The Ubiquitin-associated Domain of Cellular Inhibitor of Apoptosis Proteins Facilitates Ubiquitylation*

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Background: Many ubiquitin E3 ligases possess ubiquitin-binding modules of unknown function.

Results: The ubiquitin-binding domain from the E3 ligases cIAP1 and cIAP2 binds ubiquitin and the E2–ubiquitin conjugate. Interaction of ubiquitin with the ubiquitin-binding domain regulates ubiquitylation by cIAP proteins in a RING domain-dependent manner.

Conclusion: Mutation of the conserved motifs in the ubiquitin-associated domain causes many UBA domains to unfold.

The cellular inhibitor of apoptosis (cIAP) proteins are essential RING E3 ubiquitin ligases that regulate apoptosis and inflammatory responses. cIAPs contain a ubiquitin-associated (UBA) domain that binds ubiquitin and is implicated in the regulation of cell survival and proteasomal degradation. Here we show that mutation of the MGF and LL motifs in the UBA domain of cIAP1 caused unfolding and increased cIAP1 multimonoubiquitylation. By developing a UBA mutant that disrupted ubiquitin binding but not the structure of the UBA domain, we found that the UBA domain enhances cIAP1 and cIAP2 ubiquitylation. We demonstrate that the UBA domain binds to the Ubch5b–Ub conjugate, and this promotes RING domain-dependent monoubiquitylation. This study establishes ubiquitin-binding modules, such as the UBA domain, as important regulatory modules that can fine tune the activity of E3 ligases.

Covalent attachment of the conserved 76-amino acid polypeptide, ubiquitin, to lysine residues of substrate proteins (ubiquitylation) can activate signaling pathways, promote intracellular trafficking, and mark proteins for proteasomal degradation (1). An enzymatic cascade involving E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin-protein ligases brings about the attachment of ubiquitin to substrates. Following ATP-dependent activation of ubiquitin by the E1, ubiquitin is transferred to the active site cysteine of an E2 enzyme to form the E2–ubiquitin (E2–Ub)2 conjugate (2, 3). The E3 ligase then interacts with the E2–Ub conjugate and promotes transfer of ubiquitin from the E2 enzyme to the target lysine. In the case of the really interesting new gene (RING) E3 ligases, they bind to E2–Ub conjugates and promote direct transfer of ubiquitin to the target without forming a covalent intermediate (4, 5). Modification of substrates by either single ubiquitin protomers (monoubiquitylation) or chains of covalently linked ubiquitin molecules (polyubiquitylation) serves as a code, which is translated into specific cellular responses by a variety of structurally diverse ubiquitin-binding domains (UBDs) (6).

Recently, ubiquitin-binding modules have also been shown to facilitate protein ubiquitylation. Notably, monoubiquitylation of several UBD-containing proteins was shown to be E3-independent but relied on the presence of the UBDs (7). In other cases, the activity of E3 ligases can be modulated by UBDs. For instance, the activity of the endoplasmic reticulum-associated degradation (ERAD) pathway E3 ligase gp78 requires a functional UBD, termed the coupling of ubiquitin conjugation to endoplasmic reticulum degradation (CUE) domain (8). Similarly, the CUE domain from Cue1p enhances polyubiquitin chain elongation by other ERAD E3 ligases (9). As well as the UBD-containing RING E3 ligases, several homologous to E6AP C terminus (HECT) E3 ligases also possess a shallow ubiquitin-binding patch that is required for ubiquitin chain elongation (10, 11). Last, the C terminus of the RING-in between-RING (RBR) E3 ligase HOIP (HOIL-1-interacting protein) contains a CBR (catalytic in-between-RING) module that interacts with ubiquitin to promote formation of linear ubiquitin chains (12, 13). These studies suggest that ubiquitin-binding modules can promote ubiquitylation or fine tune the activity of E3 ligases.

Two members of the inhibitor of apoptosis (IAP) protein family, cIAP1 and cIAP2, are E3 ligases that have an important role in regulating pathways that signal through nuclear factor-κB (NF-κB) (14). In addition to a C-terminal RING domain, the multidomain cIAPs possess three baculovirus IAP repeat (BIR) domains that bind to receptor-associated proteins or sub-
strate proteins, a UBD referred to as the ubiquitin-associated (UBA) domain, and a caspase recruitment domain (CARD). RING domain dimerization is essential for E3 ligase activity of cIAPs (15–17), and cIAP1 can assume an autoinhibited monomeric form in which the RING domain is sequestered. However, autoinhibition is relieved upon binding of small molecule substrate mimicetic compounds referred to as second mitochondria-derived activator of caspases (SMAC) mimetics (SMs) to the BIR3 domain of cIAP1. As a consequence, the RING domain is free to dimerize, recruit E2–Ub conjugates, and promote ubiquitin transfer (15, 16, 18).

The UBA domain of IAP proteins binds ubiquitin (19–21) and, like other UBA domains, has a compact structure consisting of three α-helices (designated α1–α3) (Fig. 1A). Two conserved signature motifs, Met/Leu-Gly-Phe/Tyr (MGF) and Leu-Leu/Val (LL), are located at the ubiquitin binding interface of the UBA domain (Fig. 1, B–D) (15, 22, 23). These motifs are commonly mutated to disrupt ubiquitin binding, and in cIAPs, mutation of the MGF motif or deletion of the entire UBA domain resulted in impaired NF-κB activation and/or defective 26S proteasomal degradation of IAPs. As a consequence, a role for the UBA domain in binding to ubiquitin chains on other proteins, as well as to proteasome receptors was suggested (19, 20).

In this study, we show that the UBA domain of cIAP1 and cIAP2 enhances ubiquitylation by facilitating E2–Ub conjugate recruitment. This discovery relied on the identification of a UBA domain mutant that disrupted the interaction of ubiquitin but not the structural integrity of the UBA domain. Importantly, we show that mutation of the MGF and LL motifs in the UBA domain of cIAP1 and the MGF motif in the human homolog of Rad23 (HHR23A) and the Saccharomyces cerevisiae EH domain-containing and endocytosis protein 1 (EDE1) causes the UBA domain to unfold. In cIAP1, unfolding of the UBA domain affects autoubiquitylation, and the function of the UBA domain is obscured.

**EXPERIMENTAL PROCEDURES**

**Constructs and Mutagenesis**—The open reading frame of human BIRC2 (GenBank™ accession number U37547), BIRC3 (U37546), UBE2D2 (AY651263), RAD23A (D21235), and S. cerevisiae EDE1 (Z35808) were PCR-amplified with flanking BamHI and EcoRI or BamHI and XhoI restriction sites and cloned into pGEX-6P-3 (GE Healthcare) in frame with an N-terminal GST tag. As a result of cloning, the purified proteins possess five additional N-terminal residues, GPLGS, following the GST tag, and PreScission protease. The cDNA of human ubiquitin was cloned into pET3a and expressed with a tag. An expression construct encoding the ubiquitin enzyme, Uba1 (X55386), was kindly donated by Dr. H. Schindelin (Rudolf Virchow Center for Experimental Biomedicine and Institute for Structural Biology, University of Würzburg). All point mutations were introduced using QuikChange (Stratagene) site-directed mutagenesis.

**Reagents and Antibodies**—Mouse monoclonal anti-UbcH5b antibody (Y-20) was purchased from Santa Cruz Biotechnology, Inc. Rabbit polyclonal anti-cIAP1 antibody (GTX1100987) was purchased from GeneTex. Rat anti-SMAC and anti-cIAP2 antibodies were kind gifts from Dr. J. Silke (Walter and Eliza Hall Institute of Medical Research). Anti-His antibody was purchased from GE Healthcare. SM compounds were obtained from TetraLogic Pharmaceuticals as described previously (18, 24).

**Protein Expression and Purification**—Purification of cIAP1 B3UCR (residues 266–618, containing BIR3-UBA-CARD-RING domains), UbcH5b and UbcH5b-C855–Ub conjugate have been described previously (18). The longer constructs comprising the BIR2, BIR3, UBA, CARD, and RING domains of cIAP1 (B2B3UCR, residues 181–618) were expressed at 28 °C for 16 h following induction with 0.2 mM IPTG. ZnCl2 (0.1 mM) was also added into the media following IPTG induction. Following lysis, B2B3UCR was purified in the same way as the B3UCR protein. cIAP2 B2B3UCR proteins (residues 166–604) were expressed at 18 °C for 16 h and purified as described for the cIAP1 counterpart, except cells were lysed in 20 mM Tris-HCl, pH 7.5, 250 mM NaCl, 0.25% Triton X-100. Following removal of GST tag, cIAP2 was purified further using a Superdex 200 16/60 column (GE Healthcare) pre-equilibrated in 20 mM Tris-HCl, pH 7.5, 250 mM NaCl.

The isolated UBA domain from cIAP1 (residues 376–441) and UBA-CARD fragment (residues 376–554) were expressed in Escherichia coli BL21(DE3) at 37 °C for 6 h following induction with 0.4 mM IPTG. The cells were harvested, resuspended, and lysed in 50 mM Tris-HCl, 150 mM NaCl, pH 8.5. Soluble GST-fused cIAP1 fragments were bound to glutathione-Sepharose resin and washed with 50 mM Tris-HCl, pH 8.5, without salt. Following cleavage of the GST tag with PreScission protease, the untagged proteins were recovered and loaded onto a HiTrapQ HP column (GE Healthcare) pre-equilibrated in 50 mM Tris-HCl, 50 mM NaCl, pH 8.5. A linear 30-ml NaCl gradient from 50 to 500 mM was then applied. Fractions containing cIAP1 UBA or UBA-CARD were pooled and purified further using a Superdex 75 16/60 column (GE Healthcare) pre-equilibrated in 20 mM Tris-HCl, 250 mM NaCl, pH 7.5.

Wild type and Lys-less (K0) ubiquitin were expressed in E. coli BL21 (DE3) or Rosetta-2 (DE3) cells, respectively, at 37 °C for 6 h following induction with 0.2 mM IPTG and purified according a published method (25). The S. cerevisiae E1 enzyme Uba1 was expressed and purified according to Lee and Schindelin (26).

The N-terminal UBA domain from human HHR23A (residues 159–204) and the sole UBA domain from S. cerevisiae EDE1 (residues 1339–1381) were expressed in E. coli BL21 (DE3) cells at 18 °C for 16 h following induction with 0.2 mM IPTG. The cells were lysed in phosphate–buffered saline (PBS, 10 mM Na2HPO4, 1.8 mM KH2PO4, 140 mM NaCl, 2.7 mM KCl, pH 7.4). Following recovery of the soluble fractions, glutathione-Sepharose resin was added. The resin was washed with PBS. PreScission protease was then added to cleave the UBA domain from the N-terminal GST tag. Soluble UBA domains were subsequently recovered and loaded onto a Superdex 75 10/300 GL column (GE Healthcare) pre-equilibrated in PBS.

SMAC with a C-terminal His6 tag was expressed in E. coli BL21 (DE3) by autoinduction at 28 °C for 16 h. The protein was captured from crude cell lysate using Ni2⁺–nitrilotriacetic acid resin (Qiagen) in 50 mM sodium phosphate, pH 7.4, 300 mM
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NaCl, 10 mM imidazole. SMAC-His$_6$ was eluted from the resin using 300 mM imidazole and purified further using a Superdex 200 16/60 column (GE Healthcare) pre-equilibrated in PBS.

Limited Proteolysis—Stock trypsin solution was prepared by dissolving trypsin (Sigma-Aldrich) at 1 mg/ml in 1 mM HCl, 2 mM CaCl$_2$. The stock solution was diluted 1:10 in PBS directly before use. Purified cIAP1 B3UCR variants (20 or 50 µM) were incubated with trypsin (0.2 µg) at 20 °C for the indicated times. Reducing SDS-PAGE sample buffer was added to stop the reactions. The digestion products were resolved by SDS-polyacrylamide gels and visualized using Coomassie Brilliant Blue staining.

Circular Dichroism (CD) Spectroscopy—Far-UV CD spectra were recorded every 2 nm between 195 and 260 nm using an Olis DCM-10 CD spectrophotometer. The integration time was set as a function of high voltage. Measurements of diazoyzed protein samples (between 12.5 and 30 µM) in 20 mM sodium phosphate, 100 mM NaCl, pH 7.4, were recorded at 20 °C. Three spectra from each sample were averaged.

Binding Experiments—Isothermal titration calorimetry (ITC) measurements were performed using a VP-ITC (MicroCal) at 30 °C. All proteins were diazoyzed into a single stock of PBS. Wild type ubiquitin at a concentration of 410 µM was injected in 10-µl aliquots into a 1.4-ml solution of cIAP1 UBA protein variant at 25 µM in the sample cell. The data were analyzed using Origin version 7 and fit to a single site binding model.

Surface plasmon resonance (SPR) measurements was performed using a Biacore X-100 instrument (GE Healthcare) at a flow rate of 30 µl/min using 20 mM Tris-HCl, 250 mM NaCl, 0.05% Tween 20, pH 7.5, as the running buffer. Anti-GST antibody was immobilized onto a CM5 chip (GE Healthcare) using amine-coupling chemistry according to the manufacturer’s instructions up to ~15,000 response units. GST or GST-cIAP1 UBA proteins were injected into flow cells 1 and 2, respectively, and captured to ~1000 response units. At least seven concentrations of analytes were injected. Binding of ubiquitin to chip-immobilized GST-UBA exhibited fast $k_{on}$ and $k_{off}$ therefore, equilibrium binding analyses were performed using Biacore X-100 Evaluation software (GE Healthcare).

Size Exclusion Chromatography Coupled to Multianti Laser Light Scattering (SEC-MALLS)—Pure protein samples (25 µM) were loaded at a flow rate of 0.5 ml/min onto a Superdex 75 or Superdex 200 10/300 GL column (GE Healthcare) pre-equilibrated in PBS or 20 mM Tris-HCl, 250 mM NaCl, pH 7.5. The column was connected in series to a Dawn 8+ MALLS detector (Wyatt Technology) and a Waters 410 differential refractometer (Millipore). Data were analyzed using Astra software version 5.3.4 (Wyatt Technology).

GST Pull-downs—GST-fused cIAP1 UBA or B3UCR proteins were immobilized on glutathione-Sepharose resin and mixed with UbcH5b or UbcH5b-C855~Ub conjugate for 1 h at 4 °C in PBS supplemented with 0.2% Tween 20 and 1 mM DTT. The mixtures were washed with the same buffer three times before the addition of reducing SDS-PAGE sample buffer. The samples were resolved by 14.5 or 16% SDS-polyacrylamide gels. Proteins were detected by immunoblotting using anti-UbcH5b antibody.

Activity Assays—Multiple-turnover autoubiquitylation assays (see Figs. 6 (A and C), 8 (A and B), and 10B) were performed as described previously (18), except that for Fig. 8B, where 2 µM cIAP2 B2B3UCR was added.

The SMAC ubiquitylation assay (Fig. 8, C and D) was performed by incubating 12 µM SMAC, 1 µM cIAP1 B2B3UCR or 0.5 µM cIAP2 B2B3UCR, 1 µM UbcH5b, 50 µM ubiquitin, and 100 nm E1 in the same conditions as for the autoubiquitylation assays.

For single-turnover assays (Fig. 9, A and B), 5 µM UbcH5b was incubated with 50 µM WT or K0 ubiquitin in the presence of 100 mM E1 and 5 mM ATP for 15 min at 37 °C in 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 2 mM MgCl$_2$, and 1 mM DTT. After 15 min, 0.05 units/µl apyrase (Sigma) was added to deplete the remaining ATP. Subsequently, 2 µM cIAP1 B2B3UCR protein was added. The reaction mixtures were then incubated further at 37 °C, and samples were taken at different time points.

In Fig. 7, A and B, purified GST-fused cIAP1 UBA domain or untagged cIAP1 UBA-CARD fragments were incubated with 100 mM E1, 7.5 µM UbcH5b, and 50 µM ubiquitin in the multiple-turnover assay buffer.

Oxyester conjugate discharge assays were carried out by incubating 5 µM purified UbcH5b-C855~Ub conjugate with 150 µM cIAP1 UBA domain (Fig. 7C) or 2 µM cIAP1 B2B3UCR proteins (Fig. 9C) in 50 mM Tris-HCl, pH 7.5, at 37 °C.

For the E2 titration assays in Fig. 11A, varying concentrations of UbcH5b from 0 to 15 µM were charged with WT or K0 ubiquitin as described above but without apyrase treatment prior to the addition of cIAP1 B3UCR protein (total concentration of 8 µM). The reaction mixtures were then incubated further for 1 h, and reducing SDS-PAGE sample buffer was added to stop the reactions. In Fig. 11B, 0–5 µM UbcH5b was charged as in Fig. 9A, whereas cIAP2 and SMAC were incubated separately. The two mixtures were then mixed and incubated further for 20 min.

Reaction products from all assays were resolved on 12.5 or 16% Laemmli Tris-glycine SDS-polyacrylamide gels or 4–12% gradient NuPAGE gels (Novex, Invitrogen) in MES buffer according to the manufacturer’s instructions. Reaction products were visualized using Coomassie Blue staining or immunoblotting as described. National Institutes of Health ImageJ software was used for band intensity quantification, and the data from at least two independent experiments are plotted as the mean ± S.D.

RESULTS

Mutation of the MGF and LL Motifs in the UBA Domain of cIAP1 Destabilizes the Protein—Previously, we characterized a truncated form of cIAP1 that comprised the BIR3, UBA, CARD, and RING domains (B3UCR; Fig. 2) because it is readily purified and is regulated by autoinhibition (16, 18). To investigate the contribution of the UBA domain to cIAP1 ubiquitylation, we prepared B3UCR protein harboring mutations in the MGF motif of the UBA domain (M402A/F404A (MF/AA)) (Fig. 1, B and D) (19, 20). Unexpectedly, cIAP1 B3UCR-MF/AA degraded more readily during purification, and the α-helical content of B3UCR-MF/AA, as measured by CD spectroscopy,
was diminished relative to the WT protein (Fig. 2B). Furthermore, B3UCR-MF/AA was more sensitive to trypsin digestion than WT B3UCR (Fig. 2C, top).

In an attempt to identify mutations that disrupted ubiquitin binding but not the structural integrity of cIAP1 UBA domain, we next mutated the LL motif (Leu434 and Leu435) on H9251 (Fig. 1, B and D). The CD spectra of B3UCR proteins bearing L435A (L/A) or M402A/L435A (ML/AA) mutations were comparable (Fig. 2B). However, the mutants had a decreased CD signal at ~195 nm, and the proteolytic sensitivity of B3UCR-ML/AA was increased compared with the WT protein (Fig. 2B and C, bottom). This suggested that mutation of either the MGF or the LL motif in the UBA domain of multidomain cIAP1 proteins had a destabilizing effect.

**Mutation of the MGF Motif Perturbs the Structure of UBA Domains**—To further probe the effects of mutating the MGF and LL motifs, the isolated UBA domain from cIAP1 (blue, PDB code 3T6P), XIAP (green, PDB code 2KNA), Dsk2p (magenta, PDB code 1WR1), and EDE1 (yellow, PDB code 2G3Q), highlighting the relative orientations of α1–α3. B, multiple sequence alignment of representative UBA domains highlighting the conserved MGF and LL motifs (red). Elements of secondary structures are indicated below the alignment. C, the solution structure of Dsk2p UBA domain (magenta) in complex with ubiquitin (gray) (PDB code 1WR1). Residues from the Dsk2p UBA domain that interact with ubiquitin are shown as yellow sticks. Ile46-centered hydrophobic patch residues on ubiquitin are shown as blue sticks. The positions of α1–α3 of the UBA domain are indicated. D, ribbon diagram of the cIAP1 UBA domain (PDB code 3T6P) (15) showing residues mutated in this study: M402A/F404A (MF/AA), L435A (L/A), M402A/L435A (ML/AA), E401R (E/R), N428R (N/R), and E401R/428R (EN/RR).

Substitution of the MGF and LL motifs with alanine has previously been used to investigate the function of several UBA domains (19, 20, 23, 27, 28), and destabilization of the UBA domain of cIAP1 by these mutations was unexpected. To examine the possibility that the UBA domain of cIAP1 was less stable than other UBA domains, we characterized the N-terminal UBA domain (UBA1) of human HHR23A and the UBA domain from *S. cerevisiae* EDE1 (Fig. 1B). For both UBAs, we purified the WT UBA domain as well as the MGF motif mutants: HHR23A UBA1-MY/AA (bearing M173A/Y175A mutations) and EDE1 UBA-MF/AA (bearing M1351A/F1353A mutations). As observed for the cIAP1 UBA domain, alanine substitution of residues in the MGF motif resulted in loss of α-helical content and the early elution of the mutant proteins (Fig. 3, B and C). Thus, mutation of the MGF motif in UBA1 of HHR23A and the UBA of EDE1 destabilized their fold.

Our results show that mutating residues in the conserved UBA domain motifs destabilized the UBA domain from cIAP1. In addition, the structures of the UBA domains from EDE1 and HHR23A were compromised by similar mutations. We suggest that caution should be exercised when using MGF or LL mutants to investigate UBA domain function because, although these mutants no longer bind ubiquitin, the structure of the protein is altered, and this may have unexpected consequences.

**Introduction of Two Arginines Impairs Ubiquitin Binding but Does Not Disrupt the UBA Domain Fold**—To identify mutations that abrogated ubiquitin binding but did not disrupt the
ubiquitin, we used isothermal titration calorimetry (ITC) and the UBA domain. EN/RR mutation had not perturbed the structural integrity of trypsin (Fig. 4, equivalent, and the mutant was also resistant to proteolysis by EN/RR forms of the multidomain B3UCR proteins appeared equivalent, and the mutant was also resistant to proteolysis by EN/RR (Fig. 4, N428R, respectively). A double mutant containing both E401R and N428R was also generated (EN/RR). Because monoubiquitylation of cIAP1 appeared to be delayed by disruption of ubiquitin binding to the UBA domain, we performed assays that utilized ubiquitin in which all lysine residues have been mutated to arginine (K0) (Fig. 6, A (right) and B). In these assays, which precluded chain formation, monoubiquitylation of WT B3UCR was again more efficient relative to the EN/RR proteins. This suggested that ubiquitin binding by the UBA domain enhances monoubiquitylation of B3UCR (Fig. 6A, right).
Because the unfolded B3UCR-MF/AA mutant was also available to us, we compared its activity with that of B3UCR-EN/RR. Remarkably, in contrast to EN/RR, which had diminished activity, the MF/AA mutant was more extensively modified than WT B3UCR. This is apparent in both multiple-turnover assays, where more high molecular weight species were formed (Fig. 6C, left), and in the K0 ubiquitin assays, where striking multimonoubiquitylation of B3UCR-MF/AA occurred (Fig. 6C, right).
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right). The increased autoubiquitylation of the MF/AA mutant is clearly apparent when the intensity of bands across the gel lane containing the 5 min time point sample is compared for WT, EN/RR, and MF/AA (Fig. 6D). The B3UCR-ML/AA mutant was comparable with MF/AA (data not shown), suggesting that unfolding of the UBA domain increases protein flexibility, allowing lysine residues to more readily access the active site of RING domain-bound E2~Ub conjugate.

These results illustrate the importance of investigating the stability of mutant forms of proteins before drawing conclusions about their function. Importantly, in the case of cIAP1, our results indicate that when ubiquitin binding is disrupted, but the structure of the UBA domain is preserved, monoubiquitylation of cIAP1 is impaired.

**The Isolated UBA Domain of cIAP1 Does Not Promote Discharge**—Several previous studies have shown that proteins harboring UBDs can undergo “coupled monoubiquitylation” (32, 33). One way this can be achieved is through direct recruitment of E2~Ub conjugates by UBDs (7). However, in the case of the cIAP UBA domain, this seems unlikely because when we included the purified GST-UBA fusion protein in assays, no ubiquitylated products were detected (Fig. 7A). To assess the extent of non-RING-mediated ubiquitylation, we also purified a cIAP1 fragment that only comprised the UBA-CARD domains of cIAP1 (Fig. 2A) and evaluated its autoubiquitylation (Fig. 7B). The MF/AA mutant was included in this assay because it was possible that the increased activity of this mutant was due to exposure of Lys residues that were modified in a RING-independent manner. However, in our assays, no ubiquitylated products were detected for either protein (Fig. 7B).

Furthermore, we showed that the purified UBA domain did not promote hydrolysis of the oxyester UbcH5b-C85S~Ub conjugate (Fig. 7C), in contrast to RING domain-containing proteins (18) (see also Fig. 9A). We concluded that the UBA domain of cIAP1 facilitates RING-dependent ubiquitin transfer.

**Substrate Ubiquitylation and Autoubiquitylation Are Promoted by Binding of Ubiquitin to the UBA Domain**—Having established that the UBA domain enhanced autoubiquitylation of the cIAP1 B3UCR protein, we next sought to determine whether the activity of longer proteins and the related protein cIAP2, as well as ubiquitylation of substrate proteins were similarly affected. Because the full-length proteins were not well behaved, we utilized cIAP proteins that lacked just the BIR1 domain, referred here to as B2B3UCR (Fig. 2A). For cIAP2, we characterized WT and the equivalent UBA mutant in which Glu387 and Asn414 were replaced with Arg (EN/RR). For substrate ubiquitylation, we utilized SMAC that was expressed with a C-terminal His tag.

First, we characterized the stability of the WT and EN/RR B2B3UCR forms of cIAP1 and showed that they were comparable with B3UCR (data not shown). Autoubiquitylation was then assayed, and, as expected, modification of the parent band was significantly delayed for the UBA mutant, irrespective of whether WT or K0 ubiquitin were included (Fig. 8A). Likewise, for cIAP2, monoubiquitylation of the EN/RR protein was impeded, and disappearance of the parent band was delayed relative to WT UBA B2B3UCR (Fig. 8B). These results suggested that ubiquitin binding to the UBA domain of cIAP1 and cIAP2 had a similar function.
We next purified SMAC and evaluated whether ubiquitin binding to the UBA domain also enhanced ubiquitylation of substrate proteins. For both cIAP1 and cIAP2, our experiments showed that, compared with the WT protein, monoubiquitylation of SMAC was delayed for the EN/RR mutant (Fig. 8, C and D). Taken together, these results show that ubiquitin binding to the UBA domain enhances ubiquitin transfer by both cIAP1 and cIAP2 and that substrate and autoubiquitylation are both increased by ubiquitin binding.

**Single-turnover Assays Also Highlight the Importance of the UBA Domain**—To further evaluate the role of the UBA domain, we undertook a series of single-turnover assays that allowed a single round of discharge from the E2 to be measured. First, we established conditions that allowed UbcH5b to be charged with K0 and WT ubiquitin (Fig. 9A). As noted previously, charging of the E2 was always slightly delayed for K0 ubiquitin (33, 34). Therefore, for subsequent experiments, we first charged the E2 for 15 min before apyrase was added. Following a further 5-min incubation, to ensure any remaining ATP was degraded, either wild type or EN/RR cIAP1 B2B3UCR protein was added, and autoubiquitylation of cIAP1 was monitored (Fig. 9B).

In these single-turnover assays that included limited E2/Ub conjugate, the extent of cIAP autoubiquitylation was significantly reduced (Fig. 9B), yet the pattern was very comparable with the multiple-turnover assays. Notably, monoubiquitylation of the EN/RR mutant was reduced compared with WT, irrespective of whether WT or K0 ubiquitin was included (Fig. 9B). However, as observed in the multiple-turnover assays, transfer of K0 ubiquitin was slow compared with that of WT ubiquitin. The reasons for this difference remain uncertain but may relate to the binding properties of K0 ubiquitin to enzymes in the ubiquitylation cascades (33, 34).

The activity of WT and EN/RR B2B3UCR was also assessed using discharge assays, where hydrolysis of the oxyester-linked...
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**FIGURE 6.** The structure and ubiquitin binding properties of the UBA domain influence cIAP1 autoubiquitylation. *In vitro* assays comparing autoubiquitylation of WT and EN/RR cIAP1 B3UCR proteins (A) or WT and MF/AA proteins (C). Purified cIAP1 B3UCR proteins were incubated with Uba1 (E1), UbcH5b, Mg^{2+}-ATP, and either WT ubiquitin (left) or Lys-less (K0) ubiquitin (right) for the indicated times. Reactions were stopped by adding reducing SDS-PAGE sample buffer and were resolved. Coomassie Blue staining was used to visualize the reaction products. Reactions containing no E1 were included as negative controls.

**FIGURE 7.** The UBA domain alone is not sufficient to promote ubiquitin transfer or E2–Ub discharge. A, purified GST-fused cIAP1 UBA domain was incubated with UbcH5b, Mg^{2+}-ATP, and WT ubiquitin at 37 °C for 1 h in the absence (−) or presence (+) of an E1 enzyme. Reactions were resolved by SDS-PAGE and visualized by staining with Coomassie Blue. B, purified untagged WT and MF/AA cIAP1 UBA-CARD fragments were incubated with assay component as in A. C, purified cIAP1 UBA domain was added in molar excess to UbcH5b-C85S–Ub conjugate for up to 120 min.
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FIGURE 8. Ubiquitin binding by the UBA domain promotes autoubiquitylation of cIAP B2B3UCR proteins and SMAC ubiquitylation. **A**, in vitro assays comparing autoubiquitylation of WT and EN/RR cIAP1 B2B3UCR proteins. The assays were carried out in the same manner as in Fig. 6. Reactions were resolved by SDS-PAGE and visualized by staining with Coomassie Blue. **B**, autoubiquitylation assays comparing WT and EN/RR cIAP2 B2B3UCR proteins in the presence of WT or K0 ubiquitin. Reaction products were visualized with immunoblotting using anti-cIAP2 antibody. **C**, ubiquitylation of SMAC by WT or EN/RR cIAP1 B2B3UCR proteins was monitored over time by immunoblotting using anti-SMAC antibody (left). The intensity of monoubiquitylated SMAC (Ub-SMAC) was quantified and normalized against the intensity of SMAC parent band at t = 0 min. The mean average intensity values ± S.D. (error bars) from duplicate measurements were plotted against time (right). **D**, ubiquitylation of SMAC by WT or EN/RR cIAP2 B2B3UCR proteins. SMAC was probed using anti-His antibody. The intensity of Ub-SMAC was quantified as in C from triplicate measurements.
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E2−Ub conjugate is measured (16). As was observed before, the activity of the EN/RR mutant was impaired (Fig. 9C). Together, these experiments confirm the findings from the multiple-turnover assays and indicate that binding of ubiquitin to the UBA domain of cIAP1 promotes ubiquitylation.

Disruption of Ubiquitin Binding by the UBA Does Not Alter RING Dimerization—Ubiquitin transfer by cIAP1 is regulated by RING dimerization because the autoinhibited monomeric form of cIAP1 does not efficiently bind to the E2−Ub conjugate (15, 18). We therefore investigated whether differences in dimer stability accounted for the diminished monoubiquitylation of B3UCR-EN/RR.

Consistent with stabilization of the dimer, autoubiquitylation of both B3UCR proteins was increased when Compound A was
added (Fig. 10B, compare with Fig. 6A). However, even when fully dimeric, ubiquitylation of B3UCR-EN/RR was delayed relative to the WT protein, and a larger proportion of unmodified B3UCR-EN/RR remained at each time point (Fig. 10, B and C). This difference is evident irrespective of whether WT or K0 ubiquitin was included in the assays. Together, these results indicate that differences in the oligomeric state of WT and EN/RR cIAP1 B3UCR proteins are unlikely to account for the differences in the activity of these two proteins. Instead, this finding suggests that ubiquitin binding to the UBA domain promotes ubiquitin transfer.

The UBA Domain of cIAP Proteins Facilitates Ubiquitylation

Mediated SMAC ubiquitylation in the presence of a range of UbcH5b concentrations (0–5 μM) was limited. In contrast, ubiquitylation of SMAC by the WT form of cIAP2 was less sensitive to changes in the E2 concentration (Fig. 11B). Together, these results suggest that recruitment of UbcH5b–Ub conjugate was impaired in the EN/RR mutant protein.
For ubiquitin transfer to occur, the E2 of the E2–Ub conjugate must bind to a conserved face on the RING domain, and in most E3 ligases, this is thought to be the primary site of E2 recruitment (35–37). However, our data suggested that the UBA domain might also recruit conjugate, and we assessed the ability of GST-fused B3UCR proteins to bind the relatively stable oxyester UbH5b–Ub conjugate. Using pull-down assays, we observed diminished binding of the conjugate to B3UCR-EN/RR. Interestingly, mutation of RING domain residues that are required for E2 binding (V573A/R606A; VR/AA) did not completely abolish UbH5b–Ub conjugate binding. However, when coupled to the EN/RR mutations, we could not detect significant conjugate binding to cIAP1 B3UCR. This is consistent with our prediction that the UBA domain can assist in recruiting E2–Ub conjugate (Fig. 12A).

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Next, the ability of the isolated UBA domains to bind E2 and E2–Ub was assessed. As expected, UbH5b–Ub conjugate was pulled down by the isolated UBA domain (Fig. 12B, top), but the E2 alone was not (Fig. 12B, bottom). Importantly, the interaction was markedly reduced when GST-UBA-EN/RR was used as the bait. These results show that the cIAP1 UBA domain can bind to the ubiquitin moiety of E2–Ub conjugates.

The strength of the interaction between cIAP1 UBA and E2–Ub conjugate was measured using SPR. The $K_d$ for the interaction between immobilized GST-UBA and UbH5b–Ub was about 80 μM (Fig. 12C), about 2.5 times weaker than observed for ubiquitin alone (Table 1). Many E2–Ub conjugates adopt both open and closed conformations (38). In the closed conformation of the UbH5b–Ub conjugate, the Ile44-centered interface of ubiquitin required for binding to the UBA domain would be masked (35), and this may explain the diminished affinity of the conjugate compared with free ubiquitin.

**DISCUSSION**

Interaction of UBDs with ubiquitin is required to decipher ubiquitin modifications and allow different outcomes to be signaled. A role for UBDs in the attachment of ubiquitin has also started to emerge, and here we show that the UBA domain modifies the E3 ligase activity of cIAP1 and cIAP2. We observed direct binding of UbH5b–Ub conjugate to the UBA domain, and this interaction enhances monoubiquitylation (Figs. 8–12). This discovery depended on a detailed analysis of UBA domain mutants because mutation of the signature MGF and LL motifs of UBA domains, motifs that are routinely mutated to disrupt ubiquitin binding, caused the UBA domain of cIAP1 to unfold.

A number of studies have established the role of residues in the MGF and LL motifs in mediating ubiquitin binding by the UBA domain (23, 27, 28), but few of these studies include a detailed analysis of the structural stability of the mutant UBA domains. It is interesting to note that mutation of Met1041 in the MGF motif of the p62 UBA domain decreased the thermal stability of this protein (39). Furthermore, mutation of the MGF motif in the SOUBA (solenoid of overlapping UBA) domain of UBAP1 (ubiquitin-associated protein 1) resulted in a loss of structure (40). In addition to the destabilizing effect of mutations in the UBA domain of cIAP1, we also show that mutation of the MGF motif in the UBA domains from HHR23A and EDE1 compromised the structural integrity of these domains (Fig. 3, B and C). Apart from the MGF and LL motifs, the UBA domains from cIAP1, HHR23A, and EDE1 are not well conserved (Fig. 1B). Therefore, we suggest that the conserved motifs might have a common role in stabilizing the fold of other UBA domains as well as contributing to ubiquitin binding. It is also possible that for some UBA domains, dimerization, as in the case for Dsk2p and XIAP (23, 27, 41), or interactions with neighboring domains, such as the interaction between the C-terminal UBA domain and the core ubiquitin-conjugating domain of the E2 enzyme E2–25K (42), could compensate for the destabilizing effects of MGF motif mutations.

Compared with WT or EN/RR cIAP1, the unfolded MF/AA mutant displayed remarkable differences in autoubiquitylation activity. Notably, where the formation of polyubiquitin chains is precluded, cIAP1 with an unfolded UBA domain was multi-

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**FIGURE 11.** The EN/RR mutation impairs ubiquitylation at low Ubch5b–Ub concentrations. A, autoubiquitylation assays of WT and EN/RR cIAP1 B3UCR proteins with an increasing concentration of Ubch5b (indicated in μM below the gel). E1, Ubch5b, and Mg2+-ATP were incubated for 1 h at 37 °C with either WT (top) or K0 (bottom) ubiquitin prior to the addition of cIAP1 proteins and further incubation for 1 h. α-ATP were incubated for 1 h at 37 °C with either WT or EN/RR cIAP2 B2B3UCR proteins with different Ubch5b concentrations. Unmodified SMAC and ubiquitylated SMAC species were detected using anti-His antibody (top).
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monoubiquitylated, whereas the equivalent WT protein was mostly monoubiquitylated. Unfolding of the UBA domain is likely to increase the availability of lysine residues and increase protein flexibility, as well as destabilizing the autoinhibited form of cIAP1. These changes could all enhance monoubiquitylation. In addition to increased monoubiquitylation, the unfolded mutant forms of cIAP1 gave rise to extensive high molecular weight smears and not just the very high molecular weight products observed for cIAP proteins with a folded UBA domain (Fig. 6, C and D). Because the protein is modified by ubiquitin at more sites, the extensive but lower molecular weight smear may be due to the formation of shorter ubiquitin chains on a number of lysine residues of cIAP1. The formation of shorter ubiquitin chains on full-length cIAP1 MF/AA could account for the reduced interaction between MF/AA cIAP1 mutant and the proteasome reported previously (19).

Several ubiquitin-binding proteins, including those that do not harbor any apparent canonical E3 catalytic domains, have been shown to promote their own ubiquitylation or regulate the E3 ligase activity of other proteins (7, 9, 33). Here we show that E2−Ub conjugate binds the UBA domain of cIAP1, but this interaction does not directly activate ubiquitin transfer or promote RING domain dimerization. Instead, E2−Ub binding by the UBA domain promotes RING-dependent monoubiquitylation of cIAP proteins and SMAC. At the simplest level, this suggests that the UBA domain promotes interaction of the E2−Ub conjugate with the RING domain.

The role of RING domain dimerization in promoting avid interactions between the UBA domain and Lys63-linked polyubiquitin chain has been suggested (43). However, the effect of chain binding on E3 ligase activity has not been demonstrated until recently. Conceptually, a polyubiquitin chain could serve as a recruitment platform for a UBD-containing E3 ligase. This may increase the local concentration of the E3 and promote its activation by RING dimerization. Notably, recent analyses of RNF4 demonstrated that binding to poly-SUMO chains via the SUMO interaction motifs promoted RING dimerization and enhanced autoubiquitylation (5, 44). Our data did not explicitly demonstrate that polyubiquitin chains activate cIAP1. However, in our assays, considerably more unmodified cIAP1 remained at the latest time point for the EN/RR protein (Fig. 6A). This observation is consistent with the UBA domain serving to enhance the activity of cIAP1 by binding to ubiquitin chains. Further studies are required to determine whether this mode of activation occurs in cIAPs. However, given that a number of E3 ligases harbor UBDs, it is likely that polyubiquitin chains may regulate activity for at least a subset of them.

Ubiquitin-binding modules are often employed by E3 ligases to promote polyubiquitin chain elongation (10−12, 45, 46). However, disruption of ubiquitin binding by the cIAP UBA domain did not impede polyubiquitylation in our assays, and in some cases, the high molecular weight products were increased. This may be because a number of different interactions can promote the formation of polyubiquitin chains. For example, non-covalent interaction between ubiquitin and the backbone of some E2s enhances chain formation (47), and polyubiquitylation of cIAP2 is reduced when this interaction is disrupted (48). It is therefore possible that once cIAP1 is monoubiquitylated, the ubiquitin moiety attached to cIAP1 could recruit E2−Ub by binding to the backside of the E2. Such an interaction may mean that once cIAP1 is monoubiquitylated, the UBA is less important for recruitment of E2−Ub conjugate. Interactions of this type, coupled with decreased monoubiquitylation, may mean that formation of high molecular weight chains can be favored in some circumstances. A similar model has been proposed for the activation of MDM2 (mouse double minute 2) by polyubiquitin chains (49). However, further studies will be required to dissect the precise role of such interactions.

Because ubiquitin-mediated processes, including ubiquitin transfer, appear to depend on multiple weak interactions, E3 ligases employ different strategies for recruiting E2−Ub conjugate to enhance the overall rate of ubiquitin transfer or modu-
late the pattern of ubiquitylation. For example, dynamic electrostatic interactions between the E2 enzyme Cdc34 and Cullin-RING ligases promote efficient substrate ubiquitylation (50). Several RING E3 ligases also possess separate E2-binding modules that enhance ubiquitin transfer. In addition to having RING and CUE domains, the ERAD E3 ligase gp78 has a Ube2G2-binding region, which binds to the backside of its cognate E2 enzyme, Ube2G2, and increases ubiquitin transfer (51). Similarly, the Rad6-binding domain of Rad18 binds the backside of the E2 enzyme Rad6 to promote substrate monoubiquitylation (52). In other cases, the E2-binding module is found in accessory proteins that cooperate with E3 ligases. Notably, the Pex4p-binding protein, Pex22p (53), and the Ubc7p-binding region of the CUE domain-containing protein Cue1p (9, 54) possess E2-binding modules. The HECT E3 ligase E6AP also possesses two E2~Ub conjugate binding sites (55). Clearly, interactions between E2~Ub conjugates and E3 ligases are dynamic and extend beyond the canonical catalytic domains.

Our data suggest that recruitment of the E2~Ub conjugate by the UBA domain of cIAP1 may serve a similar purpose and that the UBA domain adds to the range of auxiliary E2- and ubiquitin-binding modules that have been adopted by E3 ligases to enhance the rate or modulate the outcome of ubiquitylation. In the case of the cIAPs, recruitment of the E2~Ub conjugate by the UBA domain may be of particular importance because it appeared to most dramatically influence attachment of the first ubiquitin molecule. It is this step that is often rate-limiting in the ubiquitylation cascade, and it is often tightly regulated, involves dedicated E2 enzymes, and, when disrupted, can have profound effects on cellular processes (56–59). Further studies will be required to evaluate the contribution of the UBA domain in a cellular setting.

Together, this study highlights the importance of detailed biochemical studies in discovering subtle regulatory mechanisms that depend on relatively weak interactions. In a cellular setting, where disruption of domain function can alter multiple facets of a protein, such as localization as well as substrate recruitment, the underlying function of an individual domain can be overlooked. However, for proteins such as IAPs that are involved in widespread cell proliferation and migration, cell cycle regulation, and cell apoptosis, more detailed understanding of protein function is critical.

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