Structures of the cIAP2 RING Domain Reveal Conformational Changes Associated with Ubiquitin-conjugating Enzyme (E2) Recruitment*§

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Inhibitor of apoptosis (IAP) proteins are key negative regulators of cell death that are highly expressed in many cancers. Cell death caused by antagonists that bind to IAP proteins is associated with their ubiquitylation and degradation. The RING domain at the C terminus of IAP proteins is pivotal. Here we report the crystal structures of the cIAP2 RING domain homodimer alone, and bound to the ubiquitin-conjugating (E2) enzyme UbcH5b. These structures show that small changes in the RING domain accompany E2 binding. By mutating residues at the E2-binding surface, we show that autoubiquitylation is required for regulation of IAP abundance. Dimer formation is also critical, and mutation of a single C-terminal residue abrogated dimer formation and E3 ligase activity was diminished. We further demonstrate that disruption of E2 binding, orimerization, stabilizes IAP proteins against IAP antagonists in vivo.

Apoptosis is a tightly controlled process that is required for normal development and homeostasis of cell number that when dysregulated contributes to disease (1). Mammalian “inhibitor of apoptosis” (IAP) proteins, such as XIAP, cIAP1, and cIAP2, are key negative regulators of apoptosis that can prevent apoptosis by directly inhibiting caspases or by blocking pathways that activate them (2). Amplification of the locus bearing cIAP1 and cIAP2 has been observed in liver, lung, and cervical tumors in humans, raising the possibility that inhibition of cell death by IAP proteins contributes to oncogenesis and provoking the design of drugs to antagonize IAP proteins for the treatment of cancer (3).

IAP proteins are characterized by the presence of one or more zinc-binding baculoviral IAP repeat (BIR) domains that function as protein–protein interaction domains. The N-terminal BIR domains of cIAP1 and cIAP2 interact with the TRAF-N domain of tumor necrosis factor-associated factors (4, 5). This interaction recruits these IAP proteins to TNF-receptor complexes where they modulate receptor-mediated apoptosis. The C-terminal two BIR domains of cIAP1, cIAP2, and XIAP interact with, and in the case of XIAP, inhibit caspases. Proapoptotic proteins that have an N-terminal IAP-binding motif sequence, such as Smac/Diablo also bind to these BIR domains and can displace caspases, leading to their activation (2, 6, 7). Following the BIR domains, cIAP1 and cIAP2 have a caspase recruitment domain (CARD). IAP proteins also bear a C-terminal RING domain that confers ubiquitin E3 ligase activity and is critical to their function (8–10).

To promote apoptosis of cancer cells, compounds that resemble the N-terminal IAP-binding motif residues of Smac/Diablo have been developed to relieve caspase inhibition by XIAP. Surprisingly, experiments with these “Smac-mimetic” compounds have shown that they primarily antagonize IAP activity (11–13). These studies suggest that the normal roles of cIAP1 and cIAP2 are to reduce pro-apoptotic and NF-κB activating signals from members of the TNF receptor superfamily. Consistent with this function, deletion of the gene for cIAP1 or treatment of cells with IAP antagonist compounds leads to activation of NF-κB, and in certain cell types, induction of apoptosis. IAP abundance may be critical because treatment of susceptible cells with Smac-mimetic compounds leads to rapid degradation of cIAP1 (12, 13).

The RING domains of IAP proteins are required for both dimerization and E3 ligase activity (14, 15). Attachment of ubiquitin to proteins requires the sequential action of three enzymes: an E1 ubiquitin activating enzyme, an E2 ubiquitin-conjugating (Ubc) enzyme, and a ubiquitin E3 ligase that specifies the protein to which ubiquitin is added (16). The RING domain E3s, such as IAP proteins, function as molecular scaffolds bringing together E2 and target proteins, allowing direct transfer of ubiquitin from the E2 to the target. In some cases, autoubiquitylation of RING domain containing proteins also
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occurs, and this enables E3s, including IAP proteins, to regulate their own abundance as well as that of interacting proteins. All RING domains have a conserved structure with two zinc ions coordinated by invariant cysteine and histidine residues (17). However, RING domain sequences are variable, and it is likely that this determines their selective binding properties because each RING domain interacts with a subset of the available (~100) E2 proteins. The structure of only one E2:RING-E3 complex has been solved (Protein data bank (PDB) code 2fby) (18), and it is not known how the RING domain promotes ubiquitin transfer as the binding interface is distant from the E2s catalytic site. In addition, the E2 undergoes only small changes upon binding the RING domain, and allosteric activation has been proposed (19).

To obtain a detailed understanding of the nature and regulation of the E3 ligase activity of IAP proteins and to determine whether autoubiquitylation is necessary for the pro-apoptotic activity of Smac-mimetic compounds, we determined the structure of the cIAP2 RING domain homodimer, both alone and in complex with the E2 enzyme UbcH5b. These structures show that the cIAP2 RING domain forms symmetrical dimers and that small changes in the RING domain are associated with binding to the E2. Analysis of the E2-E3 interface reveals an invariant core interface on the RING domain that binds E2, with additional contacts likely to comprise specificity determinants. Analysis of RING domain mutants that are structurally intact suggests that in vivo, the E3 ligase activity of cIAP proteins is required for their degradation and that this depends on dimerization.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Proteins for in vitro experiments and crystallization were expressed from pGEX-6P-3 as glutathione-S-transferase (GST) fusion proteins in Escherichia coli, with the exception of ubiquitin that was produced from pQE80 with an N-terminal histidine tag. Full cloning and purification details are included as supplemental data.

Crystallization—Crystals of the cIAP2 RING were grown at 18 °C by the vapor diffusion method. Sitting drops (200 nl) were set up using the Mosquito robot (TTP LabTech Ltd.) and contained a 2:1 ratio of protein (~5 mg/ml in 10 mM Tris, pH 8.5) and crystallization buffer (0.4 M NH₄(SO₄)₂, 1 M LiSO₄, 0.1 M sodium citrate, pH 6.2). Crystals formed in 1–2 days in the space group C222 with cell dimensions of a = 34.87 Å, b = 66.47 Å, c = 77.47 Å, α = β = γ = 90.0°. The complex was prepared by mixing a 1:1 ratio of cIAP2 RING:UbcH5b and concentrating to ~15 mg/ml. Crystals formed in 1–4 days in various conditions, with diffraction quality crystals growing from 3.3 M NaCl, 100 mM HEPES, pH 7.3, in space group P6₃22, with cell dimensions a = 137.2 Å, b = 137.2 Å, c = 111.9 Å, α = β = γ = 90.0°, γ = 120°.

Structure Solution and Refinement—Diffraction data from crystals of cIAP2 RING were collected at beamline BL9-2 at the Stanford Synchrotron Radiation Laboratory (SSRL) and processed with programs from the CCP4 package (20). The structure was solved using the multiple wavelength anomalous dispersion method (see supplemental data for further details).

Diffraction data from crystals of the cIAP2 RING-UbcH5b complex were collected using Cu-ka radiation, and the structure was solved by molecular replacement using Phaser (21) and the cIAP2 RING and UbcH5b (PDB code 2esk) as search models. Processing and refinement statistics are shown in Table 1. Structural figures were created using PyMOL.

Cell Culture—Cell lines were maintained at 37 °C, 10% CO₂ in Dulbecco’s modified Eagle’s culture media (Invitrogen) supplemented with 10% fetal bovine serum. cIAP1−/− (null) mouse embryonic fibroblasts were infected using a lentiviral inducible system to generate stable cell lines expressing WT mouse cIAP1 and mouse cIAP1 mutants under the control of 4-hydroxy-tamoxifen (4HT) (Sigma). Cells were lysed in DISC lysis buffer supplemented with protease inhibitors and proteins were resolved by SDS-PAGE. Other reagents used were 500 nM Compound A (Tetralogics), monoclonal α-cIAP1 (Vaux laboratory), 100 nM 4HT, and monoclonal α-β-actin (Sigma).

Pull-downs—GST pull-down experiments were used to directly measure interaction between cIAP2 and E2 enzymes. Soluble proteins and glutathione-Sepharose immobilized proteins were mixed for 60 min at 4 °C in phosphate-buffered saline buffer containing 0.2% Tween 20 and 2 mM dithiothreitol and then washed with phosphate-buffered saline containing 0.2% Triton X-100 and 2 mM dithiothreitol prior to the addition of 2× SDS-PAGE sample loading buffer. Samples were then separated on 14.5% SDS-PAGE gels and transferred onto nitrocellulose membrane (Bio-Rad) for analysis using α-His (Qiagen) antibodies.

E3 Ubiquitin Ligase Assays—Ubiquitylation reactions containing 2 μM UbcH5b, 50 nM E1, and 10 μM soluble protein were incubated at 37 °C for 40 μl of 20 mM Tris, pH 7.5, 50 mM NaCl, 60 mM His-tagged ubiquitin, 5 mM ATP, 2 mM MgCl₂, and 2 mM dithiothreitol. Samples were removed at indicated time points, and 2× SDS-PAGE sample buffer was added. Samples were resolved by 14.5% SDS-PAGE and transferred onto nitrocellulose membrane (Bio-Rad) for analysis using α-clp2 antibodies.

To identify the site of ubiquitylation in the cIAP2 RING domain, reactions contained 70 μM of soluble cIAP2 RING. Following ubiquitylation, proteins were resolved by 16% SDS-PAGE, and bands were cut out for in-gel tryptic digest. Mass spectrometry analyses were used to identify fragments that had changed by a ubiquitin specific weight adduct in ubiquitylated samples. Ubiquitylation sites were verified by tandem mass spectrometry analysis.

RESULTS

The RING Domain of cIAP2 Mediates Autoubiquitylation—To determine the minimal requirements for autoubiquitylation of cIAP2, we used purified recombinant proteins in vitro. A construct corresponding to residues 252–604 (the BIR3, CARD, and RING domains) of cIAP2 was expressed as a GST fusion protein (Fig. 1A). Following affinity purification, cleavage from GST, and elution from resin, the ubiquitin E3 ligase activity of cIAP2 BIR3-CARD-RING was apparent (Fig. 1B). No autoubiquitylation was observed when E1 was omitted from the reaction, whereas a ladder of high molecular weight products, due to autoubiquitylation of cIAP2 BIR3-CARD-RING, accu-
mulated when all the components were included (Fig. 1B). A construct containing only the RING domain of cIAP2 (residues 535–604) yielded just a single ubiquitylated band that was not clearly resolved from ubiquitin dimers by SDS-PAGE (data not shown). Analysis by mass spectrometry confirmed that a single ubiquitin chain had been added to the RING domain of cIAP2 and that Lys-563 and Lys-579 were the primary sites of ubiquitin attachment. These results indicate that the RING domain alone is both an active E3 ligase and a substrate for autoubiquitylation, but the larger BIR3-CARD-RING protein provides further target sites and a ladder of ubiquitylation products forms.

**Figure 1: Structure of the RING domain dimer from cIAP2.** A, schematic representation of cIAP2 showing the constructs used. N535 corresponds to the construct used for structural studies. B, autoubiquitylation activity assay of cIAP2 BIR3-CARD-RING (ΔN251). Reactions omitting E1 or with all components present were incubated for up to 60 min, separated by SDS-PAGE, and detected by Coomassie Blue staining, or using α-cIAP2 antibodies. C, sequence alignment of the RING domains of cIAP1, cIAP2, and XIAP. Residues that coordinate the zinc ion are indicated by boxes, hydrophobic residues at the dimer interface (panel E) are shaded yellow, and the remaining interface residues are colored gray. Secondary structural elements are indicated. D, schematic representation of the cIAP2 RING domain homodimer with zinc coordinating residues represented in stick form and zinc ions as gray spheres. E, surface representation of the dimer interface. The right monomer in D is rotated ~90°. Hydrophobic residues within 4 Å of the interface are colored yellow, and polar and charged residues are white.

**Dimer Formation Depends on Residues N- and C-terminal to the RING Domain**—The RING domains of cIAP1, cIAP2, and XIAP are highly conserved (Fig. 1C), and there is evidence to suggest that the domains are capable of forming both homodimers and heterodimers (15, 22). To further investigate the mechanism of IAP autoubiquitylation and dimerization, we determined the crystal structure of the cIAP2 RING domain (ΔN535) to 2.0 Å resolution (Fig. 1D and Table 1). The core RING domain of cIAP2 bears features common to all RING domains, with two zinc ions coordinated in a cross-braced arrangement by 1 histidine and 7 cysteine residues (ligands indicated in Fig. 1, C and D). The structure was solved in space group C222, with just one RING monomer per asymmetric unit. The RING domain dimer is generated by a crystallographic two-fold symmetry axis. Size-exclusion chromatography coupled with multiple angle light scattering (MALS) showed that the RING domain forms stable dimers in solution (Fig. 2C). The dimer interface involves both the core RING domain and residues C-terminal to it, including β3, as well as an N-terminal helix that packs against its dimeric partner, and in total, dimerization bures a surface area of 1018 Å². As observed in many protein interfaces, the dimer interface is predominantly hydrophobic in nature (Fig. 1E) but is also stabilized by two electrostatic interactions between the charged residues Arg-555 and Glu-554 near the base of the N-terminal α-helix, which are conserved among IAP RINGs.

**Relative Orientation of Dimeric RING Domains Is Conserved**—When compared with other RING domain dimers (Fig. 2A and supplemental Fig. S1), the cIAP2 RING domain structure overlays most closely (1.79 Å over 92 residues) with the crystal structure of the MDM2-MDMX RING domain heterodimer (PDB code 2vje) (23). The most obvious difference is due to the distinct conformation of residues N-terminal to the core RING domain. In cIAP2, these residues form a helix that constitutes a major part of the dimer interface, whereas in the MDM dimer structure, N-terminal interactions are less extensive, and this region is disordered. However, the β1-β2 loop of the MDM RING domains interacts much more closely with β3 of the adjacent monomer at the dimer interface than in cIAP2 (Fig. 2A). Despite these differences, the RING domains in the MDM and IAP dimers have a similar disposition, and interactions involving β3 at the C terminus of each RING domain dominate, a characteristic that is not seen in other structures of RING domain dimers. Instead, the C termini of both BRCA1-BARD
and Bmi1-Ring1b are helical (supplemental Fig. S1) and are in close proximity to the N-terminal helix, as well as the equivalent helices of their respective dimeric partners (24, 25).

Although the dimer interface is quite different, the RING domains of the cIAP2 dimer overlay well with those of the BRCA1-BARD dimer (2.71 Å over 110 residues) and the Bmi1-Ring1b dimer (3.44 Å over 99 residues). The related U-box proteins PRP19 and CHIP also overlay closely (26, 27). The conserved disposition of the RING domains in distinct dimers suggests that the relative position of the domains is of functional relevance.

The high degree of structural homology between the MDM and cIAP2 RING domain dimers suggested that many residues would have equivalent roles in both proteins. In MDM2, the C-terminal residues are required for dimerization and for activity, with a single aromatic residue located 3 residues from the C-terminus (Tyr-489 in MDM2) having a critical role (23, 28).

Likewise, in IAP proteins, the C-terminal residues are required for dimer formation as deletion of the 6 C-terminal residues from cIAP1 disrupts the cIAP1-XIAP heterodimer (15). To determine whether the equivalent aromatic C-terminal residue in IAP proteins is also required for E3 ligase activity, we mutated phenylalanine 602 to alanine in cIAP2 (F602A). Like MDM2, this mutation disrupts the E3 ligase activity of purified cIAP2 BIR3-CARD-RING and RING proteins, and ubiquitin ladders no longer form (Fig. 2B, data not shown). Analysis of the RING domain by MALS (Fig. 2C) showed that a shift from dimer to monomer is associated with the F602A mutation, suggesting that a stable RING dimer is required for formation of a ubiquitin ladder.

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The RING domains of IAP proteins are highly conserved (Fig. 1B), and we anticipate that our studies with cIAP2 provide a model for other IAP proteins. To investigate this and to show that the C-terminal aromatic is also required for activity in cells, we mutated the equivalent C-terminal residue in mouse cIAP1 (Phe-610) to alanine (supplemental Fig. S2) and expressed the
full-length protein in cIAP1−/− mouse embryonic fibroblasts (Fig. 2D). The WT and mutant forms had similar levels in healthy cells. However, when an IAP antagonist compound, which has previously been shown to promote degradation of IAP proteins, was added (11), WT cIAP1 was degraded, but the levels of the F610A mutant protein were not diminished. This shows that autoubiquitylation of IAP proteins in vivo and in vitro depends on the aromatic residue located 3 residues from the C terminus. These parallels suggest that the C-terminal residues in IAP and MDM RING domains fulfill similar functions and that ubiquitylation of target lysine residues depends upon the integrity of the C-terminal residues.

The cIAP2 RING Dimer Recruits Two UbcH5b Molecules—The affinity of cIAP2 RING for UbcH5b was measured using isothermal titration calorimetry (supplemental Fig. S3, A and B) and found to be between 19 and 43 μM. The RING domains of XIAP and cIAP1 bind UbcH5b with comparable affinity in GST pull-down experiments (data not shown), suggesting that the RING domains of these IAP proteins form similar complexes with UbcH5b. This relatively high affinity interaction allowed crystallization of the complex between the cIAP2 RING domain and the E2, UbcH5b. The structure was solved to 3.4 Å resolution by molecular replacement, using the RING monomer and UbcH5b (19) as search models. A single chain of each protein in the asymmetric unit was identified, corresponding to a relatively high solvent content of ~80%. Each RING domain interacts independently with UbcH5b, and analogous to the structure of the RING domain alone, the RING domain dimer two-fold axis coincides with a crystallographic two-fold axis. As a consequence, the cIAP2 RING homodimer, with UbcH5b bound to each RING domain, is symmetrical (Fig. 3A).

Interaction between the RING domain and the E2 involves residues in the α-β3 loop of the RING domain, as well as residues in the N-terminal helix and the loop between this and β1 (Fig. 3A and supplemental Fig. S3C). Notably, three salt bridges are formed between Asp-562 and Lys-4, Glu-553 and Lys-8, and Arg-549 and Asp-12 of cIAP2 and UbcH5b, respectively (supplemental Fig. S3D). Further H-bonds are formed between Arg-5, Lys-8, and Ser-94 of UbcH5b and main-chain atoms of Val-559, Met-561, and Pro-589 of the RING domain, respectively (supplemental Fig. S3, C and D). Analysis of the B-factors is consistent with these residues contributing to E2 recruitment as relative to the rest of the structure, a decrease in the B-factors of RING domain interface residues is observed in the complex.

Differences in the RING Domain Associated with Binding to an E2—Comparison of the polypeptide backbone of the RING domain dimer in the presence and absence of the E2 shows localized changes. In addition to the residues that directly contact UbcH5b, the C-terminal residues are also displaced (Fig. 3B). Direct interaction with the E2 probably accounts for differences in the N-terminal helix and following loop. However, the shift in the position of the C-terminal residues is likely to be due to an indirect effect, with small differences in the α-β3 loop of one monomer, which contacts the E2, affecting the position of the C terminus of the other monomer (Fig. 3C). The altered position of the C terminus may be important for activity as the residues that move include Phe-602, which is required for E3 ligase activity (Fig. 2B). The equivalent residue is also essential for the ubiquitylation of MDMX by MDM2 and has been proposed to form part of an extended surface that mediates transfer of ubiquitin from the E2 to the target lysine (20).
This is the first report of a RING domain E3 ligase structure in the presence and absence of an E2. The differences observed suggest that although binding depends on local rearrangements of interface residues to optimize interactions, ubiquitin transfer from E2 to the target lysine may depend on small changes in the RING domain, as well as the allosteric effects already documented in the E2 (19).

**Disruption of the E2 Interface Impedes IAP Homeostasis and Antagonist Action**—To probe the role of individual residues in the E2-binding surface of the RING domain (supplemental Fig. S4), site-directed mutagenesis of selected interface residues was performed. The ability of these mutants to bind UbcH5b was tested in GST pull-down experiments (Fig. 4). Consistent with the surface identified in the complex structure, three hydrophobic to alanine mutations in cIAP2 (V559A, L585A, and I590A), as well as D562A, interfered with UbcH5b binding, whereas diminished binding was observed for the mutants K558A and P589A. These residues interact with Arg-5, Pro-95, Ala-96, and Lys-4 among other UbcH5b residues (supplemental Fig. S3). The residues in UbcH5b that interact with the RING domain of cIAP2 coincide with the interface required for interaction of the BRCA1 RING domain with E2 enzymes (29), suggesting that similar contacts are involved.

To further investigate the link between E3 ligase activity and E2 recruitment, we mutated Val-567 in mouse cIAP1, which is equivalent to Val-559 in cIAP2 (supplemental Fig. S2), and expressed it in cIAP1/−/− mouse embryonic fibroblasts. cIAP1 containing V567A had slightly higher steady state protein levels than WT, and the levels were only marginally reduced when an IAP antagonist was added (Fig. 4B). This result shows that disrupting the E2-binding interface impedes E3 ligase activity in cells.

Together, these results show that autoubiquitylation and degradation of IAP proteins following the addition of IAP antagonists depends on an intact E2-binding site. In addition, these interface mutants provide a general method of specifically disrupting interface impedes E3 ligase activity in cells.

Conserved Core Features Mediate E2 Recruitment by RING Domains—Although only a limited number of E2-E3 complexes have been reported, the key features underlying binding of E2 are becoming apparent. When UbcH7, from the proto-oncogene RING protein c-Cbl complex structure (18) is overlaid onto UbcH5b, the contacting RING has a similar disposition as the cIAP2 RING domain (supplemental Fig. S5). Closer analysis of the contacts reveals a small central hydrophobic region, flanked on the left by an arginine residue and on the right by an acidic region, which have comparable positions and spacing in both RING domains (Fig. 5). An extremely similar core-binding region also mediates recruitment of Ubc13 to the U-box type E3 ligase CHIP (Fig. 5) (27). In all three complexes, additional and distinct contacts are also apparent, and it is likely that these residues confer specific binding.

Consistent with this, although no other complexes involving UbcH5b have been reported, the interface residues observed here have been predicted to be required for binding of UbcH5b to CNOT4, which contains an N-terminal RING domain (30). In addition, using NMR and chemical shift differences to assess binding Christensen et al. (29) showed that Arg-5, Lys-8, and Ala-96 of the closely related E2, UbcH5c, were located at the E2-binding site of the RING of BRCA1. They also observed that these residues are highly conserved among E2 enzymes capable of binding BRCA1. Given that these residues all form specific contacts in the cIAP2 RING-UbcH5b structure, it is likely that the BRCA1 RING domain interacts with E2 enzymes in a very similar manner to that observed for cIAP2. In addition, it is likely that the IAP RING domains use a similar subset of E2 enzymes to those identified for BRCA1 (29). Consistent with this, a limited analysis by Hu and Yang (14) showed that cIAP2 binds to UbcH5a, UbcH5b, UbcH5c, and UbcH7 but not to UbcH8.

**DISCUSSION**

Caspase inhibition by IAP proteins depends on the BIR domains of IAP proteins (2). The inhibitory activity of the IAP proteins is relieved when proapoptotic molecules, such as Smac/Diablo, are released from the mitochondria. Unexpectedly, the Smac-mimetic compounds do not induce apoptosis by relieving XIAP inhibition of caspases. Instead, they activate NF-κB signaling from TNF receptors due to removal of cIAP1, in a manner that depends on TNF and the E3 ligase activity of IAP proteins (12, 13). These results show that cIAP1 and cIAP2 regulate TNF-R1 signaling and are the major targets of the IAP antagonist compounds.

Here we show that the RING domain is essential, as well as sufficient, for both E3 ligase activity and dimerization of IAP proteins (Fig. 1). The closest structural homolog of the cIAP2 RING homodimer is the MDM RING heterodimer, with each having extensive interactions between β2 of one monomer and β3 (the C-terminal tail) from its dimeric partner. The main differences between the MDM and IAP RING structures are due to an extended N-terminal helix in cIAP2.
and a longer β1-β2 loop in the MDMs (Fig. 2A). Accordingly, IAP proteins have an additional dimer interface at the N terminus, whereas MDMs have more contacts at the C terminus. This means that mutating the aromatic residue 3 residues from the C terminus disrupts dimer formation for cIAP2 (Fig. 2C) but not for MDM2-MDMX (23, 28). However, in both cases, the equivalent mutation disrupts E3 ligase activity, suggesting that the aromatic residue contributes to both dimer formation and ubiquitin transfer.

E2-E3 interactions are often of modest affinity and difficult to detect (29). However, the RING domain of cIAP2 binds to UbcH5b with μM affinity, and the structure of the complex shows that in addition to the core interface, which includes residues in the α-helix and the zinc-chelating loops of the RING domain, interactions involving the N-terminal helix and the loop at the base of the this helix (e.g. Asp-562) mediate binding (Fig. 3A). By ensuring correct packing of the two helical N termini against one another, dimerization may facilitate E3 ligase activity and present residues on the opposite face of the helix in the correct orientation for E2 interaction. In addition, the inherent flexibility associated with the helix, which is reflected by higher B-factors, may be important to allow the RING domain to interact with a number of distinct E2 enzymes.

A major question that remains unresolved is how RING domains promote the transfer of ubiquitin from the E2 to target lysine residues in a substrate protein since the structure of the E2 is unchanged by binding a RING domain, and the RING domain is not in close proximity to the E2 active site cysteine (18). One model suggests that long range allosteric changes in the E2 promote ubiquitin transfer (19). Our structures show that subtle changes due to E2-E3 complex formation may also extend across the RING domain dimer interface, and this may be important for activity (Fig. 3, B and C). In support of this, in the MDM RING domains, the integrity of the dimer interface is required for E3 ligase activity, and ubiquitin is added to the inactive MDMX RING domain (19). The position of the targeted lysine residue (Lys-442) in MDMX is spatially conserved with the two sites we have identified in the cIAP2 RING domain (Lys-563 and Lys-579). However, in longer proteins that have additional domains, such as the BIR3-CARD-RING protein used here (Fig. 1B), more extensive ubiquitylation probably occurs because the inherent flexibility allows other lysine residues to be correctly positioned for ubiquitylation.

Despite different modes of dimerization, other dimeric RING domains and U-box structures have a similar relative arrangement (supplemental Fig. S1). This structural conservation suggests that the relative orientation of the two RING domains in a dimer is important, possibly serving to position the substrate proteins for ubiquitin transfer or to aid ubiquitin chain formation. Dimerization has been shown to promote E3 ligase activity before (21), but even heterodimers that comprise an active and an inactive E3 ligase RING domain, as in the case of the Bmi/Ring1b or BRCA1/BARD1 heterodimers, have increased activity, suggesting that RING dimerization is more important than the close proximity of two active RING E3 ligases.

Key to ubiquitylation is the selective binding of E2s by each E3 as this in part specifies the nature of the modification that occurs. Analysis of other E2-E3 structures suggests that although similar interactions underpin binding, additional contacts outside the core interface provided by the RING domain contribute to E2 recruitment (Fig. 5). Each RING domain interacts with a subset of the available E2 enzymes, and it is likely that interactions by residues outside the RING domain contribute to selective binding. Targeting these additional interactions may provide an ideal route for the development of specific inhibitors of different E3 ligases, without disrupting general ubiquitylation in the cell. However, the utility of directly targeting the E3 ligase activity of IAP proteins remains to be investigated.

In the cIAP2 RING-UbcH5b complex, both RING E3 molecules in the dimer recruit an E2. Like cIAP2 RING, the CHIP U-box bound to Ubc13 forms a symmetrical complex. However, full-length CHIP in the absence of E2 forms an asymmetric dimer, whereby the E2-binding site of one monomer is occluded by an additional TPR domain (27). This suggests that additional domains in CHIP can determine whether the E2 binds in a symmetrical or non-symmetrical manner. More recently, the C2 domain of the homologous to E6AP C terminus (HECT) type E3 ligase Smurf2 was shown to have an autoinhibitory function and prevent cycles of futile autoubiquitylation (31). These examples indicate that remote domains in a protein can control the E3 ligase activity. This suggests a possible mechanism to account for the observed increase in autoubiquitylation of IAP proteins upon binding of IAP antagonists to the N-terminal BIR domains (12).

In conclusion, the structures presented here provide the first view of the cIAP2 RING domain, and importantly, the first report of a RING domain in the presence and absence of an E2. These studies suggest that changes in the RING domain are associated with E2 binding and that these may be important for activity. The identification of a specificity interface between the RING and E2 suggests a target for development of selective antagonists. In support of this, we show that specifically disrupting E2 recruitment impedes E3 ligase function and that the currently available IAP antagonists require E3 ligase activity for their function (Fig. 4B). However, to fully understand how the Smac-mimetic antagonists function, future efforts must investigate how distant domains in IAP proteins cooperate to regulate ubiquitylation via the RING domain.
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