and 2 (TRAF1 and TRAF2) complex [9]. The induction of cIAP1 by transcription factor NFκB is also required to effectively block TNF-induced apoptosis [10].

IAP-mediated ubiquitination provides another mechanism in apoptotic regulation [11]. Ubiquitination transfers 76-residue ubiquitin protein(s) to its target protein and is involved in protein degradation, cell cycle progression, signal transduction, DNA repair, and apoptosis [12,13]. Ubiquitination requires sequential activation of ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin protein ligase (E3) [14]. cIAP1 functions as ubiquitin protein ligase (E3) in ubiquitination of death inducers, Smac (second mitochondria-derived activator of caspase) and its murine homologue DIABLO

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[15]. Thus, in addition to inhibiting caspase activity, cIAP1 may also protect cells by targeting pro-apoptotic molecules for proteasomal degradation. In addition, cIAP1 was found to direct the ubiquitination of TNF signaling molecules such as TRAF2, receptor interacting protein (RIP), and I κ B kinase- γ (IKK γ) [16–18]. The RING domains of IAPs are essential for ubiquitination. Taken together, cIAP1 regulates apoptotic process by both caspase inactivation and substrate ubiquitination. In addition to directing the ubiquitination of its substrates, cIAP1 can catalyze its own ubiquitination in response to apoptotic stimuli [19]. The polypeptide chain-releasing factor GSPT1/eRF3 promotes auto-ubiquitination of cIAP1 in vitro [20]. Ubiquitination and degradation of IAPs may be a key event in the apoptotic program. However, the mechanism of how to regulate the ubiquitination of cIAP1 is not clear.

In this study, we performed a yeast two-hybrid screen in order to identify novel proteins involved in cIAP1 regulation. We found that cIAP1 can interact with an F-box protein, Fbxo7. F-box proteins are components of E3 ubiquitin protein ligases, SCF complexes (Skp1, Cdc53/Cullin1, F-box protein) [21]. F-box proteins recruit a large number of diverse substrates to SCF complexes and allow for their ubiquitination [22]. There are at least 38 F-box proteins in humans [23]. Fbxo7 is one of those F-box proteins [24,25]. Moreover, we also showed that overexpression of Fbxo7 in human cells can promote the ubiquitination of cIAP1. Our results suggest that Fbxo7 can mediate the ubiquitination of cIAP1 by SCF ubiquitin protein ligase, thus providing important implication in cIAP1 regulation.

Materials and methods

Plasmid constructs. Human cIAP1 cDNA fragment from the second BIR domain to the C-terminus (aa 183–618) was obtained by PCR amplification from human brain cDNA (BD Biosciences). The sequences of the PCR primers used are 5'-GGAATTCACCTGAAGAAGCCAGATT-3' and 5'-GGAATTCCTTAAGAGAGAAATGTACG-3'. The PCR product was cut with *Eco*RI and inserted into the *Eco*RI site of the Gal4 DNA-binding domain vector pAS2-1 to generate pAS2-1-cIAP1-B2T as the bait for yeast two-hybrid screen. Human full-length cIAP1 cDNA fragment was obtained by PCR amplification from human placenta cDNA (BD Biosciences). The sequences of the PCR primers used are 5'-GGAATTCACCTACTCATGCACAAAAC-3' and 5'-GGAATTCGAGAGAGAAATGTACGAACAG-3'. The PCR product was cut with *Eco*RI and inserted into the *Eco*RI site of the Flag-tagged vector pRK5F to generate pRK5F-cIAP1 encoding full-length cIAP1 with a C-terminal Flag-tag. Human Fbxo7 cDNA fragment was obtained by PCR amplification from the original plasmid isolated by yeast two-hybrid screen. The sequences of the PCR primers used are 5'-GGAATTCGAGAACATGGCCGGCCT-3' and 5'-GGAATTCATGAATGACAGCCGGCC-3'. The PCR product was cut with *Eco*RI and inserted into the *Eco*RI site of the Hemagglutinin (HA)-tagged vector pcDNA3-HA to generate pcDNA3-HA-Fbxo7 encoding full-length Fbxo7 with an N-terminal HA-tag.

Yeast two-hybrid screen. Competent yeast YRG-2 cells were transformed with the bait plasmid pAS2-1-cIAP1-B2T and selected on medium lacking Trp. The resulting transformants were isolated and used to screen a human lymphocyte Matchmaker cDNA library (BD Biosciences) fol-

lowing the Matchmaker Two-Hybrid System protocol. Transformants were selected on media lacking Trp, Leu, and His. After 3–4 days, positive colonies were picked and further assessed for β -galactosidase activity by colony-lift filter assay. The pACT-derivative plasmids containing positive cDNA clones were then isolated and retransformed into the yeast strain containing the bait plasmid for confirmation of true positives. Positive cDNA clones were sequenced with the Gal4 AD sequencing primers (BD Biosciences) using the BigDye Terminator Cycle Sequencing kit and ABI 3100 genetic analyzer (Applied Biosystems). Network BLAST searches were conducted using the NCBI online service.

Co-immunoprecipitation assay. Human embryonic kidney 293T cells were maintained in DMEM supplemented with 10% fetal bovine serum, antibiotics, and 1 mM L-glutamine. Ten micrograms each of pRK5F-cIAP1 and pcDNA3-HA-Fbxo7 was co-transfected into 293T cells by electroporation. Co-transfection with pRK5F vector and pcDNA3-HA-Fbxo7 was also performed as control. Cells were collected 24 h after transfection by E1A buffer (50 mM Hepes, pH 7.6, 250 mM NaCl, 5 mM EDTA, 0.1% NP40, 1 $\times$ protease inhibitor cocktails (Roche), and 1 mM PMSF) at 4 $^{\circ}$ C for 20 min. Anti-Flag M2 antibody (Sigma) was then added and the samples were incubated at 4 $^{\circ}$ C for 1 h. Flag-tagged cIAP1 proteins were precipitated by protein A-Sepharose beads (Sigma). After washing with E1A buffer, SDS sample buffer was added and the samples were boiled and harvested for immunoblotting. The blots were probed with the anti-Flag antibody (Sigma) or with the anti-HA antibody (Roche) and detected by ECL chemiluminescence kit (Amersham).

Immunocytochemistry with confocal microscope. 293T cells were transfected with pRK5F-cIAP1 and/or pcDNA3-HA-Fbxo7 by electroporation and then plated onto dishes with glass coverslips. At 24 h after transfection, cells were fixed with 4% paraformaldehyde for 30 min. After washing with PBS, cells were permeabilized in 0.1% Triton X-100/PBS for 5 min, blocked in 1% BSA/PBS for 1 h, and then treated with mouse anti-Flag antibody and/or rat anti-HA antibody for 1 h. After washing with 0.5% Tween 20/PBS and blocking with 1% BSA/PBS, cells were treated with rhodamine-conjugated anti-mouse secondary antibody and/or FITC-conjugated anti-rat secondary antibody for 1 h. Cells were stained with DAPI for 3 min and then visualized by confocal fluorescence microscope (Olympus, FluoView).

Ubiquitination analysis. pRK5F-cIAP1 and pcDNA3-HA-Fbxo7 were co-transfected into 293T cells by electroporation. Transfections with pcDNA3-HA vector or pRK5F vector were also performed as controls. Cells were collected 24 h after transfection by E1A buffer. Flag-tagged cIAP1 proteins were immunoprecipitated with the anti-Flag antibody and analyzed by immunoblotting according to the procedures described above. The blots were probed with the anti-ubiquitin antibody (Santa Cruz) to detect the ubiquitination of cIAP1.

Results

Identification of Fbxo7 as a cIAP1 interacting protein by yeast two-hybrid screen

In order to identify novel proteins involved in cIAP1 regulation, a yeast two-hybrid screen was performed. To avoid a transcriptional activating sequence present in cIAP1 protein (our unpublished data), the bait construct consists of the cIAP1 region from the second BIR domain to the C-terminus (aa 183–618) fused to the Gal4 DNA binding domain. A human lymphocyte Matchmaker cDNA library fused to the Gal4 activation domain was screened. Yeast colonies, which grew on media lacking Trp, Leu, and His, and turned blue in β -galactosidase filter assay, were picked and further confirmed by retransforming the recovered plasmids into the yeast strain containing the bait construct (data not shown). One positive clone

isolated encodes a full-length member of the F-box protein family, Fbxo7. Two transcript variants of FBXO7 gene have been reported in sequence databases and encode two isoforms (522 and 443 aa) with a distinct N-terminal region. Our clone corresponds to the shorter transcript, which encodes the isoform 2 of Fbxo7. Since F-box proteins are specificity determining subunits of SCF ubiquitin protein ligases, we decided to further characterize the interaction between cIAP1 and Fbxo7 in human cells in the hope to reveal a novel mechanism for cIAP1 regulation.

cIAP1 interacts with Fbxo7 in human cells

To demonstrate the interaction between cIAP1 and Fbxo7 in human cells, we performed co-immunoprecipitation assay. We made two constructs pRK5F-cIAP1 and pcDNA3-HA-Fbxo7, which express a Flag-tagged full-length cIAP1 and an HA-tagged full-length Fbxo7, respectively. These two constructs were then co-transfected into human 293T cells. We also co-transfected 293T cells with pRK5F vector and pcDNA3-HA-Fbxo7 as control. Then we precipitated cIAP1 with the anti-Flag antibody and determined whether Fbxo7 was co-precipitated. We found that Fbxo7 was detected in the precipitate with cIAP1-Flag but not in the precipitate of pRK5F vector control (Fig. 1). This result indicates that cIAP1 can interact with Fbxo7 in human cells.

cIAP1 and Fbxo7 co-localize in human cells

To determine whether cIAP1 and Fbxo7 co-localize in human cells, we performed immunocytochemistry with a confocal fluorescence microscope. To visualize the cellular localization of cIAP1, 293T cells were transfected with pRK5F-cIAP1 and detected with the anti-Flag antibody and rhodamine-conjugated secondary antibody (Fig. 2,

upper panels). Most of cIAP1 were localized to the nucleus. This result is consistent with the previous reports [26,27]. To visualize the cellular localization of Fbxo7, 293T cells were transfected with pcDNA3-HA-Fbxo7 and detected with the anti-HA antibody and FITC-conjugated secondary antibody (Fig. 2, middle panels). In contrast to the result of cIAP1, most of Fbxo7 were in the cytoplasm. This result is also consistent with the previous report [28]. We then expressed both cIAP1 and Fbxo7 in 293T cells and detected them by immunocytochemical staining (Fig. 2, lower panels). The result showed that cIAP1 and Fbxo7 co-localized remarkably both in the cytoplasm and nucleus. Moreover, we noticed that considerable amounts of cIAP1 and Fbxo7 often co-localized at one or few distinct patchily stained structures close to the nucleus (Fig. 2, lower right panel, arrowhead). In conclusion, our results indicate that cIAP1 and Fbxo7 can co-localize in human cells.

Overexpression of Fbxo7 promotes the ubiquitination of cIAP1

Since Fbxo7 is one component of the SCF complex, we tried to determine whether overexpression of Fbxo7 could promote cIAP1 ubiquitination in the hope to reveal functional aspects of the cIAP1–Fbxo7 interaction. cIAP1-Flag was co-expressed with or without Fbxo7 in 293T cells. After being precipitated with the anti-Flag antibody, the ubiquitination of cIAP1 was detected by immunoblotting with the anti-ubiquitin antibody (Fig. 3). The result clearly showed that the ubiquitination of cIAP1 was greatly enhanced in the presence of overexpressed Fbxo7. The lower level of cIAP1 ubiquitination without Fbxo7 overexpression may result from the endogenous Fbxo7 or auto-ubiquitination of cIAP1. In conclusion, our results show that overexpression of Fbxo7 promotes the ubiquitination of cIAP1.

Discussion

In this paper, we demonstrate that the inhibitor of apoptosis protein cIAP1 interacts with an F-box protein, Fbxo7. Furthermore, we show that overexpression of Fbxo7 promotes the ubiquitination of cIAP1. cIAP1 has been reported to interact with several proteins, including caspases-3 and -7, TRAF2, Smac, RIP, and IKK γ [6,15–18]. All these proteins have known functions in apoptosis signaling or execution pathways, and most of them are downstream targets subjected to cIAP1 regulation. Fbxo7 has not been shown to participate in the apoptotic pathways. Some known functions of Fbxo7 include that Fbxo7 functions in the SCF complex regulating Cdk1-cyclin B-phosphorylated hepatoma up-regulated protein (HURP) proteolysis [29] and Fbxo7 possesses transforming activity mediated through regulation of cyclin D/cdk6 [28]. Our result that the ubiquitination of cIAP1 can be promoted by Fbxo7 overexpression suggests that in addition to auto-ubiquitination of cIAP1, certain SCF ubiquitin protein ligase

Fig. 1. cIAP1 interacts with Fbxo7 in human cells. Co-immunoprecipitation assay was used to detect the association of cIAP1 with Fbxo7. Human 293T cells were co-transfected with pRK5F-cIAP1 encoding cIAP1-Flag and pcDNA3-HA-Fbxo7 encoding HA-Fbxo7. Co-transfection with pRK5F vector and pcDNA3-HA-Fbxo7 was also performed as control. After 24 h, cells were lysed and the lysates were immunoprecipitated with the anti-Flag antibody. The precipitates were separated on 12% SDS–polyacrylamide gel, transferred to membrane, and detected with either anti-HA or anti-Flag antibodies.

Fig. 2. cIAP1 and Fbxo7 co-localize in human cells. Human 293T cells were transfected with pRK5F-cIAP1 alone (upper panels), pcDNA3-HA-Fbxo7 alone (middle panels) or both of them (lower panels). After 24 h, cells were fixed, immunostained, and visualized with confocal microscope. cIAP1 was detected with the anti-Flag antibody and rhodamine-conjugated secondary antibody (left panels). Fbxo7 was detected with the anti-HA antibody and FITC-conjugated secondary antibody (second left panels). Nuclei were detected with DAPI staining (second right panels). The images were merged in the right panels. Some distinct patchily-stained structure is pointed by arrowhead.

Fig. 3. Fbxo7 promotes the ubiquitination of cIAP1. Human 293T cells were co-transfected with pRK5F-cIAP1 and pcDNA3-HA-Fbxo7. Co-transfections with pRK5F-cIAP1 and pcDNA3-HA vector or pRK5F vector and pcDNA3-HA-Fbxo7 were also performed as controls. After 24 h, cells were lysed and cIAP1-Flag was immunoprecipitated with the anti-Flag antibody. The ubiquitination of cIAP1 was detected by immunoblotting with the anti-ubiquitin (Ub) antibody (upper panel). The same blot was stripped and blotted with the anti-Flag antibody to detect cIAP1-Flag (middle panel) or with the anti-HA antibody to detect HA-Fbxo7 (lower panel).

may also catalyze the ubiquitination of cIAP1. Further studies on this SCF-mediated ubiquitination of cIAP1 may reveal novel mechanism for regulation of cIAP1 function.

Our co-localization studies showed that when co-expressed in human cells, cIAP1 and Fbxo7 co-localized remarkably both in the cytoplasm and nucleus. In addition, considerable amounts of cIAP1 and Fbxo7 often co-localized at one or few distinct patchily stained structures close to the nucleus. Plenchette et al. [30] have reported that when monocytic cell lines undergo differentiation upon

phorbol ester exposure, cIAP1 translocates from the nucleus to the cytoplasm, forming a patchy staining close to the nucleus. They also showed that this patchy staining is due to the accumulation of cIAP1 to the cytoplasmic side of the Golgi apparatus. An examination of their and our confocal images shows that the patchily stained structures we saw are very similar to the structures they observed. Thus, Fbxo7 may be involved in the translocation of cIAP1 from the nucleus to the Golgi apparatus during the differentiation of hematopoietic cells.

In conclusion, we show that human inhibitor of apoptosis protein cIAP1 can interact with the F-box protein Fbxo7 in human cells. In addition, overexpression of Fbxo7 in human cells promotes the ubiquitination of cIAP1. Our results are the first report showing that the ubiquitination of cIAP1 may be mediated by SCF ubiquitin protein ligase and suggesting additional mechanism for regulation of cIAP1 function.

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