Structural Basis for UBA-mediated Dimerization of c-Cbl Ubiquitin Ligase

Received for publication, April 20, 2007, and in revised form, June 22, 2007 Published, JBC Papers in Press, July 16, 2007, DOI 10.1074/jbc.M703333200

Guennadi Kozlov‡, Pascal Peschard‡§1,2, Brandon Zimmerman‡, Tong Lin‡, Tudor Moldoveanu‡, Nura Mansur-Azzam‡, Kalle Gehring‡§3, and Morag Park‡§‡‡1,2

From the Departments of ‡Biochemistry, §Medicine, and ‡Oncology, McGill University, ‡Molecular Oncology Group, McGill University Health Center, Montréal, Québec H3G 1Y6, Canada

Ligand-induced down-regulation by the ubiquitin-protein ligases, c-Cbl and Cbl-b, controls signaling downstream from many receptor-tyrosine kinases (RTKs). Cbl proteins bind to phosphotyrosine residues on activated RTKs to affect ligand-dependent ubiquitylation of these receptors targeting them for degradation in the lysosome. Both c-Cbl and Cbl-b contain a ubiquitin-associated (UBA) domain, which is important for Cbl dimerization and tyrosine phosphorylation; however, the mechanism of UBA-mediated dimerization and its requirement for Cbl biological activity is unclear. Here, we report the crystal structure of the UBA domain of c-Cbl refined to 2.1-Å resolution. The structure reveals the protein is a symmetric dimer tightly packed along a large hydrophobic surface formed by helices 2 and 3. NMR chemical shift mapping reveals heterodimerization can occur with the related Cbl-b UBA domain via the same surface employed for homodimerization. Disruption of c-Cbl dimerization by site-directed mutagenesis impairs c-Cbl phosphorylation following activation of the Met/hepatocyte growth factor RTK and c-Cbl-dependent ubiquitination of Met. This provides direct evidence for a role of Cbl dimerization in terminating signaling following activation of RTKs.

Cbl proteins are a small class of E3 ubiquitin ligases represented by three mammalian members: c-Cbl, Cbl-b, and Cbl-3. Unlike Cbl-3, the c-Cbl and Cbl-b genes are ubiquitously expressed with the highest levels of expression in hematopoietic tissues. c-Cbl and Cbl-b are important regulators of many receptor-tyrosine kinases (RTKs), including the hepatocyte growth factor (HGF) receptor Met. Cbl proteins are recruited to many activated RTKs and promote their ubiquitination and lysosomal degradation (1). The domain architecture for the Cbl family contains a highly conserved NH2-terminal half that includes a tyrosine kinase-binding domain and a RING finger domain, which recruits the ubiquitin-conjugating enzyme and is required for Cbl-mediated down-regulation of RTKs. The COOH-terminal half of Cbl proteins is more variable. c-Cbl and Cbl-b contain multiple protein interaction domains in addition to a COOH-terminal ubiquitin-associated (UBA) domain (1). An intact UBA domain is required for efficient tyrosine phosphorylation of c-Cbl and Cbl-b proteins downstream from the EGF and insulin RTKs (2, 3). Hence, the Cbl UBA domain may play a role in the recruitment of Cbl proteins to these RTKs. This domain was originally thought to be a leucine zipper dimerization motif (2), which contains leucine/isoleucine residues spaced every seventh amino acid residue along one side of an α-helix. Several approaches, including yeast two-hybrid, far Western, and in vitro binding assays, have shown that the putative leucine zipper motif can mediate homodimerization of c-Cbl (2). Furthermore, the same motif could also mediate homodimerization of Cbl-b and heterodimerization of c-Cbl/Cbl-b (3). However, from bioinformatics analyses, the putative leucine zipper in c-Cbl and Cbl-b has the sequence pattern of a UBA domain (4).

The UBA domain is an ~40-amino acid residue motif originally identified in a variety of proteins involved in ubiquitination processes (4). UBA domains are protein-protein interaction modules that utilize hydrophobic surfaces to recognize their binding partners. The three-dimensional structures of a number of UBA domains have been determined using NMR and x-ray crystallography (5–14). Despite low sequence homology, their three-dimensional structures are very similar, consisting of a bundle of three α-helices. The majority of UBA domains studied bind to ubiquitin or ubiquitin-like domains

*This work was supported in part by operating Grants MOP-11545 (to M. P.) and MOP-14219 (to K. G.) from the Canadian Institutes of Health Research. This work is based upon research conducted at the Cornell High Energy Synchrotron Source (CHESS), which is supported by the National Science Foundation under award DMR 0225180, using the Macromolecular Diffractometer at the CHESS (MacCHESS) facility, which is supported by Grant RR-01646 from the National Institutes of Health, through its National Center for Research Resources. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ The online version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Fig. S1.

The atomic coordinates and structure factors (code 2O09) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

1 These authors contributed equally to this work.

2 Recipient of a Terry Fox research studentship from the National Cancer Institute of Canada.

3 Chercheur National of the Fonds de recherche en santé du Québec. To whom correspondence may be addressed: Biochemistry Dept., Faculty of Medicine, McGill University, 3655 Promenade Sir William Osler, Montréal, Québec H3G 1Y6, Canada. Tel.: 514-398-7287; Fax: 514-398-7384; E-mail: kalle.gehring@mcgill.ca.

4 Senior scholar of the Canadian Institutes of Health Research. To whom correspondence may be addressed: 687 Pine Ave. West, Montréal, Québec H3A 1A1, Canada. Tel.: 514-843-1479; Fax: 514-843-1478; E-mail: morag.park@mcgill.ca.

5 The abbreviations used are: RTK, receptor-tyrosine kinase; UBA, ubiquitin-associated; HGF, hepatocyte growth factor; EGF, epidermal growth factor; FKBP, FK506-binding protein; HA, hemagglutinin; HEK, human embryonic kidney.
using a hydrophobic patch of residues in the α1–α2 loop and helix α3 (10). Besides c-Cbl UBA, other UBA and UBA-like domains have been implicated in homodimerization (15–17) and heterodimerization (15).

The molecular mechanisms involved in dimerization of Cbl UBA domains are poorly understood. Here, we report the crystal structure of the human c-Cbl UBA domain. In combination with NMR, site-directed mutagenesis, and biochemical studies, it shows that Cbl UBA domains have a conserved hydrophobic surface, which is utilized for their homo- and heterodimerization. To address the function of the UBA domains in Cbl proteins, we show that an intact UBA domain is required for the tyrosine phosphorylation of Cbl proteins and ubiquitination of the Met receptor.

**EXPERIMENTAL PROCEDURES**

**Generation of DNA Constructs**—The UBA domain from c-Cbl (residues 856–895) was cloned into the pGEX-4T-1 vector (Amersham Biosciences) and expressed in *Escherichia coli* BL21(DE3) in rich (LB) medium as a fusion protein with an amino-terminal glutathione S-transferase tag. Human c-Cbl was tagged with either an HA or a FLAG epitope at their amino terminus and subcloned in the pMX139 mammalian expression vector. The c-CblΔUBA construct was generated by adding a stop codon at Pro855 in c-Cbl, using the QuikChange™ site-directed mutagenesis kit (Stratagene). Site-directed mutagenesis was performed using the same kit and confirmed by DNA sequencing. c-Cbl-FKBP was constructed by subcloning c-CblΔUBA into the pC4Fv1E vector from the ARGENT Regulated Homodimerization kit (www.ariad.com/regulation-kits, ARIAD Pharmaceuticals, Cambridge, MA) to generate FKBP fused to the COOH terminus of c-CblΔUBA.

**DNA Transfections, Immunoprecipitations, and Western Blotting**—Transient transfections in HeLa and HEK 293 cells were performed using Lipofectamine Plus reagent according to the manufacturer’s instructions (Invitrogen). HEK 293 cells were harvested 24 h post-transfection. For HGF stimulation of HEK 293 cells, they were serum-starved for 24 h. They were then stimulated with 1.5 nM HGF for the indicated amount of time. HGF was a generous gift from George Vande Woude (Van Andel Research Institute, Grand Rapids, MI). To induce the dimerization of the Cbl-FKBP fusion proteins, cells were treated with 100 nM AP20187 (ARIAD Pharmaceuticals) 30 min prior to HGF stimulation. To examine in vivo ubiquitination of Met, cells were lysed in RIPA lysis buffer (0.05% SDS, 50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.05% sodium deoxycholate) with inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 10 mg/ml aprotinin, and 10 mg/ml leupeptin). Otherwise, cells were lysed in TGH lysis buffer with inhibitors (50 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 1% Triton X-100, 10% glycerol). Immunoprecipitations were carried out at 4 °C for 2 h with gentle rotation with the indicated antibody. Proteins collected on either protein A– or G-Sepharose (Amersham Biosciences) were washed three times in their respective lysis buffers, resolved by SDS-PAGE, and transferred to a nitrocellulose membrane. Membranes were blocked in 3% bovine serum albumin in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 2.5 mM EDTA, 0.1% Tween 20) for 1 h and incubated with primary and secondary antibodies in TBST for 2 and 1 h, respectively. After four washes with TBST, bound proteins were visualized with an ECL detection kit (Amersham Biosciences). The immunoblots were quantified using ImageJ 1.36b (Wayne Rasband, National Institutes of Health).

**Protein Expression, Preparation, and Purification**—For production of a selenomethionine-labeled protein, the c-Cbl UBA expression plasmid was transformed into the E. coli methionine auxotroph strain DL4177(DE3) (18). Selenomethionine-labeled protein was produced using LeMaster medium (18). For NMR experiments, the recombinant fragments were labeled by growth of *E. coli* BL21 in M9 minimal medium with 15N-ammonium sulfate or 15N-ammonium sulfate/13C-glucose as the sole sources of nitrogen and carbon. Cells were harvested and broken in phosphate-buffered saline, pH 7.8. The glutathione S-transferase fusion proteins were purified by affinity chromatography on glutathione-Sepharose resin and tags were removed by cleavage with thrombin, leaving Gly-Ser NH₂-terminal and Ala-Ala-Ala-Ser COOH-terminal extensions for the c-Cbl UBA domain. The cleaved protein was additionally purified using C18 reverse phase chromatography. Selenomethionine-labeled protein was purified in a similar manner.

**Crystallization**—Initial crystallization conditions were identified using hanging drop vapor diffusion using sparse matrix screens (Hampton Research, Aliso Viejo, CA). Crystals of c-Cbl UBA were obtained by equilibrating a 0.6-μl drop of a protein (15 mg/ml) in 20 mM ammonium phosphate, mixed with 0.6 μl of reservoir solution containing 3.8 mM sodium formate, 0.1 M Tris, pH 8.5, and 4% (v/v) glycerol and suspended over 1 ml of reservoir solution. Crystals grew in 2–4 days at 20 °C. For data collection, crystals were picked up in a nylon loop and flash cooled in a N₂ cold stream (Oxford Cryosystem). Crystals belong to a primitive tetragonal system, space group P4₁2₁2₁ with unit cell dimensions a = b = 82.0, c = 56.2 Å. The crystals contain three molecules in the asymmetric unit (Z = 24) corresponding to Vₘ = 3.11 Å³ Da⁻¹ and a solvent content of 60.4%.

**Structure Solution and Refinement**—Diffraction data from a selenomethionine-labeled crystal of c-Cbl UBA domain were collected using a three-wavelength MAD regime on a Quantum-4 CCD detector (Area Detector Systems Corp.) at beamline F2 at the Cornell High-Energy Synchrotron Source (Table 1). Data processing and scaling were performed with HKL2000 (19). The structure was determined by SAD phasing using the program SOLVE/RESOLVE (20). Data to 2.1-Å resolution at the maximum peak wavelength (λ = 0.9790) were used to locate selenium atoms in the asymmetric unit. The asymmetric unit contains three UBA molecules. Density modification with the program ARP/wARP (21) allowed for automated model building of almost complete chains A and B. The model obtained from ARP/wARP was extended manually using program Xfit (22) and was improved by several cycles of refinement, using the program REFMAC (23). The refined chain A was used in molecular replacement program PHASER (24) to locate chain C, followed by several cycles of refinement and refitting for this chain using Xfit (22). In addition, 73 water molecules were included in the model.
Structure and Function of c-Cbl UBA Domain

The final model was subjected to translation-libration-screw refinement (25). Of 46 residues in the c-Cbl UBA construct, the final model does not include residues Glu856—Leu857 for chain C in addition to the very NH2- and COOH-terminal residues from the linkers for chains B and C. The final model has an Rwork factor of 0.216 and Rfree of 0.260 for all data to 2.1-Å resolution (Table 1) and has excellent stereochemistry computed using PROCHECK (26). Structure figures were made with PyMOL (pymol.sourceforge.net/).

NMR Spectroscopy—NMR resonance assignments of the UBA domain from c-Cbl were carried out using 15N-labeled and 13C,15N-labeled protein samples. Protein signal assignments were performed using standard techniques, including three-dimensional experiments HNCACB, CBCA(CO)NH, and 15N-nuclear Overhauser spectroscopy heteronuclear multiple quantum correlation. NMR samples contained 0.5–1.0 mM protein in 50 mM phosphate, 100 mM NaCl, pH 6.8. NMR sample preparation and signal assignments for the Cbl-b UBA domain are described elsewhere (27). For heterodimerization NMR titrations, unlabeled UBA domain from Cbl-b or c-Cbl was added to the 15N-labeled 0.25–0.5 mM c-Cbl or Cbl-b UBA, respectively, to the final molar ratio of (6 + 7) to 1. All NMR experiments were performed at 303 K using Varian 500 and 800 MHz spectrometers and a Bruker 600 MHz spectrometer. NMR self-diffusion experiments were acquired at 300 K. NMR spectra were processed using NMRPipe (28) and analyzed with XEASY (29).

RESULTS

UBA Domain Is Required for c-Cbl Dimerization—Despite their high sequence identity (Fig. 1), the UBA domains from c-Cbl and Cbl-b display remarkably different ubiquitin-binding abilities. Whereas the UBA domain of Cbl-b binds ubiquitin chains with high affinity, the UBA domain of c-Cbl shows no binding (30, 31). The UBA domain of c-Cbl and Cbl-b has been implicated in Cbl homodimerization and heterodimerization but nothing is known about their dimerization interface (2, 3). To confirm that the UBA domain is required for the homodimerization of c-Cbl, we performed co-immunoprecipitation experiments with HA and FLAG-tagged c-Cbl protein. Following transient expression, FLAG-c-Cbl efficiently coimmunoprecipitates with HA-c-Cbl, consistent with its homodimerization (Fig. 2A). In agreement with previous results (3), deletion of the c-Cbl UBA domain abrogated the ability of c-Cbl to homodimerize, whereas deletion of a proline-rich region in the carboxyl-terminal region does not affect dimerization (Fig. 2A). To establish the ability of the c-Cbl UBA domain to dimerize in solution we performed NMR self-diffusion measurements (Fig. 2B and supplemental Table S1). The UBA domain showed an apparent size of ~10 kDa, consistent with the molecular mass of a dimer.

The Structural Basis for Dimerization of the c-Cbl UBA Domain—To better understand at the molecular level how the UBA domain mediates the dimerization of c-Cbl, we determined the three-dimensional structure of the UBA domain of c-Cbl at a resolution of 2.1 Å using SAD phasing (Table 1). The crystals in symmetry space group P41212 contain three molecules in the asymmetric unit. The chains were traced from Glu856—Ser858 in chain C to Phe895. All three chains are nearly identical, as their backbone could be overlaid with root mean square deviations between 0.29 and 0.45 Å. Despite the odd number of molecules in the asymmetric unit, the crystal packing

TABLE 1

| Data collection and refinement statistics | c-Cbl UBA domain |
|----------------------------------------|----------------|
| Cell dimensions                        | P41212         |
| a, b, c (Å)                            | 82.03, 82.03, 56.19 |
| Resolution (Å)                        | 58.0–2.10 (2.18–2.10)* |
| Rmerge (%)                            | 0.061 (0.313) |
| l/d                                   | 18.2 (4.9) |
| Completeness (%)                      | 99.3 (95.9) |
| Redundancy                            | 7.3 (5.7) |
| Refinement                             |                |
| Resolution (Å)                        | 19.60–2.10     |
| No. reflections                       | 11010          |
| Rmerge/Rfree                          | 0.216/0.260    |
| No. atoms                             | 1092           |
| Protein                               | 1019           |
| Water                                 | 73             |
| B-factors                             |                |
| Protein                               | 32.18          |
| Water                                 | 47.58          |
| Root mean square deviations           |                |
| Bond lengths (Å)                      | 0.023          |
| Bond angles (°)                       | 1.63           |
| Ramachandran statistics (%)           |                |
| Most favored regions                  | 93.4           |
| Additional allowed regions            | 6.6            |

*Highest resolution shell is shown in parentheses.

FIGURE 1. Alignment of Cbl-b and c-Cbl sequences from various species. The clustering of the two most divergent sequences, from fruit fly and sea squirt, reflects their greater similarity to ubiquitin-binding Cbl-b sequences or to non-binding c-Cbl sequences. Below the sequences, the curve of the alignment (identity) score shows an alternating pattern of conserved, hydrophobic residues in the NH2-terminal half of the domain and overall greater identity in the COOH-terminal half. Sequences were clustered and the figure prepared with Clustal X. Position of helices, referring to the c-Cbl UBA structure, is shown above the alignment. Hydrophobic residues involved in c-Cbl UBA homodimerization are marked with asterisks.
Structure and Function of c-Cbl UBA Domain

A

B

FIGURE 2. Dimerization of the c-Cbl UBA domain. A, the UBA domain of c-Cbl is required for its homodimerization. HEK 293 cells were co-transfected with FLAG-tagged c-Cbl wild-type (wt) and HA-tagged c-Cbl WT or mutants (c-CblΔUBA, deletion of prolines 542 to 548, c-CblΔLys472–645). FLAG-tagged c-Cbl was immunoprecipitated (IP) and resolved by SDS-PAGE. The presence of HA-c-Cbl proteins in the immunoprecipitate was detected by Western blotting (IB). B, c-Cbl UBA forms a dimer in solution. Pulsed-field gradient (PFG) NMR self-diffusion experiments for the UBA domain from c-Cbl (UBA-wt), its I880E/I891E mutant (UBA-I880E/I891E), chicken lysozyme (lys), and bovine ubiquitin (Ub) at 300 K. Dependence of the signal intensity ($I_0$) on diffusion coefficient ($D$) results in slopes that are equal to diffusion coefficients. The gradient strength ($G$) results in slopes that are equal to diffusion coefficients. The gradient strength was calibrated by the PFG experiment for lysozyme ($D = 1.34 \times 10^{-6} \text{ at 303 K}$) (40). All samples contained 1 mM protein in 50 mM sodium phosphate buffer, pH 6.8, 100 mM NaCl.

clearly displayed dimeric species. Whereas chains A and B within the asymmetric unit formed a tightly packed dimer, chain C makes a similar dimer with its counterpart from the adjacent asymmetric unit across the crystallographic axis. As in the case with single chains, overlay of both dimers results in a low root mean square deviation value of 0.93 Å.

Each chain has a typical UBA fold consisting of a three-helical bundle ($\alpha_1$–$\alpha_3$) (Fig. 3A). The domain has a well defined hydrophobic core that includes Leu$^{857}$, Ile$^{861}$, Ile$^{874}$, Leu$^{875}$, Ile$^{880}$, and Leu$^{892}$. Noticeably, Tyr$^{869}$ from the $\alpha_1$–$\alpha_2$ loop is a part of the core stacking against Leu$^{892}$ (Fig. 3B). Most of the remaining hydrophobic residues are located on the $\alpha_2$–$\alpha_3$ surface and participate in dimer formation. Two pro-tomers form a symmetric dimer through hydrophobic contacts of Ile$^{880}$, Met$^{887}$, Ile$^{891}$, and Phe$^{895}$ (Fig. 3C). Whereas both helices, $\alpha_2$ and $\alpha_3$, make close intermolecular contacts in the dimer, helix 3 is particularly important as it provides three of four hydrophobic residues for dimerization. Interestingly, the side chain of Met$^{887}$ from one protomer contacts the same residue from the second protomer (Fig. 3C). Intermolecular salt bridges between Lys$^{857}$ and Glu$^{894}$ further stabilize the dimer (Fig. 3D). Apart from Met$^{887}$, the residues forming contacts in the c-Cbl UBA homodimer are conserved in Cbl-b (Fig. 1). Overall, the dimerization surface observed in the crystal structure corresponds to the most conserved regions of Cbl UBA domains (helix $\alpha_3$ and the loop between helices $\alpha_2$ and $\alpha_3$) (Fig. 1). The recently released solution structure of the UBA domain from mouse c-Cbl (Protein Data Bank code 2D9S) is largely similar to our crystal structure, confirming that the dimerization interface observed in the crystal does not result from crystal packing.

c-Cbl UBA Employs the Same Surface to Homo- and Heterodimerize in Solution.—To study heterodimerization between c-Cbl and Cbl-b UBA domains we used NMR titration of $^{15}$N-labeled c-Cbl UBA with its unlabeled counterpart. The heteronuclear single quantum correlation spectrum of the $^{15}$N-labeled c-Cbl UBA domain showed a good dispersion of signals characteristic of well folded domains (Fig. 4A). These were assigned using standard heteronuclear techniques. Heterodimerization experiments resulted in a slow-exchange mode as signals for both c-Cbl UBA homodimers and c-Cbl/ Cbl-b heterodimers were present during the NMR titration (Fig. 4B). The c-Cbl UBA domain showed specific spectral changes, identifying the regions affected by heterodimerization (Fig. 4, C and D). These regions coincide with the homodimerization surface of the c-Cbl UBA. Based on the slow-exchange mode in heterodimerization experiments (Fig. 4B), we estimate the high limit of $K_d$ for c-Cbl UBA homodimerization to be in a low micromolar range.

Likewise, titration of the $^{15}$N-labeled Cbl-b UBA domain with unlabeled c-Cbl UBA also resulted in similar chemical shift changes. When mapped to the Cbl-b UBA structure (PDB ID code 2OOA), the same $\alpha_2$–$\alpha_3$ surface of Cbl-b UBA was affected (Fig. 4, E and F). This shows that the surface employed for homodimerization in c-Cbl UBA is also used for heterodimerization by both c-Cbl and Cbl-b UBA domains.

Because NMR is very sensitive to even low-affinity interactions, and because the Cbl-b UBA domain, but not c-Cbl UBA domain, has been shown to bind to ubiquitin in vitro (30), we probed binding between the c-Cbl UBA domain and ubiquitin using NMR titrations. Even a high (6:1) ratio of unlabeled ubiquitin to $^{15}$N-labeled c-Cbl UBA failed to produce detectable chemical shift changes in the UBA heteronuclear single quantum correlation spectrum (data not shown), confirming the lack of binding between these molecules.

Disruption of the Dimerization Interface of UBA Impairs Cbl Functions.—Recruitment of c-Cbl and Cbl-b proteins to the Met receptor promotes its ubiquitination and is required for efficient receptor degradation and suppression of its transforming activity (32, 33). To test the biological role of UBA domains in Cbl proteins, we performed functional studies (Fig. 5). When transiently co-expressed with wild-type c-Cbl, the Met receptor becomes ubiquitinated (Fig. 5B). In contrast, a mutant of c-Cbl lacking the UBA domain (c-CblΔUBA) is unable to induce efficient ubiquitination of Met (Fig. 5B), demonstrating a requirement for the UBA domain in this process. An intact UBA
domain was previously shown to be required for tyrosine phosphorylation of Cbl downstream of the EGF and insulin RTKs (2, 3). Hence, the CblΔUBA proteins may be inefficient at inducing Met receptor ubiquitination as a result of decreased recruitment to Met. Because tyrosine phosphorylation of Cbl by RTKs is considered to reflect the recruitment of Cbl, we examined tyrosine phosphorylation of c-CblΔUBA in response to activation of the Met receptor by its ligand, HGF. Whereas wild-type c-Cbl is efficiently tyrosine phosphorylated at 2 and 10 min following stimulation, c-CblΔUBA is not, supporting a role for an intact UBA domain for Cbl recruitment to the Met receptor (Fig. 5C).

To confirm the physiological relevance of the c-Cbl dimerization mechanism, and its requirement for tyrosine phosphorylation of c-Cbl, and ubiquitination of the Met RTK, we substituted Ile880 and Ile891 for glutamic acid residues. The mutant was structured as verified by the one-dimensional NMR spectrum (supplemental materials Fig. 1). According to the c-Cbl UBA crystal structure, these substitutions should disrupt the dimerization interface of the UBA homodimer. In agreement with this, NMR self-diffusion measurements showed that this mutant has an apparent smaller size than the wild-type domain (Fig. 2B). Following transient transfections, the HA-c-Cbl I880E/I891E mutant does not coimmunoprecipitate with wild-type c-Cbl (Fig. 2B). Moreover, the c-Cbl I880E/I891E mutant was also unable to induce ubiquitination of the Met receptor to the same extent as wild-type Cbl proteins and promoted ubiquitination of Met to levels similar to that of the c-CblΔUBA domain protein (Fig. 5B). These data further support the importance of UBA-mediated dimerization of Cbl proteins to function as ubiquitin ligases. To formally establish that dimerization of c-Cbl is required for its tyrosine phosphorylation downstream of the Met RTK, we fused a modified FKBP (FK506-binding protein) dimerization domain to the carboxyl terminus of c-CblΔUBA. HeLa cells transiently expressing the c-CblΔUBA-FKBP fusion protein revealed that it was tyrosine phosphorylated following HGF stimulation in the presence, but not in the absence, of the synthetic dimerizer, AP20187 (Fig. 5D). Hence, replacing the UBA domain with a dimerization domain rescues the ability of c-Cbl to be recruited to the Met receptor. Overall, the data presented demonstrate that the UBA domain of c-Cbl acts as a dimerization domain.

DISCUSSION

Here we present the high-resolution crystal structure of the c-Cbl UBA domain and provide detailed characterization of its homo- and heterodimerization properties with the Cbl-b UBA domain. We show that an intact c-Cbl UBA domain and its dimerization interface are required for efficient recruitment of c-Cbl and for ubiquitination of a c-Cbl substrate, the Met RTK.

This work provides structural support for previous studies showing that the UBA domain, then called a leucine zipper, was required for the coimmunoprecipitation and proposed homo- and heterodimerization of Cbl proteins (2, 3). Deletion of the UBA domain decreased c-Cbl recruitment to the EGFR and decreased phosphorylation of c-Cbl in response to EGF (2). Similarly, an intact UBA domain is required for phosphorylation of c-Cbl downstream from the insulin receptor (3). As observed downstream of the EGF and insulin receptors, the UBA domain in c-Cbl is also required for efficient phosphorylation of c-Cbl proteins downstream from the Met receptor-tyrosine kinase (Fig. 5C). In direct support of a requirement for dimerization for robust tyrosine phosphorylation of c-Cbl downstream from these RTKs, the addition of a regulated dimerization domain to a c-CblΔUBA protein promotes efficient tyrosine phosphorylation of c-Cbl downstream from the Met RTK only in the presence of the dimerizing agent, AP20187 (Fig. 5D). Importantly, an intact c-Cbl UBA domain, and dimerization of c-Cbl mediated by the UBA domain, is required for ubiquitination of the c-Cbl

FIGURE 3. Structure of the UBA domain from c-Cbl. A, ribbon representation of the UBA dimer. One protomer is shown in magenta, another in yellow. Helices are labeled as α1–α3 in one protomer and α1’–α3’ in another protomer. B, hydrophobic core of the c-Cbl UBA domain. Respective residues are shown as sticks and labeled. C, the enlarged view of the hydrophobic dimer interface. Key residues in the dimer are shown as sticks and labeled. D, intermolecular hydrogen bonds (dotted line) involving Lys876 and Glu894 from both c-Cbl UBA protomers.
substrate, the Met RTK (Fig. 5B). Hence this provides direct evidence for a role for c-Cbl dimerization for efficient recruitment and biological activity of c-Cbl.

The UBA domains from several other proteins, Dsk2, Rad23, and Cbl-b have been shown to dimerize (3, 15, 17). The latter two were also shown to heterodimerize with UBA domains.
Structure and Function of c-Cbl UBA Domain

from Ddi1 and c-Cbl, respectively (3, 15). However, no structural details are published for their dimerization surface. The c-Cbl UBA domain dimer reveals a novel dimerization interface with a roughly antiparallel arrangement of the helix 2 of one protomer with the helix 3 of the other protomer (Fig. 6A). Helix 3 is crucial for dimerization as it provides three of four hydrophobic residues important for the dimerization interface (Fig. 3). Notably, sequence identity is highest in the helix 3 regions between the related c-Cbl and Cbl-b UBA domains (Fig. 1). Not surprisingly, helix 3 is also responsible for dimerization of Cbl-b UBA (Fig. 6B) (27). In contrast to the c-Cbl UBA domain, in the Cbl-b UBA dimer, the helices 3 of both protomers stack almost parallel to each other, whereas second helices provide a much smaller contribution to the dimerization interface. This leads to significant differences in the buried surface for the Cbl-b and c-Cbl UBA dimers, which are 491 and 1050 Å², respectively. In agreement with the lower buried surface, Cbl-b UBA dimerizes weakly in solution with Kd of ~230 μM (27), whereas c-Cbl shows a stronger dimerization with Kd likely to be in low micromolar to high nanomolar range. In vitro, dimerization of the Cbl-b UBA domain is promoted by polyubiquitin binding (27), demonstrating that dimerization is a general property of Cbl UBA domains.

The dimerization domain of doublesex (Dsx) protein displays a UBA-like fold, although this is not a UBA domain (16). The dimerization domain of Dsx (16) also uses helices 2 and 3 to dimerize, however, the dimerization interface of Dsx is arranged differently from that of the c-Cbl UBA domain (Fig. 6C). Whereas helix 3 contributes predominantly to dimerization by the Cbl UBA domains, helix 2 contributes predominantly to the dimerization of the Dsx protein, as it fits into the groove formed by helices 2 and 3 of the other protomer. As a result, helices 2 of both doublesex protomers stack against each other at about a 30° angle making extensive contacts with each other as well as with helix 3 from the opposing protomer.
protomer. The longer COOH-terminal helix in the Dsx dimerization domain makes this dimerization mode difficult to apply to a canonical UBA domain, such as Dsk2 and Rad23. Hence from these differences, more structural information is needed for better understanding of dimerization of other UBA domains.

Our results provide a molecular characterization for the previous observation that c-Cbl and Cbl-b heterodimerize (3). The c-Cbl and Cbl-b UBA domains employ the same surface to homo- and heterodimerize. One important implication of our data is that heterodimerization is able to compete with homodimerization for these UBA domains, at least in vitro. Thus, heterodimers of c-Cbl and Cbl-b could be recruited to RTKs. Moreover, c-Cbl could be recruited in a ubiquitin-dependent manner through its ability to heterodimerize with Cbl-b. Because c-Cbl and Cbl-b also act as scaffold proteins and have the capacity to interact with different downstream signaling proteins (34), this would provide a mechanism to expand the repertoire of Cbl-dependent signals recruited to RTKs, and would be dependent on the relative levels of c-Cbl and Cbl-b present in cells. Also, c-Cbl and Cbl-b UBA domains could potentially form heterodimers with other UBA domains from Cbl-interacting proteins. For example, the central UBA domain from the DNA repair enzyme Rad23 mediates its heterodimerization with the DNA damage-induced protein Ddl1 (15).

Upon activation, RTKs cluster either as dimers or oligomers and this process is required for structural changes leading to full activation of the kinase and transphosphorylation of the receptor, providing binding sites for downstream signaling molecules (35). Cbl proteins are recruited to several activated RTKs, including EGFR and Met, via the Grb2 adaptor protein (36). From the crystal structure of the Grb2 SH2 domain together with a phosphopeptide corresponding to its binding site on the Met receptor, the Grb2 SH2 domain forms swapped dimers and may interact with these RTKs as a dimer (37). Moreover, in the case of insulin RTK, an intact UBA domain in c-Cbl is required for its association with the adaptor protein APS, which binds to the insulin RTK as a dimer (38). Hence the requirement for c-Cbl proteins to have the capacity to form dimers for efficient recruitment to and tyrosine phosphorylation by the EGF, insulin, and Met RTKs, supports a general model, whereby Cbl proteins must dimerize for efficient recruitment to RTKs via adaptor proteins, Grb2 or APS, which themselves are recruited to RTKs as dimers. Interestingly, a truncated form of c-Cbl lacking its UBA domain is present in a T-cell lymphoma cell line (39) and may contribute to the transformed phenotype through a decreased recruitment to and capacity to promote down-modulation of c-Cbl substrates, providing further evidence for an in vivo requirement for UBA-mediated dimerization of c-Cbl.

Acknowledgments—We thank I. A. Mirza and A. M. Berghuis for help in structure determination and L. Gabrielli for technical assistance. We acknowledge S. Lipkowitz for valuable discussions.

REFERENCES

1. Thien, C. B., and Langdon, W. Y. (2005) *Biochem. J.* **391**, 153–166

2. Bartkiewicz, M., Houghton, A., and Baron, R. (1999) *J. Biol. Chem.* **274**, 30887–30895

3. Liu, J., DeYoung, S. M., Hwang, J. B., O’Leary, E. E., and Saltiel, A. R. (2003) *J. Biol. Chem.* **278**, 36754–36762

4. Hofmann, K., and Bucher, P. (1996) *Trends Biochem. Sci.* **21**, 172–173

5. Chang, Y. G., Song, A. X., Gao, Y. G., Shi, Y. H., Lin, X. J., Cao, X. T., Lin, D. H., and Hu, H. Y. (2006) *Protein Sci.* **15**, 1248–1259

6. Chim, N., Gall, W. E., Xiao, J., Harris, M. P., Graham, T. R., and Krezel, A. M. (2004) *Proteins* **54**, 784–793

7. Ciani, B., Layfield, R., Cavey, J. R., Sheppard, P. W., and Searle, M. S. (2003) *J. Biol. Chem.* **278**, 37409–37412

8. Dieckmann, T., Withers-Ward, E. S., Jarosinski, M. A., Liu, C. F., Shen, I. S., and Feigon, J. (1998) *Nat. Struct. Biol.* **5**, 1042–1047

9. Lowe, E. D., Hasan, N., Trempe, J. F., Fonso, L., Noble, M. E., Endicott, J. A., Johnson, L. N., and Brown, N. R. (2006) *Acta Crystallogr. Sect. D Biol. Crystallogr.* **62**, 177–188

10. Mueller, T. D., and Feigon, J. (2002) *J. Mol. Biol.* **319**, 1243–1255

11. Panneerselvam, S., Marx, A., Mandelkow, E. M., and Mandelkow, E. (2006) *Structure* **14**, 173–183

12. Trempe, J. F., Brown, N. R., Lowe, E. D., Gordon, C., Campbell, I. D., Noble, M. E., and Endicott, J. A. (2005) *EMBO J.* **24**, 3178–3189

13. Withers-Ward, E. S., Mueller, T. D., Chen, I. S., and Feigon, J. (2000) *Biochemistry* **39**, 14103–14112

14. Yuan, X., Simpson, P., McKeown, C., Kondo, H., Uchiyama, K., Wallis, R., Drevenyi, I., Keetch, C., Zhang, X., Robinson, C., Freemont, P., and Matthews, S. (2004) *EMBO J.* **23**, 1463–1473

15. Bertolaet, B. L., Clarke, D. J., Wolff, M., Watson, M. H., Henze, M., Divita, G., and Reed, S. I. (2001) *J. Mol. Biol.* **313**, 955–963

16. Bayerer, J. R., Zhang, W., and Weiss, M. A. (2005) *J. Biol. Chem.* **280**, 32989–32996

17. Sasaki, T., Funakoshi, M., Endicott, J. A., and Kobayashi, H. (2005) *Biochem. Biophys. Res. Commun.* **336**, 530–535

18. Hendrickson, W. A., Horton, J. R., and LeMaster, D. M. (1990) *EMBO J.* **9**, 1665–1672
19. Otwinowski, Z., and Minor, W. (1997) Methods Enzymol. 276, 307–326
20. Terwilliger, T. C. (2000) Acta Crystallogr. Sect. D Biol. Crystallogr. 56, 965–972
21. Perrakis, A., Sixma, T. K., Wilson, K. S., and Lamzin, V. S. (1997) Acta Crystallogr. Sect. D Biol. Crystallogr. 53, 448–455
22. McRee, D. E. (1999) J. Struct. Biol. 125, 156–165
23. Murshudov, G. N., Vagin, A. A., Lebedev, A., Wilson, K. S., and Dodson, E. J. (1999) Acta Crystallogr. Sect. D Biol. Crystallogr. 55, 247–255
24. Read, R. J. (2001) Acta Crystallogr. Sect. D Biol. Crystallogr. 57, 1373–1382
25. Winn, M. D., Murshudov, G. N., and Papiz, M. Z. (2003) Methods Enzymol. 374, 300–321
26. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 283–291
27. Peschard, P., Kozlov, G., Lin, T., Mirza, I. A., Berghuis, A. M., Lipkowitz, S., Park, M., and Gehring, K. (2007) Mol. Cell 27, 474–485
28. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) J. Biomol. NMR 6, 277–293
29. Bartels, C., Xia, T. H., Billeter, M., Guntert, P., and Wuthrich, K. (1995) J. Biomol. NMR 6, 1–10
30. Davies, G. C., Ettenberg, S. A., Coats, A. O., Mussante, M., Ravichandran, S., Collins, J., Nau, M. M., and Lipkowitz, S. (2004) Oncogene 23, 7104–7115
31. Raasi, S., Varadan, R., Fushman, D., and Pickart, C. M. (2005) Nat. Struct. Mol. Biol. 12, 708–714
32. Peschard, P., Fournier, T. M., Lamorte, L., Naujokas, M. A., Band, H., Langdon, W. Y., and Park, M. (2001) Mol. Cell 8, 995–1004
33. Peschard, P., Ishiyama, N., Lin, T., Lipkowitz, S., and Park, M. (2004) J. Biol. Chem. 279, 29565–29571
34. Dikic, I., Szymkiewicz, I., and Soubeyran, P. (2003) Cell Mol. Life Sci. 60, 1805–1827
35. Hubbard, S. R. (2002) Front. Biosci. 7, d330–340
36. Peschard, P., and Park, M. (2007) Oncogene 26, 1276–1285
37. Schiering, N., Casale, E., Caccia, P., Giordano, P., and Battistini, C. (2000) Biochemistry 39, 13376–13382
38. Hu, J., Liu, J., Ghirlando, R., Saltiel, A. R., and Hubbard, S. R. (2003) Mol. Cell 12, 1379–1389
39. Blak, T. T., and Langdon, W. Y. (1992) Oncogene 7, 757–762
40. Ekiel, I., Abrahamson, M., Fulton, D. B., Lindahl, P., Storer, A. C., Lavaloux, W., Lafrance, M., Labelle, S., Pomerleau, Y., Groleau, D., LeSauteur, L., and Gehring, K. (1997) J. Mol. Biol. 271, 266–277