Identification of a Novel Proline-Arginine Motif Involved in CIN85-dependent Clustering of Cbl and Down-regulation of Epidermal Growth Factor Receptors*

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CIN85 is a multidomain adaptor protein implicated in Cbl-mediated down-regulation of receptor tyrosine kinases. CIN85 binding to Cbl is increased after growth factor stimulation and is critical for targeting receptor tyrosine kinases to clathrin-mediated endocytosis. Here we report the identification of a novel polyproline-arginine motif (PXXXPR), specifically recognized by the SH3 domains of CIN85 and its homologue CMS/CD2AP. This motif was indispensable for CIN85 binding to Cbl/Cbl-b, to other CIN85 SH3 domains’ effectors, and for mediating an intramolecular interaction between the SH3-A domain and the proline-rich region of CIN85. Individual SH3 domains of CIN85 bound to PXXXPR peptides of Cbl/Cbl-b with micromolar affinities, whereas an extended structure of two or three SH3 domains bound with higher stoichiometry and increased affinity to the same peptides. This enabled full size CIN85 to simultaneously interact with multiple Cbl molecules, promoting their clustering in mammalian cells. The ability of CIN85 to cluster Cbl was important for ligand-induced stabilization of CIN85-Cbl-epidermal growth factor receptor complexes, as well as for epidermal growth factor receptor degradation in the lysosome. Thus, specific interactions of CIN85 SH3 domains with the PXXXPR motif in Cbl play multiple roles in down-regulation of receptor tyrosine kinases.

Growth factor binding to receptor tyrosine kinases (RTKs) promotes receptor autophosphorylation, association of intracellular signaling proteins with receptors, and phosphorylation of multiple substrates (1). Subsequent changes in receptor substrates, including modulations of enzymatic activities and modifications of proteins and lipids, lead to the assembly of signaling networks that ultimately control cellular responses including cell proliferation, migration, or differentiation (2). Activated RTKs are also rapidly relocated from the cell surface into the endosomal compartment, from where they can be recycled back to the plasma membrane or alternatively sorted to the lysosome for degradation. The processes of receptor internalization and endocytosis are regulated via a network of protein-protein and protein-lipid interactions, as well as protein post-translational modifications, such as phosphorylation or ubiquitination (3, 4). Several RTKs, including epidermal growth factor (EGF) receptors, are ubiquitinated and down-regulated upon interactions with the Cbl family of ubiquitin ligases (5, 6). Cbl binding to EGF receptors occurs at the plasma membrane (7, 8), and following receptor internalization they remain associated throughout the endocytic compartment (8, 9). Recent data (10) have indicated that attachment of a single ubiquitin, rather than polyubiquitin chains, to EGF and platelet-derived growth factor receptors is sufficient for receptor endocytosis and that multiple monoubiquitination events ensure proper receptor sorting and subsequent degradation in the lysosome.

Cbl can also promote receptor endocytosis via a pathway that is functionally separable from its ubiquitin ligase activity and is dependent on Cbl interactions with the adaptor protein CIN85 (11, 12). CIN85, also known as Ruk and SETA, is a broadly expressed protein containing three SH3 domains, a proline-rich region, and a coiled-coil domain (13–15). The multidomain structure of CIN85 and its homologue CMS/CD2AP enables them to associate with various proteins including Cbl, Grb2, p85 subunit of phosphatidylinositol 3-kinase, CD2 receptors, SETA-binding protein 1 (SB-1), SLPI-65/BLNK, Alg2-interacting protein 1 (AIP1), and p130Cas (16). These interactions promote formation of CIN85-linked protein networks that are implicated in the control of RTK signaling, actin reorganization, T cell functions, kidney architecture, and apoptotic signals (16). CIN85 binds to Cbl via its SH3 domains, and their association is enhanced by growth factor-induced tyrosine phosphorylation of Cbl (15, 17), whereas the proline-rich region of CIN85 constitutively interacts with endophilins (11, 12), regulatory components of clathrin-coated pits (18). Based on these features, CIN85 can rapidly recruit endophilins to complexes with activated receptors, thus controlling receptor inter-
nalization (11, 12). Importantly, an analogous mechanism has been shown recently (19) for CMS/CD2AP. Interestingly, the CMS/CD2AP proline-rich region also interacts with cortactin in a ligand-dependent manner, recruiting it to the CD2AP-Cbl/EGFR complex. These data open an intriguing possibility that CIN85/CD2AP adapter proteins link receptor endocytosis with cytoskeletal rearrangements (19). CIN85 associates with two members of the Cbl family, Cbl and Cbl-b, but not Cbl-3, and this interaction depends on the presence of a minimal binding domain in their distal carboxyl-terminal tails (17). Surprisingly, binding of SH3 domains of CIN85 or CMS to Cbl/Cbl-b is not mediated via a PXXP motif (17, 20), known to be critical for ligand recognition by the majority of SH3 domains (21).

Proline-rich sequences are among the most abundant motifs found in signaling proteins (22). Numerous protein interaction modules including the SH3 domain, the WW domain, the GYF domain have been shown to bind to distinct proline-based sequences found in various signaling proteins (21–26). The classical SH3 domain ligands adopt an extended conformation known as the polyproline-II (PPII) helix (22) and bind to SH3 domains in either of two orientations, depending on the position of a positively charged residue in the peptide sequence (23). The ligand binding surface of SH3 domains contains three shallow grooves defined by conserved aromatic residues. Two prolines in the PXXP motif occupy the hydrophobic pockets, whereas the third binding pocket associates with a positively charged residue flanking the PXXP motif (21). In addition, several reports have indicated that unconventional proline-based sequences, which are distinct from PXXP motifs, can functionally interact with various SH3 domains (27–30).

Here we identified a novel atypical polproline motif present in the distal tail of Cbl/Cbl-b, which serves as a high affinity binding site for the SH3 domains of CIN85/CMS adaptor molecules. We also show that CIN85, via its three SH3 domains, clusters Cbl molecules, which is a critical step for oligomerization of CIN85-Cbl/EGFR complexes leading to internalization and efficient degradation of activated EGFR proteins. CIN85 can thus function as an RTK scaffold molecule, like G protein-coupled receptor specific β-arrestins, controlling receptor endocytosis and degradation.

MATERIALS AND METHODS

Products, Antibodies, and Expression Vectors—EGF was purchased from Intergen; antibodies recognizing Cbl-b (C-20), phosphotyrosine (PY99), autophosphorylated EGFR receptor (phosphosine 1173, anti-pEGFR), or extracellular signal-regulated kinase 2 (C14) were from Santa Cruz Biotechnology, Inc., mouse anti-β (92C6) was from Roche Applied Science, mouse anti-FLAG M2 and M5 antibodies were from Sigma, and rabbit anti-GFP antibodies were from Molecular Probes. Anti-Cbl (RF), anti-CIN85 (CT), and anti-EGFR receptor (RK2) antibodies were used as described previously (17, 31). Constructs of Cbl-b, FLAG-CMS, FLAG-CIN85, FLAG-CIN85–SH3, FLAG-CIN85–PCX, GST fusion proteins encoding the SH3 domains of CIN85, and FLAG-tagged ubiquitin were described previously (12, 17, 31). FLAG-CIN85–3A construct encodes amino acids 78 to 661 of CIN85, and FLAG-CIN85–3AB encodes amino acids 211 to 661. Expression vectors encoding for Ruk SH3-A domain in pGEX vector was provided by Vladimir Buchman, HA-SLP-65 in pGK vector was provided by Juerger Wienands, and EGFP-Cbl was provided by Nancy Lill.

Site-directed Mutagenesis—All mutant constructs were generated by PCR using QuiChange (Stratagene). The following tryptophan residues in pcDNA-FLAG-CIN85 were mutated to alanine: SH3-A*-W36A, SH3-B*-W135A, SH3-C*-W306A, SH3-AB*-W36A, W135A, SH3-BC*-W135A, W306A, and SH3-ABC*-W36A, W135A, W306A. The arginine residues were mutated to alanines in the following proteins: Cbl-b-R829A, Cbl-b-R911A, SL65-R248A, R313A, CIN85-R40A, Cbl-b-P906A, P906A, and P910A constructs have respective proline residues mutated to alanines. The constructs were verified by sequencing. The sequences of the oligonucleotides used are available upon request.

Cell Culture and Transfections—HEK293T and CHO-EGFR cells were used as described previously (12, 17, 31). Cells were transfected with Lipofectamine reagent (Invitrogen) following the manufacturer’s instructions. 30 h after transfection the cells were starved for an additional 12 h and stimulated with 100 ng/ml EGF for indicated times. Cells were lysed in ice-cold 1% Triton X-100 lysis buffer (pH 7.4, 50 mM Hepes, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol) containing a mixture of protease and phosphatase inhibitors. The lysates were cleared by centrifugation at 13000 rpm for 20 min at 4 °C.

Peptide Synthesis and Peptide Binding Assays—All peptides were synthesized following the Fmoc (N-[9-fluorenyl]methoxycarbonyl) strategy using Fmoc amide resin resulting in carboxy-terminally amidated peptides. Peptides were purified by reverse phase high pressure liquid chromatography using a C18 column. Matrix-assisted laser desorption ionization time-of-flight-mass spectrometry analysis confirmed the correct molecular weight for each peptide. Each peptide had a single amine-terminal cysteine residue that was used for coupling to a SulfoLink resin (following the product instruction; Pierce). In peptide binding assays, 5 nmol of each SulfoLink-immobilized peptide was incubated with cell lysates for 2 h, and after washing three times in the lysis buffer the protein complex was resolved by SDS-PAGE. In peptide competition experiments, the lysates were incubated with increasing amounts of peptides 1 h before and during immunoprecipitation.

Isothermal Calorimetry (ITC) Measurements—ITC experiments were conducted on a VP ITC (Microcal Inc., Northampton, MA) as described previously (32, 33). Experiments were performed at 25 °C in 10−4 M phosphate-buffered saline at pH 7.4. The data were analyzed using the ORIGIN software supplied with the calorimeter. In a typical titration ~1.5 ml of 25 μM SH3 domain was placed in the calorimeter cell, and 250 μM peptide was injected in 25 × 10−6 M injections. The reported data for the stoichiometry and Kd are based on at least two repetitions of the titrations. The actual measurement data for these experiments are available upon request.

Ligand Internalization Assays—Ligand internalization assays were performed as described previously (12). Briefly, cell monolayers of transfected CHO cells were incubated for 1 h at 4 °C with 125I-labeled EGF, washed twice with binding buffers, and then incubated at 37 °C in serum-free medium. Cells were then incubated with either cold phosphate-buffered saline or mild acidic buffer to remove surface-bound radiolabeled EGF. The remaining radioactivity in cells was quantified by a γ-counter following cell lysis. Each point was measured in quadruplicate and expressed as a percentage (average ± S.D.) of internalized versus total cell-associated radioligand EGF. The amounts of transfected proteins were monitored by Western blotting (data not shown).

Biochemical Assays—Lysates with adjusted protein concentration (Bradford assay; Bio-Rad) were incubated with antibody for 2 h at 4 °C. Immune complexes were precipitated following 1-h incubation with protein A-agarose beads. After washing in cold lysis buffer, the complexes were resuspended in Laemmli sample buffer (Bio-Rad), boiled, and resolved by SDS-PAGE. For GST binding assays, GST fusion proteins were adsorbed on glutathione-Sepharose beads, incubated with the lysates for 2 h at 4 °C, washed in the lysis buffer, and proceeded for Western blotting as above. In vitro translation was performed with the TNT transcription/translation system (Promega), according to the manufacturer’s instructions. EGFR degradation assays were performed as described previously (31). Briefly, HEK293T were transfected with EGFR, Cbl, FLAG-ubiquitin, and CIN85 or indicated CIN85 mutants, serum-starved, and stimulated with EGF (100 ng/ml) for indicated times. The cell lysates were subjected to immunoblotting with anti-EGFR (RK2) and anti-extracellular signal-regulated kinase 2 antisera. The levels of EGF receptor (ERK) were quantified for each experiment from three independent experiments by using the NIH Image program (1.62) and expressed as the percentage of remaining EGFR for every time point as compared with unstimulated cells in the same experiment.

RESULTS

Identification of the Consensus Binding Motif for the SH3 Domains of CIN85/CMS—To identify the binding site responsible for selective recognition of Cbl proteins by CIN85/CMS molecules, we synthesized all proline-rich peptides found in the distal tail of Cbl-b (Fig. 1A) and tested their binding to CIN85 (Fig. 1B). Peptide 893 bound strongly to CIN85, peptide 968 bound much less, and the other Cbl-b-derived polproline peptides failed to interact with CIN85 (Fig. 1B). This is consistent with the fact that peptide 893 is located in the minimal binding
domain of Cbl-b (residues 891–927), which is critical for interactions with CIN85 (17). Furthermore, addition of increasing amounts of peptide 893, but not peptide 768, to the cell lysates, efficiently competed against co-precipitation between Cbl-b and CIN85 (Fig. 1C). The amino acid sequence of peptide 893 of Cbl-b (PARPPKPRPR) is similar to peptide 814 of Cbl (PERP-PKPFPR) (Fig. 1A). Consistently, peptide 814 of Cbl also bound very potently to CIN85 in peptide binding assays and competed effectively against Cbl/CIN85 binding in co-precipitation assays (Fig. 1, B and D). Close inspection of these sequences revealed a double PXXP motif followed by a charged arginine residue. However, a similar double PXXP motif found in peptide 768 of Cbl-b (PLPPARP) was not involved in binding to CIN85 (Fig. 1, A and B). To identify amino acids critical for CIN85 recognition, we synthesized peptides with proline and arginine residues in the PXXP motif substituted to alanines (Fig. 2A). The peptide containing mutations of three prolines (3A peptide) involved in the formation of double PXXP motifs (AXXAPXAXPR), bound as strongly as wild type 814 peptide, whereas mutation of two additional prolines in 5A peptide (AXXAAAAAXPR) completely blocked binding to CIN85 (Fig. 2B, left panel). More detailed analysis indicated that mutation of proline 906 in Cbl-b peptide (PXXPAXPXPR) significantly reduced its ability to associate with CIN85, and the
Fig. 2. Identification of the consensus binding site for the SH3 domains of CIN85. Different mutants of peptide 814 from Cbl and peptide 893, 968, or 500 from Cbl-b are listed (A) and were used for pull down assays with lysates of HEK293T cells transfected with CIN85 (B) or CMS (C). After recovery of the bound material, detection was performed by immunoblotting with anti-CIN85 or anti-FLAG (CMS) antibodies. D, HEK-293T cells were transiently transfected with CIN85 or CMS together with Cbl or Cbl-b or their mutants Cbl-R829A or Cbl-b-R911A. Lysates were subjected to immunoprecipitation (IP) with anti-Cbl or anti-Cbl-b antibodies and immunoblotting (IB) with anti-FLAG, anti-CIN85, or anti-Cbl/Cbl-b antibodies. TCL, total cell lysate. E, HEK293T cells were transiently transfected with CIN85 and HA-Cbl-b or indicated Cbl-b proline mutants. Lysates were subjected to immunoprecipitation (IP) with anti-HA antibodies and immunoblotting (IB) with anti-CIN85 or anti-FLAG antibodies. F, sequences from several CIN85-binding proteins. The consensus PXXPR motifs are emphasized in bold, and amino acid positions of arginines in each motif in SLP-65 sequence are indicated. G, HEK293T cells were transiently transfected with CIN85 and HA-SLP-65 or HA-SLP-65 arginine mutants (R248A, R313A, and R248,313A). Lysates were subjected to immunoprecipitation (IP) with anti-HA or anti-CIN85 antibodies and immunoblotting (IB) with indicated antibodies.

Because peptide analysis indicated that the arginine in the PXXPR motif is essential for binding to CIN85 and CMS, we further tested whether the same residue is necessary for interactions between CIN85/CMS and Cbl/Cbl-b in the context of full size molecules. Mutation of arginine 829 in Cbl or arginine 911 in Cbl-b abolished their co-precipitation with CIN85 or CMS (Fig. 2D). Mutations of proline residues within the high affinity PXXPR motif of Cbl-b led to similar results as our peptide pull-down assays, with the first proline residue (Pro-906) being the most important for the association (Fig. 2E). Substituting the last proline (Pro-910) to alanine significantly affected the binding to CIN85, whereas the middle (Pro-908) could be exchanged for alanine without any effect on the interaction (Fig. 2E). Interestingly, several proteins shown previously to interact with the SH3 domains of CIN85 and CMS, including the CD2 receptor and AIP1, contained the conserved PXXPR motif, whereas the SETA-binding protein 1 (SB1) and B cell linker adaptor protein SLP-65 had two conserved binding sites (Fig. 2F) (16). Furthermore, mutation of individual arginine residues in the corresponding motifs in SLP-65, as well as in SB1, reduced their association with CIN85, whereas mutation of both arginines completely blocked co-precipitation with CIN85 (Fig. 2F). Taken together, our data demonstrate that the PXXPR motif found in numerous signaling proteins is indispensable for interactions with the SH3 domains of CIN85/CMS adaptor molecules.

Binding of the SH3 Domains of CIN85 to the PXXPR Motif in Cbl Proteins Leads to Clustering of Cbl—The three SH3 domains of CIN85/CMS lead to clustering of Cbl proteins, as shown by immunofluorescence microscopy. In Cbl-Cbl-b heterodimers, clustering with CIN85/CMS was reduced when arginine 911 in Cbl-b was mutated to alanine (Fig. 3). Interestingly, mutating arginine 979 in Cbl enhanced clustering with CIN85 and CMS, whereas mutating arginine 979 in Cbl-b abolished their co-precipitation with CIN85 or CMS (Fig. 3). These results suggest that the SH3 domains of CIN85/CMS are involved in clustering of Cbl proteins. The effect was even more prominent when proline 906 was mutated together with proline 910 in Cbl-b sequence (Pro-906, Pro-910) completely abrogated their ability to bind to CIN85 or CMS (Fig. 2, B and C), indicating that the minimal recognition sequence for the SH3 domains of CIN85 may contain the PXXPR motif. However, additional peptides containing the PXXPR core sequence were found in Cbl-b, such as peptide 968 (PpvsPR) and peptide 500 (PpypPR) (Fig. 2A). Peptide 968 bound much less efficiently to CIN85 as compared with peptide 893, and this low affinity binding was also dependent on the intact arginine 979, whereas peptide 500 did not bind to CIN85 (Fig. 2B, right panel). Peptide 968 may represent the low affinity binding site, associating only in vitro binding assays, as in the context of the full size Cbl-b molecule, introduction of a stop codon at a residue 927, which preserves only the motif corresponding to the peptide 893 but not 968 of Cbl-b, did not affect the binding to CIN85 (17).
domains of CIN85/CMS share high amino acid sequence similarity with each other. To investigate potential overlapping specificity of CIN85 SH3 domains for the interacting peptides we determined their affinity and stoichiometry of binding using ITC. All individual SH3 domains of CIN85 bound to peptide 893 of Cbl-b or 814 of Cbl with stoichiometries 1:1 and with affinities in the range of 3 to 14 μM (Fig. 3A), which is similar to many SH3 domain-polyproline peptide interactions reported previously (34). The combined double SH3-BC construct bound two Cbl-b peptides with two different affinities. The tighter binding event was ~20 nM whereas the weaker was ~12 μM (Fig. 3A). Similarly, interaction of Cbl peptide 814 with the SH3-BC domain displayed two distinct binding events, the first with an affinity of 30 nM and the second with an affinity of 6.6 μM (Fig. 3A). Furthermore, binding of Cbl or Cbl-b peptides to the SH3-ABC construct gave a complex isotherm, with at least three individual binding events, occurring with different affinities and thermodynamic parameters associated with them (data not shown). These results support the notion that the SH3 domains of CIN85 bind the PXXXPR peptide with different affinities in the context of larger constructs. It is not clear why this occurs; however, the increased size of the construct possibly provides an increased site for interaction with the peptide, i.e. the linker region between the domains might be important for additional formation of non-covalent bonds with the peptide.

Because each SH3 domain of CIN85 can bind the same single peptide in Cbl or Cbl-b, and the double CIN85-SH3 domain construct can bind two peptides, we investigated whether the three SH3 domains present in the full size CIN85 are involved
The SH3 domains of CIN85 simultaneously bind multiple Cbl molecules. A, the binding affinities and stoichiometry of the SH3 domains of CIN85 for the PXXPR peptides were determined by isothermal calorimetry as described under “Materials and Methods.” Sequences of peptides 893 and 814 are shown in Fig. 1A. B, equal amounts of lysates from HEK293T cells transiently transfected with Cbl or HA-Cbl-b were incubated with GST alone or different GST fusion proteins encoding the single (SH3-A, SH3-B, SH3-C), double (SH3-AB and SH3-BC), or triple (SH3-ABC) SH3 domains of CIN85. Amounts of precipitated Cbl or Cbl-b are indicated by arrows in the upper panel, whereas the lower panel shows the levels of GST constructs used stained with PonceauS. C, HEK293T cells were transiently transfected with the wild type Cbl and FLAG-tagged CIN85 or its indicated tryptophan mutants (single SH3 domains: SH3-A*, SH3-B*, SH3-C*; double SH3 domains: SH3-AB* and SH3-BC*; and triple SH3 domains: SH3-ABC*). All transfections were performed in duplicate. Lysates were immunoprecipitated with anti-Cbl antibodies and immunoblotted with anti-Cbl or anti-CIN85 antibodies. Expression of proteins was monitored in total cell lysates (TCL). D, HEK293T cells were transiently transfected with HA-tagged and GFP-tagged Cbl and WT CIN85 or CIN85-PCc constructs. Lysates were subjected to immunoprecipitation (IP) with anti-GFP antibodies, followed by immunoblotting (IB) with anti-GFP, anti-HA, or anti-CIN85 antibodies. Expression of proteins in HEK293T cells was monitored in total cell lysates (TCL). E, HEK293T cells were transiently transfected with GFP-tagged Cbl and CIN85 and HA-tagged Cbl, either wild type or the R829A mutant. Lysates were subjected to immunoprecipitation (IP) with anti-GFP antibodies, followed by immunoblotting (IB) with anti-GFP, anti-HA, or anti-CIN85 antibodies.

| SH3 domain construct | Stoichiometry (N1) | Stoichiometry (N2) | $K_D_1$ (μM) | $K_D_2$ (μM) |
|----------------------|-------------------|-------------------|--------------|--------------|
| Cbl-b peptide 893    |                   |                   |              |              |
| SH3A                 | 1.09              | -                 | 8.4          | -            |
| SH3B                 | 1.06              | -                 | 2.7          | -            |
| SH3C                 | 0.95              | -                 | 14.5         | -            |
| SH3BC                | 0.79              | ~1                | 0.02         | 12.2         |
| Cbl peptide 814      |                   |                   |              |              |
| SH3A                 | 0.92              | ~1                | 6.3          | -            |
| SH3BC                | 0.95              | ~1                | 0.03         | 6.6          |

Fig. 3. The SH3 domains of CIN85 simultaneously bind multiple Cbl molecules. A, the binding affinities and stoichiometry of the SH3 domains of CIN85 for the PXXPR peptides were determined by isothermal calorimetry as described under “Materials and Methods.” Sequences of peptides 893 and 814 are shown in Fig. 1A.
in interactions with multiple Cbl molecules. Consistent with this hypothesis, we were able to show that GST fusion proteins encoding individual CIN85 SH3 domains (SH3-A, SH3-B, or SH3-C) precipitated similar amounts of Cbl or Cbl-b, whereas GST fusion proteins containing SH3-AB, SH3-BC, or SH3-ABC domains bound significantly more of Cbl or Cbl-b (Fig. 3B), thus leading to Cbl clustering by CIN85. Subsequently, we mutated the conserved tryptophan, shown to be important for SH3 domain binding to polyproline ligands (23), in each of the three SH3 domains in the context of the full size CIN85, individually or in combinations of two or all three SH3 domains, and further analyzed their requirement for Cbl clustering in mammalian cells. CIN85 molecules with mutations in single SH3 domains (CIN85-SH3-A*, CIN85-SH3-B*, CIN85-SH3-C*) bound less efficiently to Cbl than the wild type CIN85 (Fig. 3C). Moreover, mutations in two SH3 domains (CIN85-SH3-AB* and CIN85-SH3-BC*) decreased the binding even further, whereas the triple SH3 domain mutant of CIN85 (CIN85-SH3-ABC*) was completely impaired in its ability to associate with Cbl (Fig. 3C), suggesting that all three SH3 domains of CIN85 play important roles in binding to Cbl.

To further demonstrate that CIN85 acts as a scaffold for Cbl molecules, we co-transfected HA- or GFP-tagged Cbl in the absence or presence of full size CIN85 or the CIN85-PCc construct (proline and coiled-coil fragment). Expression of CIN85, but not CIN85-PCc, led to increased co-precipitation of HA-tagged Cbl with anti-GFP antibodies (Fig. 3D). The effect was abolished when HA Cbl R829A mutant, unable to interact with CIN85, was used instead of the wild type HA-Cbl (Fig. 3E). Taken together, these results indicate that CIN85 via its three SH3 domains may act as a platform bridging multiple Cbl molecules in mammalian cells.

Interactions between the SH3-A Domain and the Proline-rich Region of CIN85—Growth factor stimulation leads to increased association between CIN85/CMS and Cbl that appears to be regulated by tyrosine phosphorylation of Cbl (12, 15, 17, 20). Mutation of principal tyrosine phosphorylation sites, which are present in close proximity to the PXXXPR motif of Cbl, reduces its phosphorylation and inhibits ligand-induced binding of CIN85/CMS (15, 20). It was postulated that phosphorylation of Cbl promotes a conformational change in its carboxyl terminus, leading to the exposure of the high affinity binding site (12, 15, 20). Yet, co-precipitation between individual SH3 domains of CIN85 and Cbl is only slightly increased upon EGF stimulation (data not shown). Therefore, phosphorylation-independent mechanisms may also contribute to ligand-induced interactions between Cbl and CIN85.

One possibility is that intramolecular changes in CIN85 may enable the SH3 domains of CIN85 to bind to Cbl more efficiently following growth factor stimulation. Indeed, an intramolecular interaction between the SH3 domains and the proline-rich region of CIN85 has been reported recently (35).
Here we show that this association is dependent on a PPKKPR sequence in the proline-rich region of CIN85. We have found that the isolated SH3-A of CIN85 or its rat homologue Ruk, but not SH3-B or SH3-C, bound to full size CIN85 (Fig. 4A) and CIN85-PCc (Fig. 4B). This binding was of low affinity, because a 20-fold excess of GST-SH3-A was needed to detect association comparable with binding of GST-SH3-A to Cbl (see Fig. 3B and Fig. 4A). Mutation of arginine 404 in the corresponding motif of CIN85 was sufficient to block interactions between SH3-A and CIN85 (Fig. 4A). Moreover, addition of increasing amounts of Cbl peptide 814, but not of its R829A mutant, efficiently blocked SH3-A binding to CIN85-PCc, indicating that interactions between SH3-A and a proline-rich region of CIN85 can be competed by high affinity ligands present in Cbl (Fig. 4B). On the other hand, no intramolecular interaction was detected for CIN85 homologue CMS (35), which lacks this PXXPR motif. These results raise an interesting possibility that SH3-A engages in low affinity autoinhibitory interactions with the carboxyl terminus of CIN85 and that Cbl binding may elicit conformational changes in CIN85, thus allowing the other SH3 domains of CIN85 to associate with their effectors. Accordingly, deletion mutants of CD2BP3, a splice variant of CIN85, that encode only SH3 domains or lack the proline-rich region bind Cbl much more efficiently (35).

**CIN85 Clustering of Cbl Stabilizes Cbl-EGF Receptor Complexes upon Ligand Stimulation**—The specificity in SH3 domain-ligand interactions and their biological importance are often regulated by compartmentalization of interacting partners and assembly of multiprotein complexes (21). Previous studies (9, 12, 31) showed that Cbl and CIN85 are spatially partitioned with activated EGF receptors in the endocytic compartments of EGF-treated cells. We therefore tested whether the ability of CIN85 to bind multiple Cbl molecules contributes to the formation of larger protein complexes upon cell stimulation, containing activated EGFR. To this aim, we compared co-precipitation between CIN85-Cbl-EGFR in the presence of

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**Fig. 4. Intramolecular interactions of CIN85.** A, equal amounts of lysates from HEK293T cells transiently transfected with FLAG-CIN85 or FLAG-CIN85-R404A were incubated with GST alone or GST fusion proteins encoding the single SH3-A, SH3-B, or SH3-C domains of CIN85 or the SH3-A domain of the CIN85 rat orthologue Ruk. Amounts of precipitated CIN85 proteins are visualized in the upper panel, whereas the lower panel shows the levels of used GST constructs stained by PonceauS. The sequence of proline-rich region of CIN85 in which the R404A mutation was introduced is presented below. B, reticulate lysates of in vitro translated CIN85-PCc or lysates of HEK293T cells transfected with CIN85-PCc were incubated with GST-CIN85-SH3-A alone or together with indicated peptides. Subsequently, bound proteins were analyzed by immunoblotting (IB) with anti-CIN85 antibodies.
wild type CIN85 or of CIN85 constructs that are lacking individual SH3 domains and are thus incapable of efficiently clustering Cbl. As expected, CIN85 was found in complexes with Cbl and activated EGF receptors upon EGF stimulation in HEK293T and CHO cells (Fig. 5, A and B), whereas CIN85 isoforms with deletions of the SH3-A, SH3-AB, or SH3-ABC domains (CIN85-PCc) were gradually impaired in their ability to associate with Cbl and activated EGF receptors (Fig. 5, A and B). Interestingly, Cbl co-precipitated more activated EGF receptors in cells expressing wild type CIN85 as compared with cells expressing CIN85-ΔA or CIN85-PCc (Fig. 5, A and B). These data indicate that CIN85-mediated clustering of Cbl can stabilize Cbl-EGF receptor complexes after growth factor stimulation, possibly ensuring their proper trafficking.

CIN85 SH3 Domains/Cbl Interaction Is Required for Efficient EGFR Internalization and Degradation—Cbl and CIN85 have been implicated previously (12) in regulation of EGF receptor internalization. Cbl and CIN85 are also co-localized with the EGF receptor along the endocytic pathway and are degraded together in the lysosome (8, 31, 38, 39). We further examined whether the ability of CIN85 to cluster Cbl-EGF receptor complexes is important for receptor internalization and degradation. First we investigated whether the Cbl R829A mutant, unable to bind to CIN85, is impaired in mediating EGF receptor endocytosis, measured by ligand internalization assays. We have shown previously (12, 17) that the expression of wild type Cbl, together with EGF receptors, led to a significant increase in the rate of EGF internalization when compared with cells transfected with EGF receptor alone. However, Cbl R829A was significantly impaired in its ability to promote internalization of EGF receptors when compared with cells transfected with EGF receptor alone. However, Cbl R829A was significantly impaired in its ability to promote internalization of EGF receptors (Fig. 6A), thus emphasizing a functional role for the PXXXPR motif of Cbl for RTKs down-regulation.

Subsequently, we used CIN85 isoforms lacking individual SH3 domains or CIN85 mutants impaired in their SH3 domain functions to determine whether SH3 domain binding events are critical to promote degradation of EGF receptors in the lysosome. HEK293T cells were transfected with EGF receptors,
A

![Graph showing EGF internalization in CHO cells](image)

**FIG. 6.** CIN85-SH3/Cbl interaction is critical for EGFR internalization and degradation. A, ligand internalization assays were performed as described under “Materials and Methods.” EGF internalization in CHO cells shows effect of Cbl R829A as compared with the wild type Cbl on EGFR internalization. B, quantification of three independent experiments monitoring degradation of EGF receptors in the presence of CIN85, CIN85-ΔA, CIN85-ΔAB, CIN85-PCc, and CIN85-SH3-ABC* was performed as described under “Materials and Methods.” The data are expressed as a percentage ± S.D. of EGF receptors remaining in total cell lysates for each time point.
Cbl, ubiquitin, and wild type CIN85 or its mutant forms, and the levels of EGF receptors were monitored following prolonged stimulation with EGF. As shown in Fig. 6B, degradation of EGF receptors was significantly and similarly reduced in cells expressing CIN85 mutants impaired in their SH3 domains’ functions (CIN85-ΔAB, CIN85-Pcc, and CIN85-SH3-ABC) when compared with kinetics of EGF receptor degradation in the presence of wild type CIN85 or CIN85-ΔΔ. Thus, CIN85-dependent clustering of Cbl-EGF receptor complexes also contributes to regulation of receptor sorting for efficient degradation in mammalian cells.

DISCUSSION

Clathrin-mediated endocytosis of RTKs and their degradation in the lysosome are major determinants of ligand-induced receptor down-regulation (3). Data presented in this report indicate a model whereby CIN85 acts as a scaffolding molecule involved in several steps of Cbl-directed down-regulation of RTKs. CIN85 contains three SH3 domains endowed with the ability to bind to multiple Cbl molecules, thus promoting clustering of Cbl-EGFR complexes upon ligand engagement (see Figs. 3 and 5). CIN85-SH3 domains are also important for efficient EGF receptor internalization and degradation (Fig. 6).

Interactions between CIN85 and Cbl/Cbl-b are mediated by binding of CIN85 SH3 domains to the PXXXPXR motif present in the distal carboxyl terminus of Cbl/Cbl-b (Fig. 1). This is an atypical polyproline motif preferentially recognized by the SH3 domains of CIN85 and CMS (Figs. 1 and 6) but not by other SH3 domain-containing proteins including Grb2, Src, Crk, Abl, CAP, or ArgBP2 (data not shown). The affinity of individual CIN85 SH3 domain-PXXXPXR interactions is in the micromolar range, analogous to the affinity of other SH3 domains for binding to classical polyproline peptides (21, 34). Similarly, the amino-terminal SH3 domain of CIN85 and CMS has been shown recently (42) to bind with micromolar affinities to the peptide CD2 receptor that contains the PXXXPXR motif. Although the PXXXPXR motif resembles the canonical PXXPPP motifs recognized by the 2R class of SH3 domains (23), it is quite clear that CIN85-SH3 domains are selective in binding to only a limited spectrum of cellular effectors, all containing the consensus PXXXPXR motif (Fig. 2F) but not PXXPPP motifs (see Figs. 1 and 2). It is unknown whether PXXXPXR peptides acquire a novel conformational fold that can interact with distinct binding surfaces of CIN85-SH3 domains or whether they form a polyproline-II helix, as shown for PXXP-dependent and some PXXP-independent peptides (23, 27).

The recognition motifs found in Cbl/Cbl-b and many other CIN85 effectors contain PXXPXR sequences (Fig. 2F). However, the middle proline in the motif can be exchanged to alanine and still mediate complete binding between Cbl and CIN85 both in vitro and in vivo (see Fig. 2, B (left panel) and E). Consistent with these findings, several novel CIN85-SH3 targets have been identified recently to contain a PXXXPXR motif, with other residues, such as alanine or lysine, instead of the middle proline (Fig. 4). Thus, the core consensus binding sequence can be defined as a PXXXPXR motif. Variations within and around this core motif may further determine binding specificities for distinct CIN85-SH3 domains. For example, the SH3-A domain binds preferentially to PPKKPXR sequence in CIN85 (Fig. 4A), whereas SH3-A and SH3-C domains were shown to associate with several intracellular signaling proteins containing different PXXXPXR motifs. Interestingly, variations in these motifs can also lead to modulation of CIN85-SH3 domain binding affinities. Peptide binding experiments have shown that the PXXXPXR motif in the context of Cbl/Cbl-b peptides could represent the high affinity ligand for all three SH3 domains of CIN85/CMS, whereas binding of CIN85-SH3 domains to the PPVSPR of Cbl-b (Fig. 1) or PPKKPXR of CIN85 (Fig. 4) was apparently of lower affinity. Taken collectively, these data point to overlapping but distinct specificities and affinities of individual CIN85 SH3 domains in binding to their target PXXXPXR sequences.

CIN85 association with Cbl is enhanced following growth factor stimulation in mammalian cells (12, 43), and initial studies have proposed that ligand-induced tyrosine phosphorylation of Cbl promotes a conformational change in its carboxyl terminus, thus exposing the high affinity peptide for CIN85/CMS binding (12, 17, 20, 43). The PXXXPXR sequence of Cbl is indeed located adjacent to known phosphorylated tyrosine residues in its carboxyl terminus (44). Consistent with this hypothesis, serine or tyrosine phosphorylation of sequences close to the proline-rich motif were shown to modulate the association of SH3 or WW domains with their peptide ligands (45, 46). On the other hand, serine phosphorylation of WW domains could also influence their ability to associate with their target sequences (47). CIN85 contains a cluster of potential serine/threonine phosphorylation sites in proximity to the SH3-B and SH3-C domains (16) and is strongly phosphorylated on serine/threonine, but not tyrosine, residues in mammalian cells. Work in progress should shed additional light on the role of serine/threonine phosphorylation of CIN85 in regulating its binding to Cbl.

Furthermore, ligand binding can also cause conformational changes in SH3 domain-containing proteins leading to their activation, as shown for Src family kinases (48). In the case of CIN85, we demonstrated that CIN85 SH3-A domain is engaged in low affinity interactions with the PPKKPXR peptide of CIN85 (Fig. 4). The exact function of SH3-A-PPKKPR interactions in vivo is still obscure. One possibility is that these interactions may hold the protein in its repressed conformation preventing SH3-B and SH3-C domains from interacting with their ligands. The proximity of high affinity binding sites, such as those found in Cbl, may lead to opening of the CIN85 structure enabling all three SH3 domains to interact with locally available effectors. In addition, it is not yet clear whether SH3-A participates only in intramolecular CIN85 interactions or whether it is also capable of associating with other CIN85 molecules, thus stabilizing larger oligomeric complexes.

Affinities of SH3 domains for their natural ligands are quite low (micromolar range), and selectivity of binding to a particular target is generally modest (21, 34). Therefore, the biological significance of SH3 domain-peptide interactions is accomplished by additional mechanisms, including compartmentalization of interacting partners, multiple separate interactions, and co-operative assembly of multiprotein complexes (21). In the case of CIN85-Cbl, their interactions in mammalian cells are enhanced via SH3 domain-dependent clustering of Cbl (Fig. 3). These data argue that the extended structure of three SH3 domains of CIN85 could aggregate Cbl molecules when the local concentration of tyrosine-phosphorylated Cbl is high enough, for example around internalizing EGF receptor complexes. Accordingly, we were able to show that the presence of CIN85 leads to increased co-precipitation between differently tagged Cbl molecules (Fig. 3D). Importantly, CIN85 SH3 domain-mediated clustering of Cbl promotes stabilization of ligand-induced EGF-Cbl-CIN85 complexes (Fig. 5) and is critical for EGF receptor internalization and efficient receptor degradation in the lysosome (Fig. 6).

Taken together, our findings support the concept whereby

2 K. Husnjak and I. Dikic, unpublished results.
3 I. Dikic, unpublished observations.
EGF-induced association between CIN85 and Cbl is regulated by multiple steps, including phosphorylation-dependent exposure of specific PXXXP peptides in Cbl, opening of the autorepressed conformation of CIN85, recruitment of CIN85 to Cbl-enriched cellular microdomains, and subsequent clustering of Cbl molecules by the three SH3 domains of CIN85. It is likely that in living cells all these mechanisms simultaneously contribute to formation of EGF-ER associated complexes that are critical for regulation of EGF receptor endocytosis and degradation.

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