The Adapter Type Protein CMS/CD2AP Binds to the Proto-oncogenic Protein c-Cbl through a Tyrosine Phosphorylation-regulated Src Homology 3 Domain Interaction*

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CMS/CD2AP is a cytoplasmic protein critical for the integrity of the kidney glomerular filtration and the T cell function. CMS contains domains and motifs characteristic for protein-protein interactions, and it is involved in the regulation of the actin cytoskeleton. We report here that the individual SH3 domains of CMS bind to phosphotyrosine proteins of ~80, 90, and 180 kDa in cell lysates stimulated with epidermal growth factor. The second SH3 domain of CMS bound specifically to a tyrosine-phosphorylated protein of 120 kDa, which we identified as the proto-oncoprotein c-Cbl. The c-Cbl-binding site for CMS mapped to the carboxyl terminus of c-Cbl and is different from the proline-rich region known to bind SH3-containing proteins. CMS binding to c-Cbl was markedly attenuated in a tyrosine phosphorylation-defective c-Cbl mutant indicating that this interaction is dependent on the tyrosine phosphorylation of CMS. It also implies that CMS interacts with c-Cbl in an inducible fashion upon stimulation of a variety of cell-surface receptors. Immunofluorescence analysis revealed that both proteins colocalize at lamellipodia and leading edges of cells, and we propose that the interaction of CMS with c-Cbl offers a mechanism by which c-Cbl associates and regulates the actin cytoskeleton.

Adapter type molecules are composed of noncatalytic protein-protein interaction domains. They are important components of integrated signal transduction pathways and of the cytoskeleton that organizes the structure of eukaryotic cells (reviewed in Refs. 1 and 2). These molecules selectively control the spatial and temporal assembly of multiprotein complexes that transmit intracellular signals that regulate cell proliferation, differentiation, and survival (reviewed in Refs. 3–5).

CMS/CD2AP (p130*caus ligand with multiple SH3 domains/CD2-associated protein) is an adapter-type molecule composed of three SH3 domains, a proline-rich region, and a coiled-coil domain. Originally, we identified CMS as a molecule that binds via its proline-rich region to the SH3 domain of p130*caus (6). We have also shown that CMS can associate with and become phosphorylated by nonreceptor tyrosine kinases. These interactions are mediated also by the SH3 domain of the tyrosine kinase and the proline-rich region in CMS. The coiled-coil domain in the carboxyl terminus of CMS mediates its dimerization (6).

CMS plays a role in the regulation of the actin cytoskeleton. Overexpression studies in COS-7 cells demonstrated that full-length CMS colocalizes with F-actin to membrane ruffles and leading edges of cells (6). Interestingly, the first SH3 domain of the mouse homologue of CMS, named CD2AP, has been shown to bind to the cell adhesion molecule CD2 (7). This interaction enhances CD2 clustering and anchors CD2 at sites of cell contacts. Besides CD2, no other molecules have been identified to date that associate with the SH3 domains of CMS. CMS/CD2AP-deficient mice exhibit defects in glomeruli of the kidney, develop nephrotic syndrome, and die of renal failure (8). Glomerular epithelial cells (podocytes) display effacement of their foot processes accompanied by mesangial hyperplasia and extracellular matrix deposition.

The proto-oncogene c-cbl is the cellular homologue of v-cbl, the transforming oncogene of the Cas NS-1 retrovirus (9). c-Cbl is a component of protein tyrosine kinase signaling pathways where it has been established as a negative regulator (10). It is an adapter protein that contains many structural domains involved in protein-protein interactions. The amino terminus is highly conserved among the Cbl family members that harbor a tyrosine kinase binding domain. This domain binds to the activated nonreceptor tyrosine kinases ZAP-70 and Syk (11–13) and to the epidermal growth factor receptor (EGFR), platelet-derived growth factor, and colony-stimulating growth factor-1 receptor tyrosine kinases (14–18). The center region of c-Cbl contains a conserved RING finger domain, which recruits and activates an E2 ubiquitin-conjugating enzyme (19, 20). c-Cbl contains in its carboxyl terminus many PXXP motifs that can bind to SH3 domains as well as tyrosine residues, which when phosphorylated form binding sites for SH2 domains. These motifs serve as binding sites for Grb2, 14-3-3, phosphatidylinositol 3-kinase, Crk, Nck, Vav, and Src-like tyrosine kinases (reviewed in Ref. 10). A leucine zipper is located in its carboxyl terminus, which could mediate oligomerization.

c-Cbl is a widely expressed molecule, and it is localized exclusively in the cytoplasm. Endogenous c-Cbl is localized in osteoclasts to some vesicular structures in the perinuclear areas and at the cell periphery (21), whereas overexpressed c-Cbl

* This work was supported by National Institutes of Health Grants CA44356 and GM55760. 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.

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§ Supported by NCI Fellowship CA09673 from the National Institutes of Health.

† The abbreviations used are: SH, Src homology; CD, cluster of differentiation; aa, amino acid residue; EGF, epithelial growth factor; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; HA, hemagglutinin; GST, glutathione S-transferase; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum.

Published, JBC Papers in Press, November 6, 2000, DOI 10.1074/jbc.M005784200
in NIH 3T3 fibroblasts is targeted to membrane ruffle-associated actin lamellae (22). This association requires specific SH3-binding sequences localized in the carboxyl-terminal half of c-Cbl.

We report here that tyrosine-phosphorylated c-Cbl associates with CMS in vitro and in vivo. This interaction was further characterized, and the binding region for CMS in c-Cbl was determined. Moreover, we found in our studies that both molecules colocalize to actin structures in membrane ruffles, suggesting that CMS may link c-Cbl to the actin cytoskeleton.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Construction of FLAG-tagged full-length CMS (639 aa) and the GST fusion protein constructs for the individual SH3 domains of CMS were described (6). The full-length CMS construct was subcloned in frame with a Myc tag in the retroviral vector pCX (a gift from T. Agaki) (23). The CMSΔP construct lacking the proline-rich region (aa 332–426) was constructed by standard polymerase chain reaction (PCR) of the 5' and 3' cDNA fragments flanking the sequences encoding the proline-rich region. A PstⅠ restriction site was introduced resulting in a single amino acid change at position 427 from asparagine to glutamine to ligate both amplified fragments and cloned in frame with the Myc tag into the pCX vector. The constructs of HA-tagged Cbl and GST-tagged Cbl (Cbl(437–647), and Cbl(648-end) were described (24). GST-tagged Cbl(1–436) was created by introducing a stop codon at aa position 436. HA-tagged Cbl8F was generated by introducing Tyr to Phe changes at positions Tyr-552, Tyr-674, Tyr-700, Tyr-731, Tyr-735, Tyr-774, Tyr-869, and Tyr-871. The FLAG-tagged constructs F-Cbl(457–647) and F-Cbl(648-end) were generated by standard PCR and cloned into the pFLAG-CMV2 vector (Eastman Kodak Co.). Proline to alanine mutations were introduced into F-Cbl(684-end) as described above. All PCR products were verified by DNA sequencing.

Cell Lines, Retroviral Infection, Transient Transfection, EGF Treatment of 293T Cells, and Cell Lysis—Human 293T kidney epithelial (293T) cells and COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Cellgro) containing 10% fetal calf serum (FCS, HyClone). Immortalized mouse podocytes (a gift from P. Mundel) were cultured in RPMI 1640 supplemented with 10% FCS and 10 ng/ml interferon γ (Life Technologies, Inc.) at 33 °C. Immortalized mouse podocytes were used for generating cell lines stably expressing CMS and CMSΔP. B232 retrovirus-packaging cells were maintained in DMEM supplemented with 10% FCS and were cotransfected in 6-cm dishes by using Fugene™ 6 transfection reagent (Roche Molecular Biochemicals) with 1 μg of the various CMS-containing plasmids and 1 μg of the pCL-Eco plasmid (Imgenex, San Diego). After 48 h, the virus-loaded supernatants were transferred to exponentially growing podocytes and supplemented with 10 ng/ml interferon γ and Polybrene (4 μg/ml; Sigma). Infected podocytes were transferred to new culture dishes and grown in selection medium containing 10 μg/ml blasticidin (Invitrogen). Stably transfected podocytes were obtained after ~8 days and further cultured in medium containing 5 μg/ml blasticidin. 293T cells seeded in 6-cm plates were transiently transfected as described above with 1 μg each of the plasmids indicated. After 36 h the cell were lysed as described (25). Subconfluent 293T cells were washed twice with serum-free medium and cultured in DMEM containing 0.5% FCS for 16 h. Subsequently the cells were treated for 5 min with 50 ng/ml EGF (Life Technologies, Inc.), washed, and lysed as described.

Expression of GST Fusion Peptides and in Vitro Binding Assay—Expression and affinity purification of GST fusion proteins as well as in vitro binding assay were carried out as described (6).

Immunoprecipitation and Immunoblotting—Proteins were immunoprecipitated by incubating 250 μg of total cell lysate with the specific antibodies for 2 h and further collected on GammaBind™ G-Sepharose™ (Amersham Pharmacia Biotech) for 1 h at 4 °C. The antibodies and antisera were as follows: anti-FLAG (M2, monoclonal) (Sigma); anti-HA (polyclonal) and anti-GST (polyclonal), (Santa Cruz Biotechnology); anti-Cbl (17, monoclonal) (Transduction Laboratories); anti-Cbl (C-15, polyclonal), (Santa Cruz Biotechnology); anti-phosphotyrosine (Tyr(P), 4G10, monoclonal) (Upstate Biotechnology, Inc.). The immunocomplexes were washed three times in lysis buffer, denatured by boiling for 5 min in double-strength sample buffer, and resolved by SDS-polyacrylamide gel electrophoresis (PAGE). Immunoblotting was performed as described (25).

Immunofluorescence—COS-7 cells and podocytes grown on polyl-lysine-coated coverslips were transiently transfected with 0.5 μg of the indicated plasmid DNA. For immunofluorescence analysis 48 h post-transfection, the cells were prepared as described (6). Cells were inspected by microscopy using a Nikon Eclipse 800 instrument attached to a digital camera.

RESULTS

In Vitro and in Vivo Association of the SH3 Domains of CMS with Cbl—CMS contains multiple protein-protein interaction sites (shown in Fig. 1A). To investigate the signaling potential of CMS, we analyzed the ability of the individual SH3 domains of CMS fused to glutathione S-transferase (GST) to bind to tyrosine-phosphorylated proteins. We performed an in vitro pull-down assay where the GST fusion peptides and control GST proteins were incubated with cell lysates from EGF-treated 293T cells. Immunoblotting with an anti-phosphotyrosine antibody revealed that all three SH3 domains of CMS bound proteins with the molecular masses of ~180, 90, and 80 kDa. In addition, the second SH3 domain associated with a protein of ~120 kDa (Fig. 1B). A candidate protein of 120 kDa was c-Cbl. c-Cbl is a component of tyrosine kinase signaling pathways, and it becomes highly phosphorylated in response to treatment of cells with EGF. Reprobing of the same membrane with an anti-c-Cbl antibody did indeed identify the 120-kDa phosphotyrosine protein as c-Cbl (Fig. 1C). In addition, also the first and the third SH3 domain of CMS had a much weaker interaction with c-Cbl. In contrast, when we used lysates from cells transiently trans-
Proline-rich region), and we were also able to detect the interaction of CMS with c-Cbl. In this assay, CMS binding to c-Cbl was abolished in the presence of c-Src as source for phosphotyrosine proteins in this assay, only the second SH3 domain of CMS associated with c-Cbl.

CMS is highly expressed in podocytes of the kidney suggesting a specific role in these specialized epithelial cells (8). We generated stable cell lines of immortalized podocytes expressing Myc-tagged versions of CMS and CMSΔPP (lacking the proline-rich region), and we were also able to detect in vivo interaction of endogenous c-Cbl with CMS and CMSΔPP (Fig. 2). We estimate that 5% of the endogenous c-Cbl was associated with CMS in this assay.

**CMS and c-Cbl Colocalize to Actin Structures in Lamellipodia**—The association between CMS and c-Cbl prompted us to analyze the intracellular localization and distribution of CMS and c-Cbl. We have previously shown that CMS can be found at the leading edge of cells and that it is colocalized with F-actin in lamellipodia (6). Moreover, immunofluorescence analysis of podocyte cell lines expressing CMS or CMSΔPP revealed that both peptides are localized to membrane ruffles and leading edges of cells (not shown). The expression pattern of HA-tagged c-Cbl was investigated by immunofluorescence analysis. As expected, c-Cbl is expressed in the cytoplasm, and it could be found rather like CMS at the leading edge of migrating cells. There it colocalized with F-actin similarly to CMS (Fig. 3). In addition, we investigated whether CMS and c-Cbl colocalize to these structures by coexpressing FLAG-tagged CMS with HA-tagged c-Cbl. Indeed both molecules could be found simultaneously in membrane ruffles and leading edges of motile cells (Fig. 3).

**The Association of CMS with c-Cbl Is Regulated by Tyrosine Phosphorylation of c-Cbl**—Since the in vitro interaction of the individual SH3 domains with c-Cbl varied when we used different methods to phosphorylate c-Cbl, we were interested to investigate the importance of c-Cbl phosphorylation in this interaction. In randomly growing cells, a small fraction of cellular proteins including c-Cbl is phosphorylated on tyrosine residues. Moreover, we found that transient expression of c-Cbl in 293T cells results in its tyrosine phosphorylation and, in addition, we investigated whether CMS and c-Cbl colocalize to membrane ruffles. A, randomly growing COS-7 cells, plated on poly-κ-lactide-coated coverslips, were transfected with HA-tagged c-Cbl. Cells were fixed and stained with rhodamine-labeled phalloidin for visualizing F-actin and anti-HA antibodies for monitoring c-Cbl. Membrane ruffles are indicated by arrows. B, COS-7 cells coexpressing HA-tagged c-Cbl and CMS were analyzed with anti-HA antibodies and anti-CMS rabbit serum. The cells were inspected with a Nikon Eclipse 800 instrument (×60). Colocalizations of CMS and c-Cbl to membrane structure are indicated by arrows.

Phosphorylation-dependent Association of CMS with c-Cbl

The c-Cbl proline-rich domain. Thus our results suggest that the interaction of CMS with c-Cbl is rather regulated than constitutive. Moreover, the tyrosine phosphorylation of c-Cbl proposes the involvement of structural changes for the association of CMS with c-Cbl.

If only structural changes were responsible for the inducible interaction of CMS with Cbl, then a smaller peptide such as an individual SH3 domain might be able to bind to the phosphorylation-defective c-Cbl. We tested this hypothesis by assessing the ability of the second SH3 domain of CMS to pull down HA-tagged wild-type c-Cbl and Cbl8F from cell lysates. In this assay, we found that the individual SH3 domain of CMS was able to associate with Cbl8F. However, compared with wild-type c-Cbl, this association was much weaker (Fig. 4D). This finding suggests that the inducible interaction of CMS with c-Cbl is not solely mediated by conformational changes of c-Cbl.

**CMS Associates with the PXXP Motifs in the Carboxyl-terminal Region in Cbl**—The site of interaction of CMS with c-Cbl was investigated by assessing the ability of FLAG-tagged CMS to associate with GST-tagged Cbl constructs coexpressed in 293T cells. Cbl, Cbl(1-436) (encoding the amino-terminal half of c-Cbl), Cbl(437-647) (encoding the proline-rich region), Cbl(648-end), and vector control were used for this assay. As expected, full-length Cbl was efficiently associated with CMS (Fig. 5A, upper panel). We observed similar efficient binding of CMS only to the Cbl(648-end) construct and not to Cbl(437-647). In contrast, to the latter we detected only marginal binding of CMS, although this construct is composed of the entire proline-rich region (aa 480–555) of Cbl defined in the literature for binding SH3 domain-containing proteins (22). The Cbl(468-end) fragment contains six partially overlapping PXXP motifs that resemble putative SH3 domain binding sites (Table I). These PXXP motifs are situated outside the defined c-Cbl proline-rich domain. Thus our results suggest that the major interaction site of CMS in c-Cbl lies carboxyl-terminal of the c-Cbl proline-rich region. No binding of CMS to the amino-terminal fragment Cbl(1-436) was detectable (Fig. 5C).
Fig. 4. CMS associates with c-Cbl in a tyrosine-dependent manner. 293T cells were cotransfected with FLAG-tagged CMS and vector control, HA-tagged Cbl, or with HA-tagged Cbl8F. Tyrosine phosphorylation was induced by cotransfecting Fyn. A, tyrosine phosphorylation was analyzed by immunoprecipitation with anti-phosphotyrosine (Tyr(P)) antibodies and immunoblotting with anti-Tyr(P), anti-HA, anti-FLAG and anti-Fyn antibodies. B, cell lysates were incubated with anti-FLAG antibodies, subsequently the precipitates were collected on Sepharose beads and subjected to SDS-PAGE. Coprecipitated (IP) wild-type and mutant c-Cbl were identified with anti-HA antibodies (upper panel). We controlled for immunoprecipitation of CMS with anti-FLAG antibodies (lower panel). C, the expression levels were analyzed in total cell lysates by immunoblotting (IB) with anti-HA, anti-FLAG, and anti-Fyn antibodies. D, GST-SH3/2 fusion peptide coupled to glutathione beads was incubated with increasing amounts (20–320 μg) of clarified lysates of 293T cells expressing HA-tagged c-Cbl or Cbl8F. Precipitates were analyzed by SDS-PAGE and probed with an anti-HA antibody (upper right panel). The blot was reprobed with an anti-GST antibody (lower right panel). The expression levels were analyzed in total cell lysates by immunoblotting with anti-HA antibody (left panel). wt, wild type.

We identified the carboxyl-terminal region of c-Cbl as a major binding site for CMS. This prompted us to investigate the intracellular localization of coexpressed CMS and Cbl(648-end). Immunofluorescence analysis of mouse podocytes revealed that both molecules did colocalize. The expression pattern observed for Cbl(648-end) was similar to that observed for full-length c-Cbl (Fig. 6). Cbl(648-end) was expressed throughout the cytoplasm and at the cell periphery. Moreover, we also noticed prominent colocalization of CMS and Cbl peptides to vesicular structures formed in cells overexpressing CMS and different versions of Cbl. In our previous studies (6), we found that ectopic expression of CMS in COS-7 cells induces vesicle formation. We noticed increased vesicle formation also in podocytes coexpressing Cbl(648-end) with CMS. These structures were spread throughout the entire cell (not shown).

Initially we observed differences in the binding potential of the individual SH3 domains toward c-Cbl. Moreover, we found that the major recognition site for CMS in c-Cbl lies outside of the ascribed c-Cbl proline-rich region. In an in vitro pull-down assay, we analyzed the binding of the individual SH3 domains to FLAG-tagged Cbl(437–647) and Cbl(648-end) (Fig. 5B). This assay also revealed the region of aa 648–906 as a major interaction site. The second SH3 domain bound predominantly, and by this assay we established the second SH3 domain of CMS as a major structural element for the CMS/c-Cbl association. We obtained similar results when we coexpressed in 293T cells c-Cbl with mutant CMS constructs containing point mutations in the individual SH3 domains that abolish their binding capacity (not shown).

To characterize further the CMS-binding motif in c-Cbl, we mutated the core PXXP sequences by introducing Pro → Ala point mutants in FLAG-tagged Cbl(648-end). The mutants were introduced at positions P864A, P706A, P778A/P779A, and P822A, which are underlined in Table I. We disrupted the PXXP motifs 10–14 individually. These constructs and the wild-type counterpart were transiently expressed in 293T cells, and total cell lysates were used in a GST pull-down assay by using the second SH3 domain of CMS, since this domain initially gave the strongest signal in the GST pull-down assay. However, we did not see any change in the binding capacity of CMS to the mutated constructs compared with the wild-type positive control (not shown). Alignment of SH3-binding motifs comprising the proline-rich region in c-Cbl (aa 347–647) and the carboxyl-terminal peptide sequence of c-Cbl (aa 648–906) are displayed in Table I. This alignment revealed great similarity in the PXXP-binding motifs in the carboxyl-terminal fragment. Five out of six partially overlapping binding motifs contain a positively charged amino acid. It has been shown that amino acids adjacent to the core consensus binding motif contribute to the specificity of SH3-PXXP interactions (26, 27). However, this high similarity may explain why the disruption of the individual SH3-binding motifs in c-Cbl did not abolish the binding of the second SH3 domain of CMS to c-Cbl. This suggested that the second SH3 domain of CMS can bind to more than one PXXP motif in the carboxyl-terminal region of c-Cbl.

**DISCUSSION**

The modular structure of CMS/CD2AP indicates a function in assembling intracellular molecules into selective complexes. Previously we have shown that CMS interacts with signaling molecules such as p130Cas, Src-like kinases, and the p85 subunit of the phosphatidylinositol 3-kinase via PXXP-SH3 interactions (6). Furthermore, CD2AP, which is the mouse homologue of CMS, was first identified as CD2-associated protein (7) where the binding to CD2 was mediated by the first SH3 domain of CMS.

Treatment of cell lines with growth factors or phorbol ester is known to activate tyrosine kinases that result in an overall increase in tyrosine-phosphorylated cellular proteins and to induce the reorganization of the actin cytoskeleton. A prominent feature of cytoskeletal rearrangements is the induction of membrane ruffles and lamellipodia (sheet-like extensions of the plasma membrane that contain a meshwork of F-actin). We have shown that CMS colocalizes with F-actin in such phorbol ester-induced membrane ruffles (6). To identify further molecules of the signal transduction pathways involving CMS, we searched for EGF-induced tyrosine-phosphorylated proteins that interact with the three individual SH3 domains of CMS. One of the molecules identified was the proto-oncogene c-erit, which is a substrate of protein tyrosine kinases that is rapidly phosphorylated following stimulation by growth factors, antigen, and integrins (28–31). c-Cbl is a widely expressed protein with higher expression in the thymus and hematopoietic cell lines (9). Rather like c-Cbl, the mRNA expression level of CMS is elevated in the thymus (6). Interestingly, in T cells, Dustin et al. (7) suggested a function for CMS/CD2AP in the process antigen recognition in particular in the formation of the
"immunological synapse," which is a specialized junction between a T lymphocyte and an antigen-presenting cell. The immunological synapse consists of a central cluster of T cell receptors and costimulatory molecules surrounded by a ring of adhesion molecules. The first SH3 domain of CMS/CD2AP has been shown to bind to CD2. We found here that the second SH3 domain of CMS binds to c-Cbl. c-Cbl has been shown to associate with the T cell receptor complex (reviewed in Refs. 10 and 32) and to be localized to the immunological synapse (33). It is likely that CMS could act as a bridging molecule between the cell adhesion molecule CD2 and the ternary complex formed by c-Cbl, the T cell receptor, and ZAP-70. Overexpression of a dominant-negative form of CMS formed by the first two SH3 domains blocked CD2-triggered cytoskeletal polarization and the formation of the immunological synapse. CMS has the potential to homodimerize via its carboxyl-terminally located leucine zipper (6). We suggest that dimerization of CMS mediates the clustering of CD2 molecules and that the truncated CMS acts as a dominant-negative molecule by titrating out CD2 and c-Cbl from the central region of the immunological synapse. However, further work is needed to study this hypothesis.

Although higher expression levels in the thymus have been reported for CMS and c-Cbl, both molecules are ubiquitously expressed. A specialized role for CMS in the dynamic regulation of the actin cytoskeleton has been underscored by the phenotype of CMS/CD2AP-deficient mice (8) and overexpression studies in COS-7 cells (6). Interestingly, CMS/CD2AP−/− mice develop congenital nephrotic syndrome due to the impaired function of specialized epithelial cells, podocytes, in the glomerulus of the kidney. The dynamic regulation of the actin cytoskeleton seems to be affected, because of the observed effacement of the actin-rich foot processes formed by the podocytes. Interestingly, recently c-Cbl has also been identified as an important regulator of the actin cytoskeleton (22). Scaife and Langdon (22) have shown that c-Cbl localizes to lamellipodia and leading edges of cells. Furthermore, they suggested that c-Cbl localization to actin-rich structures requires the interaction with a SH3 domain-containing molecule. Here we found that CMS interacts with c-Cbl via SH3 domain-PXXP motif binding, and CMS might be the link between c-Cbl and F-actin since at least four putative actin-binding sites are localized in the carboxyl terminus of CMS (6).

Most SH3 domains bind proline-rich motifs with the core consensus sequence PXXP (34). It is widely believed that this interaction is rather constitutive, and preformed complexes

**FIG. 5. CMS binds to proline-rich sequences in the carboxyl terminus of c-Cbl.** A, 293T were transfected with the vectors alone or vectors containing cDNAs of FLAG-tagged CMS and GST-tagged Cbl, Cbl(1–436), Cbl(437–647), or Cbl(648-end). Precipitation was carried out by capturing the GST fusion peptides on glutathione beads. Precipitates were subjected to SDS-PAGE, and blots were probed with anti-FLAG antibodies to detect bound CMS (upper panel) and with anti-GST antibodies to monitor the amounts of captured GST-Cbl peptides (middle panel). Expression levels of transiently expressed CMS were analyzed in 25 μg of total cell lysate (lower panel). B, GST fusion peptides coupled to glutathione-Sepharose beads were incubated with lysates of 293T cell-expressing FLAG-tagged Cbl(437–647), Cbl(648-end), or vector control. Precipitates were analyzed for c-Cbl interaction with anti-FLAG antibodies (left panel). Immunoblot analysis of 25 μg of total lysate was carried out for monitoring the expression levels of the transiently expressed c-Cbl peptides with anti-FLAG antibodies. C, schematic representation of the GST-tagged expression constructs used in the binding assay. Amino acid residues of the boundaries are indicated. TKB, tyrosine kinase binding domain; RF, RING finger domain; PR, proline-rich region; LZ, leucine zipper; arrows indicate Tyr → Phe point mutations introduced in the Cbl8F construct. Multiple + signs indicate strong binding to CMS; +/−, weak binding; −, no binding of full-length CMS to the Cbl peptides indicated in A.

**TABLE I**

| Construct       | Position* | Sequence          | Motif* |
|-----------------|-----------|-------------------|--------|
| Cbl(437–647)    | 477       | VERGPHSPFSM       | 1      |
|                 | 490       | ASLPPVPPRL        | 2 + 3  |
|                 | 530       | KPLLVPFTPRL       | 4      |
|                 | 542       | PPPDPRSYV         | 5      |
|                 | 562       | RELPCTPDDC        | 6      |
|                 | 575       | DKLPPVPSRR        | 7      |
|                 | 599       | RPEKQPVPSA        | 8      |
|                 | 628       | EPRDVPFPG         | 9      |
| Cbl(648-end)    | 681       | LPVPKLPFPGE       | 10     |
|                 | 703       | SSSRPPLDPT        | 11     |
|                 | 775       | VPKEPPVPVAVL      | 12 + 13|
|                 | 819       | PPEKPFPFR         | 14 + 15|

* Amino acid position in respect to the full-length c-Cbl protein of the first amino acid of the shown sequence. Positions were mutated to A.

* The motifs of Cbl(437–647) are in the proline-rich region in c-Cbl.
Randomly growing podocytes, plated on carboxyl-terminal region of c-Cbl. Phosphorylation of c-Cbl generates binding sites for SH2 domain-containing proteins such as Vav, p85 subunit of phosphatidylinositol 3-kinase (p85), and Crk (35–37). One possibility could be that the interaction of CMS with c-Cbl is indirect by binding to one of the above-mentioned proteins. However, this is very unlikely since we have also shown that isolated SH3 domains of CMS are capable of binding c-Cbl. The possibility that we favor is an induced conformational change of c-Cbl from a closed to an open conformation and thereby unmasking putative SH3-domain binding motifs in the carboxyl terminus of c-Cbl. Support for this idea comes from the recently identified molecule CIN85, which is structurally related to CMS (38) and as CMS interacts with c-Cbl in a phosphorylation-dependent manner. Furthermore, phosphorylation-induced changes of the c-Cbl conformation have been suggested (38). Recently, it has been demonstrated that c-Cbl can form a closed structure that prevents Src for binding to it (24). These structural restrictions can be overcome by binding of Abi to c-Cbl which then allows Src binding. Conformational changes of c-Cbl could also lead to the generation of a nonlinear ligand-binding site for CMS as described for the p53-p53BP2 interaction (39). The interaction of the SH3 domain of p53BP2 with p53 is determined by the global structure of the p53 core domain.

Although we cannot rule out the contribution of another protein, we clearly demonstrated that the tyrosine phosphorylation of c-Cbl positively regulates its association with CMS. In addition, it could also be conceivable that the SH3 domains of CMS recognize a novel consensus sequence that contains a tyrosine phosphorylation site in a linear sequence motif as identified for the SH3 domain of Eps8 (40). Cbl contains in its center a proline-rich region with at least nine overlapping SH3 domain recognition motifs for SH3 domains to which the binding of Grb2, Nck, Cap, and Src family kinases has been demonstrated (37, 41–45). Here we provide evidence that CMS binds preferentially to consensus sequences outside the defined proline-rich region in c-Cbl. Experiments using deletion mutants of c-Cbl clearly identified the region of aa 648–906 as a major interaction site. CMS is the first protein identified to associate via SH3-PXXP interaction with the carboxyl terminus of c-Cbl.

In summary, we have identified c-Cbl as CMS-associated protein. Our results suggest that CMS can bind to multiple sites in c-Cbl and thus be able to contribute to the assembly of larger protein networks upon stimulation by growth factors and survival signals. It will be of great interest to examine the role of the CMS-c-Cbl complex formation during lymphocyte activation and for the integrity of the podocytes in the kidney glomerulus. Findings derived from such studies will have substantial implications for the understanding of important physiological processes such as cell differentiation and apoptosis.

Acknowledgments—We are grateful to Dr. P. Mundel for providing us with the immortalized podocytes, to Dr. T. Agaki for the pCX vector, and to Dr. J.E. Fajardo for assistance with the manuscript. We also thank P. Kaloudis for assistance with microscopy. We are grateful to Dr. M. Nussenzweig for accommodating KHK in his group.

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