CIN85 Interacting Proteins in B Cells-Specific Role for SHIP-1

Tom Büchse‡**, Nikolaus Horras‡, Eva Lenfert‡, Gerald Krystal§, Sandra Körbel‡, Michael Schümann¶, Eberhard Krause¶, Stefan Mikkat¶, and Markus Tiedge‡

The Cbl-interacting 85-kDa protein (CIN85) plays an important role as a negative regulator of signaling pathways induced by receptor tyrosine kinases. By assembling multi-protein complexes this versatile adaptor enhances receptor tyrosine kinase-activated clathrin-mediated endocytosis and reduces phosphatidylinositol-3-kinase-induced phosphatidylinositol-3,4,5-trisphosphate production. Here we report the expression of CIN85 in primary splenic B lymphocytes and the B-lymphoma cell lines WEHI 231 and Ba/F3. Cross-linking of the B cell antigen receptor resulted in an increased association of CIN85 with the ubiquitin ligase Cbl. Through a systematic pull-down proteomics approach we identified 51 proteins that interact with CIN85 in B cells, including proteins not shown previously to be CIN85-associated. Among these proteins, the SH2-containing inositol phosphatase 1 (SHIP-1) colocalized with both the full-length CIN85 and each of its three SH3 domains. We also showed that this association is constitutive and depends on a region of 79 amino acids near the carboxyl terminus of SHIP-1, a region rich in potential SH3 domain binding sites. Because SHIP-1 is a major negative regulator of the phosphatidylinositol-3-kinase pathway in lymphocytes, we hypothesize that the interaction between SHIP-1 and CIN85 might synergistically facilitate the down-regulation of phosphatidylinositol-3,4,5-trisphosphate levels. Molecular & Cellular Proteomics 10: 10.1074/mcp.M110.006239, 1–12, 2011.

B lymphocytes require a precise regulation of their activation status, because aberrances may lead to severe dysfunctions associated with hyper- or hyposensitivity. For this regulation, inhibitory mechanisms are as important as activation signals. They not only terminate, and therefore temporally limit, the signaling initiated by extracellular mediators but set the threshold required to trigger a cellular response. This could be achieved through variation of the receptor density on the cell surface or modulation of intracellular signaling, like the generation of phosphatidylinositol-3,4,5-trisphosphate (PIP3) by class I phosphoinositide 3-kinases (PI3K). It is intriguing that both strategies are used by the adaptor protein Cbl-interacting 85-kDa protein (CIN85) (1–4). Via binding with the proto-oncogene Casitas B-lineage lymphoma (Cbl), an E3 ubiquitin ligase, CIN85 is recruited to activated membrane receptors (1, 5). Through its constitutive association with endophilin it facilitates the formation of endocytotic vesicles leading to internalization and degradation of receptor-ligand complexes. This has been demonstrated for several receptor tyrosine kinases (RTKs), including the epidermal growth factor receptor (EGFR), the hepatocyte growth factor receptor (HGF) and the platelet-derived growth factor receptor (PDGFR) (1, 2, 5). In addition, overexpression of CIN85 decreases the survival of cultured primary neurons by inhibition of the PI3K pathway (4). This pathway is of special importance for B cells. Inactivation of the PI3K blocks B cell antigen receptor (BCR) signaling, impairs cell activation, reduces the number of immature and mature B cells, and lowers immunoglobulin concentrations in mice (6–9). However, the mechanism by which CIN85 negatively affects the PI3K pathway remains unknown.

CIN85 is composed of three SH3 domains, a proline-arginine rich region and a carboxy-terminal coiled-coil domain (10), whereas the SH3 domains prefer binding to the atypical proline-arginine motifs PxxPR and Px(P/A)xxR (11, 12). CIN85 expression could be detected in a variety of tissues, including brain, heart, skeletal muscle, liver, kidney, pancreas, placenta, and lung (13, 14), lymphocytes (15), and mast cells (3). Related to this, CIN85 was recently shown to promote the ligand-dependent endocytosis of the IgE receptor as well as the degradation of relevant signaling molecules in mast cells (3, 16). In T cells, CIN85 was found to bind to the adaptor protein complexes this versatile adaptor enhances receptor tyrosine kinase.

The abbreviations used are: PIP3, phosphatidylinositol-3,4,5-trisphosphate; CIN85, Cbl-interacting 85-kDa protein; Ab, Antibody; ARAP1, Arf-GAP, Rho-GAP, Ankyrin repeat, and Pleckstrin homology domain containing protein 1; BCR, B cell antigen receptor; CMV, Cytomegalovirus; E. coli, Escherichia coli; EGFP, Enhanced Green Fluorescent Protein; GTP, Guanosine-5’-triphosphate; LC-ESI-MS/MS, Liquid chromatography electrospray ionization tandem mass spectrometry; RIPA, radioimmunoprecipitation assay; RTK, receptor tyrosine kinase.
molecule SH3 domain-binding protein 2 (3BP2), which is involved in leukocyte signaling downstream of Src/Syk-kinase coupled immunoreceptors and formation of the immunological synapse (15).

In contrast to T cells, little is known of the role of CIN85 in B cells other than a report showing that CIN85 binds to the B-cell linker protein (BLNK) through its SH3 domains (17). As an essential component of BCR-mediated signaling events, BLNK is indispensable for the development of mature B cells (18). Despite this potential importance, the molecular processes that CIN85 is involved in within B cells are still unknown.

Although CIN85 itself lacks any enzymatic activity, it takes part in intracellular signaling through its multiple protein interaction sites (19). Hence, the identification of CIN85's binding partners is a key step in understanding its action, and in this study we sought to identify CIN85-associated proteins in B cells. Especially we searched for a potential mechanism to explain CIN85's ability to inhibit the PI3K pathway. To meet this challenge, we employed a pull-down approach to screen for CIN85 binding partners in WEHI 231 B cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Stimulation, and Transfection—**Murine Ba/F3 B cells (#ACC 300, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were cultured in RPMI 1640 (Biochrom AG, Berlin, Germany) supplemented with 10% fetal calf serum (FCS) (Invitrogen, Karlsruhe, Germany), 50 μM β-mercaptoethanol and 25 μM recombinant murine IL-3 (rIL-3, Biomol, Hamburg, Germany) (20). Murine WEHI 231 B cells (#85022107, European Collection of Cell Cultures) were grown in high glucose Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS and 10% FCS (25). The murine WEHI 231 B cells were transfected in suspension by electroporation with 10 μg of plasmid DNA. Human Jurkat T cells, clone E6.1 (#88042803, European Collection of Cell Cultures) were grown in Iscove's modified Dulbecco's medium (DMEM, Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS and 10% FCS (25). Additionally, human Jurkat T cells were transfected with 10 μg of plasmid DNA. Simian COS-7 cells (#ACC 60, German Collection of Microorganisms and Cell Cultures) were grown in high glucose Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS and 50 μM β-mercaptoethanol (21). For BCR cross-linking WEHI 231 cells were incubated with 20 μg/ml goat anti-mouse IgM antibodies (Abs) (Dianova GmbH, Hamburg, Germany) for 5 min. For expression of recombinant protein 1 × 10⁷ WEHI 231 cells were transfected by electroporation with 10 μg of plasmid DNA. Simian COS-7 cells (#ACC 60, German Collection of Microorganisms and Cell Cultures) were cultured in DMEM containing 10% FCS (22). For expression of recombinant protein 1 × 10⁷ COS-7 cells at 80% confluence were transfected in suspension by electroporation with 10 μg of plasmid DNA. Human Jurkat T cells, clone E6.1 (#88042803, European Collection of Cell Cultures), were grown in RPMI 1640 supplemented with 10% FCS (23). Murine FDCP-1 myeloid precursor cells (#ACC 368, German Collection of Microorganisms and Cell Cultures) were cultured in Iscove's modified Dulbecco's medium (IMDM, Invitrogen, Karlsruhe, Germany) containing 10% FCS and 25 μM recombinant murine IL-3 (24). Murine erythroleukemia EL4-I-1 cells (#ACC 44, German Collection of Microorganisms and Cell Cultures) were cultured out in alpha Minimum Essential Medium (α-MEM, Invitrogen, Karlsruhe, Germany) and 10% FCS (25). The murine erythroid cell line J2E (a kind gift from S. Peter Klinken, Laboratory for Cancer Medicine, Western Australian Institute for Medical Research, Perth, Australia) was grown in DMEM supplemented with 10% FCS (26). Incubation of all cell lines was performed in a 5% CO₂ atmosphere at 37 °C.

**Retroviral Infection of WEHI 231 Cells and Generation of Transgenic Cell Clones—**Retroviral particles were generated using cDNAs subcloned into the pMSCVpuro retroviral expression vector (Clontech, Saint-Germain-en-Laye, France) as described earlier (27). Two days post infection, WEHI 231 cells were selected for resistance against puromycin (0.4 μg/ml). Stable cell clones obtained by limiting dilution were maintained in the presence of puromycin (0.2 μg/ml) and verified for protein expression by immunoblotting and flow cytometry.

**Preparation of Primary Splenic B Cells—**Primary splenic B cells were isolated from male 6–8 week old C57BL/6 mice by negative selection using the MACS® technique in accordance with the manufacturer's instructions (B Cell Isolation Kit, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Purity was >90% as confirmed by expression levels of CD19 and CD45R (B cell markers).

**DNA Constructs and Recombinant Proteins—**Amino-terminally myc-tagged CIN85 cDNA (myc-CIN85) was subcloned into the pCMV-Tag3 mammalian expression vector (Stratagene Europe, Amsterdam, Netherlands) and transfected into COS-7 cells. After 48 h cells (2–3 × 10⁷) were lysed in 1 ml PBS containing 1% (v/v) Triton X-100 and protease inhibitors (“Complete”, Roche Applied Science, Mannheim, Germany) for 30 min at 4 °C. Nuclei were pelleted by centrifugation (18,000 × g, 20 min). The total protein concentration of the lysate was adjusted to 2 mg/ml based on the QuantiPro™ BCA Assay (Sigma-Aldrich, Munich, Germany). Recombinant myc-CIN85 protein (Sigma) was purified from 1 ml of lysate by binding to anti-myc Ab-coupled agarose (Santa Cruz Biotechnology, Heidelberg, Germany) for 3 h at 4 °C. Molecular weight and purity of the immobilized myc-CIN85 were verified by SDS-PAGE and silver-staining. GST-fusion proteins containing either the SH3A, SH3B, or SH3C domain of CIN85 were generated in BL21 (DE3) bacteria (Stratagene Europe, Amsterdam, Netherlands) using the pGEX-2TK expression vector (GE Healthcare, Munich, Germany). Lysis of bacteria was performed using the Bacterial Protein Extraction Reagent (THERMO FISHER SCIENTIFIC, Bonn, Germany) supplemented with 1 mg/ml Lysozyme and protease inhibitors (“Complete”, Roche Applied Science, Mannheim, Germany) following the manufacturer's instructions. GST-tagged proteins were purified by binding to glutathione-linked agarose. Molecular weight, purity, and yield of recombinant proteins were verified by SDS-PAGE and Coomassie-staining.

**Affinity Chromatography, Gel Electrophoresis, and Protein Staining—**5 × 10⁶ WEHI 231 or Ba/F3 cells were lysed in 5 ml PBS containing 1% (v/v) Triton X-100 and “Complete” mixture of protease inhibitors for 30 min at 4 °C. Nuclei were pelleted by centrifugation (18,000 × g, 20 min) and supernatants were adjusted to 2 mg/ml based on the QuantiPro™ BCA Assay (Sigma-Aldrich, Munich, Germany). For GST-tag pull-downs 1 ml of lysates were precleared with agarose coupled to the pure GST-tag for 1 h (4 °C), and supernatants were mixed with 100 μg agarose-linked GST-SH3A, SH3B, or SH3C proteins. For myc-tag pull-downs 1 ml of lysates were precleared with agarose beads coupled to anti-myc antibodies for 1 h (4 °C), and supernatants were mixed with immobilized myc-CIN85 protein as bait. Additional pull-downs from precleared lysates using beads without any CIN85 protein sequences served as negative controls and were analyzed in parallel. After 3 h of gentle agitation (4 °C) unbound molecules were washed off, and proteins were extracted by heating at 98 °C for 5 min in 80 μl of sample buffer (2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, 0.05% (w/v) bromphenol blue). Proteins were separated by SDS-PAGE and visualized by silver staining using a protocol compatible with mass spectrometric protein identification (28). Each lane was cut into 32 gel pieces for subsequent protein identification.

**Liquid Chromatography (LC)-Electrospray Ionization (ESI)-Tandem Mass Spectrometry (MS/MS)—**Protein bands were excised from silver-stained gels, washed with 50% (v/v) acetonitrile in 50 mM ammonium bicarbonate, dehydrated in acetonitrile, and dried in a vacuum centrifuge. The dried gel pieces were reswollen in 15 μl of 50 mM ammonium bicarbonate containing 60 ng trypsin (sequencing grade, Promega, Mannheim, Germany). After 17 h incubation at 37 °C, 15 μl of
of 0.3% trifluoroacetic acid in acetonitrile was added and the separated supernatant was dried under vacuum. For nanoLC-MS/MS, the samples were dissolved in 6 μl of 0.1% (v/v) trifluoroacetic acid, 5% (v/v) acetonitrile in water.

LC-MS measurements were performed on a quadrupole orthogonal acceleration time-of-flight mass spectrometer Q-TOF Ultima (Micromass, Manchester, UK) equipped with a Z-spray nanoelectrospray source and a CapLC liquid chromatography system (Waters GmbH, Eschborn, Germany). 2–5 μl of the sample were injected and concentrated on a precolumn (PepMap C18, 5 μm, 100 Å, 5 mm × 300 μm i.d., Dionex, Idstein, Germany). LC-separations were performed on a capillary column (Atlantis dC18, 3 μm, 100 Å, 150 mm × 75 μm i.d., Waters GmbH) at an eluent flow rate of 200 nl/min using a linear gradient of 4–70% B in 100 min. Mobile phase A was 0.1% formic acid (v/v) in acetonitrile-water (3:97, v/v) and B was 0.1% formic acid in acetonitrile-water (8:2, v/v). The mass spectrometer was operated in the positive ion mode using PicoTip spray capillaries (New Objective, Woburn, MA, USA). MS/MS data were acquired in a data-dependent mode using MS survey scanning followed by MS/MS of the most abundant peak. Data analysis was performed using MassLynx version 4.0 software (Micromass-Waters).

RESULTS

BCR Cross-linking Increases Binding of CIN85 to Cbl—At first we determined if CIN85-related events, such as the RTK-induced association between CIN85 and the ubiquitin ligase Cbl, also occur in B cells. Using increasing concentrations of anti-IgM Abs, BCRs were cross-linked in WEHI 231 cells and Cbl was immunoprecipitated from cytosolic extracts and subjected to Western blot analysis using various Abs. Cell stimulation was verified by probing the tyrosine phosphorylation status of Cbl (Fig. 1). Re-probing the blot with anti-Cbl Abs confirmed similar precipitation efficiencies. Association of CIN85 and Cbl was low in unstimulated cells, but increased with BCR activation. As a control, the ligand-induced binding of the adaptor protein Grb2 to Cbl was also positive in agreement with previous observations (30). These data suggest that CIN85 might play a role in B cell signaling.

CIN85 could be Overexpressed in COS-7 Monkey Cells but not in WEHI 231 Mouse Cells—To gain insight into which pathways CIN85 might affect after BCR activation we wanted to identify proteins that interacted with it in B cells. We first planned to directly overexpress a myc-epitope tagged version of CIN85 (Fig. 2) by transfection of WEHI 231 cells. Surprisingly, we could not detect any myc-tagged protein in these cells. To verify this observation, we transfected WEHI 231 cells with different cDNAs coding for CIN85 along with positive and negative controls. To exclude an impact of the position of the myc-tag we used cDNAs coding for an untagged CIN85 and a C-terminally tagged CIN85 in addition to the N-terminally tagged protein (Fig. 2). As a proof of successful transfection we also included an EGFP-coding reporter vector (pEGFP-N2, Clontech). 48 h post transfection total cell extracts were subjected to immunoblotting. As shown in Fig. 3,

Molecular & Cellular Proteomics 10.10

CIN85 Interacts with SHIP-1 in B Cells

![CIN85 Interacts with SHIP-1 in B Cells](image-url)

**Fig. 1. Binding of CIN85 to Cbl correlates with activation of the BCR in WEHI 231 B cells.** The BCR of WEHI 231 cells was activated through cross-linking with the indicated concentrations of anti-IgM Abs for 5 min. Cellular extracts were immunoprecipitated for Cbl and protein complexes subjected to immunoblotting for phosphorylase (to detect phosphorylated Cbl), Cbl (reprobe), CIN85, and Grb2.

**Table 1. Binding of CIN85 to Cbl correlates with activation of the BCR in WEHI 231 B cells.**
CIN85 expression constructs worked well when transfected in COS-7 cells by the same transfection method (Fig. 3). Equal loading of immunoblots was verified by probing for β-actin. The failure to overexpress CIN85 in WEHI 231 B cells could result from growth inhibition and/or from apoptosis of the successfully transfected portion of cells, that might be because of the negative regulating effect of CIN85 described earlier in other cells (4).

**Identification of CIN85-Associated Proteins by Mass Spectrometry**—Although the failure to overexpress CIN85 in WEHI 231 cells further points to a particular role of this protein in B cells, its direct overexpression was not applicable for identification of interacting proteins in these cells. Instead, we expressed N-terminally myc-tagged CIN85 (Fig. 2) in COS-7 cells and immobilized it to anti-myc Abs coupled to agarose beads. This recombinant myc-CIN85 was used as bait in pull-down assays with lysates from WEHI 231 B cells. The coprecipitated proteins were separated by SDS-PAGE, silver stained and identified by peptide sequencing using LC-ESI-MS/MS (Fig. 4). Pull-downs and analyses were repeated independently and only proteins were listed that were identified in two experiments. A negative control was performed to account for potential unspecific binding of proteins to the agarose beads as well as interactions between B cell lysates and proteins from COS-7 cells potentially bound to the beads. For this control α-myc-antibody linked agarose incubated with lysates from untransfected COS-7 cells was used as bait. When the number of unique peptides identified using CIN85 as bait was not at least 5 times greater compared with the control, proteins were excluded from the hit list.

Through this approach we could identify 25 CIN85-interacting proteins including the SH2-containing inositol phosphatase 1 (SHIP-1). Furthermore, Myosin 9 heavy chain, hematopoietic progenitor kinase 1 (HPK1), Rhotekin-2, Spectrin α, α-Actin 4, RAD50, and IMP-dehydrogenase 1 were found as novel binding partners of CIN85 (Table I).

To exclude that the binding partners solely arose from COS-7 cells used for myc-CIN85 expression, amino acid sequences of identified peptides were additionally checked for their species affiliation. For this analysis it was considered that the UniProtKB/Swiss-Prot protein database is biased toward entries for mouse or human proteins as compared with African green monkey, which is the source of the COS-7 cells. Assuming that the African green monkey is genetically closer related to humans compared with mice, we checked for each binding protein, if it was identified by peptides that match to a mouse protein but not to its human orthologous counterpart. Such peptides would clearly derive from mouse cells and most likely not from the COS-7 host cells used for myc-CIN85 expression. The detailed species analysis showed that all identified proteins pulled-down from WEHI 231 cell extracts with myc-CIN85 as bait originated from mice. In a control experiment the purity of the bait was checked by SDS-PAGE and silver staining (supplemental Fig. S2). Besides the ex-
pected proteins of the bait complex (CIN85, 85 kDa and light/heavy immunoglobulin chains, 25/50 kDa) we detected a noticeable band of ~160 kDa. We assume that additional faint bands observed below 50 kDa mostly result from degradation of the antibody because of the large amount of protein. The 160 kDa protein could be identified as ARAP1, originated from COS-7 cells. However, a detailed sequence analysis of the proteins pulled down from WEHI 231 cell lysates revealed that both the murine and the simian orthologous protein of ARAP1 (based on a close genetic relationship to humans) was present in precipitated complexes. Therefore, it can be concluded that in murine B cells endogenous ARAP1 readily associates with CIN85.

Identification of Proteins Binding to Individual SH3 Domains of CIN85 by Mass Spectrometry—It has been reported that CIN85 can homodimerize, not only through its coiled-coil region but also via its SH3A and SH3B domains to proline-rich segments within CIN85 (31). We therefore postulated that the SH3 domains of other proteins may displace CIN85’s SH3 domains. This in turn would allow the SH3 domains of CIN85 to interact with other proteins, as shown for the p85 subunit of the PI-3-kinase (32). Therefore, it would be conceivable that a broader spectrum of proteins could interact with the free SH3 domains of CIN85, compared with the dimerized full-length molecule. To test this possibility we generated different GST fusion proteins, each containing one of the three SH3 domains of CIN85 (Fig. 2). Pull-downs from WEHI 231 lysates and identification of coprecipitated proteins were carried out as described for the full-length CIN85. Again, pull-downs and analyses were repeated independently and only proteins were listed that were identified in two experiments. A control was included using glutathione-linked agarose coupled to the GST domain alone without any CIN85 protein sequences to verify that proteins did not bind unspecific to the agarose beads. When the number of unique peptides identified using the bait was not at least 5 times greater compared with the control, proteins were excluded from the hit list. Through this approach we could identify 50 proteins, which confirm CIN85 itself, Cbl, Alix, ARAP1, Dynamin-2, BLNK, and SHIP-1 as binding partners of CIN85 as known from other cellular systems, for example (14, 17, 32–35). Moreover, Net1, BCAP, HPK1, Rhotekin-2, PP1α, and CHTF8 were identified as novel interaction partners for CIN85’s SH3 domains (Table I).

Because the SH3 domains of CIN85 were shown to bind to PxxxPR and Px(P/A)xxR motifs (11, 12), one may expect an enrichment of proteins containing these sequence motifs. To check this, a statistical analysis of the relative frequency distribution of both motifs was done for the proteins pulled down

![Diagram of proteomics approach](image-url)
### Table I

List of proteins defined as potential interaction partners of full-length CIN85 or individual SH3 domains. Recombinant myc-tagged CIN85 was immobilized on agarose beads as bait for pulling down of proteins from cell lysates. For the identification of proteins binding to the individual SH3 domains A, B, and C of CIN85 (Fig. 2), GST-tagged SH3 domains were immobilized on glutathione-coated agarose beads. Interacting proteins were pulled down from extracts of WEHI 231 cells, separated on polyacrylamide gels, and identified by mass spectrometry. Pull-downs and analyses were repeated independently and only proteins were listed that were identified in two experiments. To verify that proteins did not bind unspecific to the agarose beads, control pull-downs were performed in parallel using the respective coated agarose matrix, but without any CIN85 protein sequences. When the number of unique peptides identified using the bait was not at least five times greater compared to the control, proteins were excluded from the hit list. Proteins were grouped according to their primary functions as known so far. The number of the predicted CIN85-SH3 binding motifs is given for each protein listed.

| No. | Protein name | Swiss-Prot accession | myc-CIN85 (full-length) | GST-SH3 A | GST-SH3 B | GST-SH3 C | CIN85-SH3 binding motifs |
|-----|--------------|----------------------|-------------------------|-----------|-----------|-----------|--------------------------|
| 1   | SHIP-1       | Q9ES52               | x                       | x         | x         | x         | 6                        |
| 2   | SHIP-2       | Q6P549               | x                       | x         | x         | x         | 6                        |
| 3   | Cbl          | Q3U527               | x                       | x         | x         | x         | 4                        |
| 4   | Cbl-b        | Q3TTA7               | x                       | x         | x         | x         | 9                        |
| 5   | BLNK         | Q9QUN3               | x                       | x         | x         | x         | 5                        |
| 6   | HPK1         | P70218               | x                       | x         | x         | x         | 5                        |
| 7   | STAP1        | Q8JNM90              | x                       | x         | x         | x         | 1                        |
| 8   | Sam68        | Q60749               | x                       | x         | x         | x         | 4                        |
| 9   | BCAP         | Q9EQ32               | x                       | x         | x         | x         | 3                        |
| 10  | PI3-kinase, p85α | P26450           | x                       | x         | x         | x         | 1                        |
| 11  | PI3-kinase, p110β | Q8BS514       | x                       | x         | x         | x         | 1                        |
|     |              |                      |                         |           |           |           |                          |
|     | Receptor linked signal transduction |               |                         |           |           |           |                          |
| 12  | B99/Gtse1    | Q542Q3               | x                       | x         | x         | x         | 5                        |
| 13  | PP1α         | P62137               | x                       | x         | x         | x         | 1                        |
|     | Signaling of the cytoskeleton and membrane traffic |               |                         |           |           |           |                          |
| 14  | ARAP1        | Q4LDD4               | x                       | x         | x         | x         | 10                       |
| 15  | ASAP1        | Q505F0               | x                       | x         | x         | x         | 2                        |
| 16  | Nadrin       | Q3UIA2               | x                       | x         | x         | x         | 4                        |
| 17  | 3BP-1        | P55194               | x                       | x         | x         | x         | 3                        |
| 18  | Net1 (Argef8) | Q6Z206              | x                       | x         | x         | x         | 4                        |
| 19  | Rhotekin-2   | Q14B46               | x                       | x         | x         | x         | 2                        |
| 20  | Spectrin α   | P16546               | x                       | x         | x         | x         | 0                        |
| 21  | α-Actinin 4  | P57780               | x                       | x         | x         | x         | 0                        |
|     | Vesicle formation/vesicular transport/endocytosis |               |                         |           |           |           |                          |
| 22  | CIN85        | Q8R550               | x                       | x         | x         | x         | 2                        |
| 23  | Myosin-9 heavy chain (Ila) | Q6VD55         | x                       | x         | x         | x         | 0                        |
| 24  | CIN85        | Q8R550               | x                       | x         | x         | x         | 2                        |
| 25  | Hip1-R       | Q6Q77                 | x                       | x         | x         | x         | 2                        |
| 26  | Alix         | Q6ZPS9                | x                       | x         | x         | x         | 2                        |
| 27  | AP-2 α1      | P17426                | x                       | x         | x         | x         | 0                        |
| 28  | AP-2 α2      | P17427                | x                       | x         | x         | x         | 1                        |
| 29  | AP-2 β1      | Q6DB3G                | x                       | x         | x         | x         | 1                        |
| 30  | Ap-2 µ1      | Q6AC9                 | x                       | x         | x         | x         | 0                        |
| 31  | MIRab13      | Q8BG76                | x                       | x         | x         | x         | 3                        |
| 32  | Septin-9 (Sint1) | Q8UOG5           | x                       | x         | x         | x         | 3                        |
| 33  | Clathrin heavy chain | Q6FDS6            | x                       | x         | x         | x         | 0                        |
| 34  | Tsg101       | Q3UCW0                | x                       | x         | x         | x         | 0                        |
|     | Cytoskeletal rearrangement |               |                         |           |           |           |                          |
| 35  | WIP          | Q8K117                | x                       | x         | x         | x         | 9                        |
| 36  | N-WASP       | Q91YD9                | x                       | x         | x         | x         | 6                        |
| 37  | Septin-2     | P42208                | x                       | x         | x         | x         | 0                        |
| 38  | Septin-6     | Q9R1T4                | x                       | x         | x         | x         | 0                        |
| 39  | Septin-7     | Q8C23A                | x                       | x         | x         | x         | 1                        |
|     | Expression control |               |                         |           |           |           |                          |
| 40  | UBF-1        | Q8DBH1                | x                       | x         | x         | x         | 0                        |
|     | General metabolism |               |                         |           |           |           |                          |
| 41  | Dbt          | Q7TND9                | x                       | x         | x         | x         | 2                        |
| 42  | IMP-dehydrogenase 1 | P50096           | x                       | x         | x         | x         | 0                        |
| 43  | Glutaryl-CoA-DH| Q60759              | x                       | x         | x         | x         | 0                        |
| 44  | Isocitrate-DH 3β | Q91VA7              | x                       | x         | x         | x         | 0                        |
| 45  | Fatty acyl-CoA reductase 1 | Q6S22J9         | x                       | x         | x         | x         | 0                        |
|     | Protein repair |               |                         |           |           |           |                          |
| 46  | RAD50        | P70388                | x                       | x         | x         | x         | 0                        |
|     | Transmembrane transport |               |                         |           |           |           |                          |
| 47  | Aralar1      | Q6BH59                | x                       | x         | x         | x         | 1                        |
| 48  | TIM50        | Q8DBD0               | x                       | x         | x         | x         | 0                        |
|     | Unknown function |               |                         |           |           |           |                          |
| 49  | SETA-binding protein 1 | Q6P72W2        | x                       | x         | x         | x         | 5                        |
| 50  | CHTF8        | P0CG14                | x                       | x         | x         | x         | 12                       |
| 51  | Irf2bp1      | Q8R3Y8                | x                       | x         | x         | x         | 3                        |
CIN85 Interacts with SHIP-1 in B Cells

CIN85-Associated Proteins Show Different Binding Capabilities for Individual SH3 Domains—Next we studied which of the different SH3 domains of CIN85 conferred binding to the discovered interacting proteins. Using GST fusion proteins with individual SH3 domains of CIN85, 10 proteins with profound function in B-cell signaling were examined for their interaction by immunoblotting (Fig. 6A). In control experiments none of the proteins bound to the GST-linked agarose alone (negative control). CIN85 could be pulled down by any of its three SH3 domains, but binding affinity to the amino-terminal SH3A domain was much higher than to SH3B and SH3C (Fig. 6A). This binding hierarchy was also found for the B-cell adaptor for PI3K (BCAP) (Fig. 6A), a protein that links PI3K to the activated BCR (36). The Neural Wiskott-Aldrich syndrome protein (N-WASP), an adaptor protein involved in signal transduction and RNA metabolism (41), mostly interacted with the SH3A domain; whereas a paralog of N-WASP, WASP, was preferentially bound by CIN85’s SH3A and SH3B domains (Fig. 6A). Both WASP and N-WASP are components of signaling pathways that control organization of the actin cytoskeleton and RNA metabolism (41), mostly interacted with the SH3A domain; whereas a paralog of N-WASP, WASP, was preferentially bound by CIN85’s SH3A and SH3B domains (Fig. 6A). Both WASP and N-WASP are components of signaling pathways that control organization of the actin cytoskeleton.

CIN85 and SHIP-1 are Constitutively Associated in B cells—Several of the proteins that could be identified as CIN85 binding partners are involved in pathways of actin remodeling, vesicle trafficking, cytoskeletal rearrangement, and surface receptor linked signal transduction (Table I). In particular, we were interested in the observed association of CIN85 with SHIP-1.

by the CIN85-SH3 domains (altogether), and for all mouse entries of the UniProtKB/Swiss-Prot protein database (release 2010_09 of 10-Aug-10, contains 519348 sequence entries) (Fig. 5). In the latter case, as expected, analysis of the entire mouse proteome showed a nearly evenly declining distribution for both of the PxxxPR or Px(P/A)xR motifs. By contrast, frequencies were clearly elevated for the proteins we identified in our CIN85-SH3 pull-down experiments using GST-CIN85-SH3A, -B and -C domain fusion proteins (continuous lines). For a direct comparison the relative frequency distributions were determined by normalizing the frequency values to the number of proteins in the data sets.

CIN85-SH3 domain interacting proteins comprise PxxxPR and Px(P/A)xR motifs. The frequency distributions of the CIN85-SH3 domain binding motifs PxxxPR and Px(P/A)xR were calculated for all mouse proteins of the UniProtKB/Swiss-Prot protein database (release 2010_09 of 10-Aug-10, contains 519348 sequence entries, dashed lines). In the next step these data were compared with the identified proteins from the pull down experiments using GST-CIN85-SH3A, -B and -C domain fusion proteins (continuous lines). For a direct comparison the relative frequency distributions were determined by normalizing the frequency values to the number of proteins in the data sets.
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Fig. 7. Expression and interaction of CIN85 and SHIP-1 in hematopoietic cells. A. Expression of CIN85 and SHIP-1 proteins in myeloid FDCP-1 cells, erythroid J2E and ELM-I-1 cells, B cells (Ba/F3, WEHI 231, primary mouse splenic B cells) and Jurkat T cells. Additionally, β-actin was detected as loading control. B, SHIP-1 was immunoprecipitated from cytosolic fractions of WEHI 231 cells incubated with or without anti-IgM Abs (20 μg/ml) for 5 min. Association with CIN85 was proved by immunoblotting. Comparable amounts of SHIP-1 showed equal pull-down efficiencies. BCR activation was verified by detection of phosphorylated SHIP-1 in the precipitates and of phosphorylated Erk in crude cytosolic lysates. C, Proteins from cytosolic fractions of untreated WEHI 231 cells were immunoprecipitated using Abs recognizing different epitopes of CIN85. The precipitation of CIN85 and SHIP-1 were checked by immunoblotting.

To elucidate if the CIN85-SHIP-1 interaction is regulated in activated B cells, we stimulated WEHI 231 cells by BCR cross-linking with anti-IgM Abs and immunoprecipitated SHIP-1. Western analysis revealed that BCR cross-linking induced rapid intracellular signaling as demonstrated by phosphorylation of the precipitated SHIP-1 and phosphorylation of Erk (Fig. 7B). Importantly, SHIP-1 coprecipitated constant levels of CIN85 irrespective of BCR activation (Fig. 7B). This constitutive interaction is in agreement with observations we made using transformed mast cells, where CIN85 co-precipitated with exogenously expressed HA-tagged SHIP-1 at constant ratios, regardless of steel factor stimulation (unpublished observation, G. Krystal).

Immunoprecipitation of CIN85 from WEHI 231 cells by two different anti-CIN85 Abs confirmed the interaction with SHIP-1 by immunoblotting (Fig. 7C). Noteworthy, the 179.1.E1 anti-CIN85 monoclonal Ab, which specifically binds to the SH3 domains (45), failed to coprecipitate SHIP-1. This finding provides cumulative evidence that interaction between CIN85 and SHIP-1 occurs via SH3 domains.

CIN85 Binds to the Carboxyl Terminus of SHIP-1—To determine which motif(s) of the SHIP-1 protein were responsible for binding to CIN85, we first examined murine SHIP-1 for the presence of the atypical PxxPxR or Pxx(P/A)xxR motifs preferred by the SH3 domains of CIN85 (11, 12). Two of these motifs could be identified at the amino terminus and four at the carboxyl terminus of SHIP-1. To localize the region essential for interacting with CIN85 we employed a series of SHIP-1 truncation mutants expressed in WEHI 231 cells (Fig. 8). To distinguish overexpressed from endogenous protein, the ectopic expressed SHIP-1 was fused to an HA-tag at its amino terminus and an EGFP-tag at its carboxyl terminus. From this double-tagged wild-type (WT) form mutant proteins were derived: (1) the 2NPXF mutant possesses two phenylalanines in lieu of the two tyrosines within the two NPxY sites of SHIP-1, disabling the recruitment of phosphotyrosine binding domains (PTB). (2) The T5 mutant lacks the carboxy-terminal CIN85-SH3 domain binding motif PxxPxxR. (3) The T4 mutant lacks two more of these PxxPxxR motifs. (4) Finally, the T3 mutant lacks all four potential CIN85 binding sites at SHIP-1’s carboxyl terminus.

These wild-type or mutant forms of SHIP-1 were expressed in WEHI 231 cells by retroviral infection generating stable cell clones after antibiotic selection. All stable cell clones used in this study expressed the recombinant SHIP-1 protein as demonstrated by immunoblotting for the HA- and the EGFP-tag (data not shown). Exogenous SHIP-1 was immunoprecipitated with agarose-linked anti-HA Abs. As expected, the full-length protein and its truncated mutants could be discriminated by their molecular weight when immunoblotted for the EGFP-tag (Fig. 9). However, CIN85 could only be found in protein complexes containing the full-length variants of SHIP-1 (WT and 2NPXF) as well as the truncation mutant T5. In contrast, the truncation mutants T4 and T3 showed no
It was the aim of this study to identify CIN85-associated proteins in B cells. By combining a pull-down approach and protein mass spectrometry, we have identified several CIN85-associated proteins that may play a role in regulating B cell functions. Specifically, we have identified 51 proteins that bind to full-length CIN85 and/or to one or more of its SH3 domains (Table I, Fig. 6). These proteins confirm previous observations in other cellular systems like Cbl, BLNK, Alix, ARAP1, Dynamin-2, Sam68, and Irf2bp1 (14, 17, 33, 35). In addition, we found several proteins not specified as CIN85-associated proteins before, such as Myosin 9 heavy chain, HPK1, Rhoetkin-2, Spectrin α, α-Actinin 4, Net1, and BCAP.

Several of the proteins binding to the SH3 domains of CIN85 in WEHI 231 B cells confirm previous observations in HeLa cells published by Havrylov and coworkers (35). The remarkable consensus for the identification of ARAP1, ASAP1, Dynamin-2, MIRab13, N-WASP, WIP, Septin family proteins (-2, -6, -7, -9), Sam68, Irf2bp1, GTSE1, and SHIP-2 support the significance of the proteomics approach. However, notable differences of protein identifications between these studies also exist and certainly reflect the fundamental different properties of the cellular model used. We verified our mass spectrometry results for 10 of the identified CIN85-binding proteins with profound function in B-cell signaling by immunoblotting (Fig. 6.) using Ba/F3 cells. The obtaining of consistent results with this second B-cell line strengthened the validity of our data.

**Functional Classification of CIN85 Binding Proteins**—The majority of the identified CIN85 interactors in B cells are involved in formation and transport of vesicles, including endosomes or in processes regulating the cytoskeleton and membrane traffic (Table I). In addition, the SH3 domains of CIN85 associate with proteins known for their role in signal transduction of transmembrane receptors (Table I). Proteins of this category show a remarkable accumulation of CIN85-SH3 domain binding motifs (average of 4.1 per protein). This fact clearly suggests a functional role for CIN85 in B cells.

Several proteins that we identified as CIN85 interactors in B cells are regulators of the Ras superfamily of small GTPases, including GTPase activating proteins, GAPs (ARAP1, Nadrin, 3BP-1, Myosin-9, ASAP1), and GDP/GTP exchange factors,
GEFs (Net1). Although the Ras superfamily consists of at least five subfamilies affecting different cellular functions (46), all GTPase regulators in complex with CIN85 control cytoskeletal rearrangement (Rho-GTPases) or vesicle transport (Arf- and Rab-GTPases) (47, 48). In addition, we identified several CIN85 binding proteins that may point to a role of CIN85 in Rho GTPase-regulated actin dynamics including Rhotekin-2, Septin family proteins (e.g. Septin-9), the Wiskott-Aldrich syndrome protein (WASP), and the Neural WASP (N-WASP) (49–52) (Table I, Fig. 6). Both WASP and N-WASP have been shown to alter the actin cytoskeleton by binding the Rho GTPase Cdc42 (49) and to control actin-driven vesicle movement (53). Stability, localization, and functioning of both WASP and N-WASP critically depend on binding to the WASP-interacting protein (WIP) (54, 55), which we also identified in complexes with CIN85 (Table I). It has been shown that WIP and N-WASP couple to activated membrane receptors by the SH3 domain-containing adaptor proteins Nck and Grb2 (56, 57). Intriguingly, N-WASP and WIP comprise six and nine binding motifs specific for CIN85’s SH3 domains, respectively. In the present study we showed that both proteins specifically interact with the most amino-terminal SH3 domain of CIN85 (Fig. 6, Table I). Hence, it is tempting to presume that CIN85 also plays a role in directing WIP and N-WASP to the sites of their action.

Several of the identified CIN85 interactors proved to be crucial for B cell signaling, like SHIP-1, HPK1, Sam68, BCAP and PP1α (40, 58–61). Our study revealed for the first time the interaction of these proteins to CIN85’s SH3 domains in cells naturally expressing them (Fig. 6). In the case of Sam68 it has been demonstrated previously that its function in B cell signal pathways is conferred through its proline-rich domain (59). Notably, this region contains four potential CIN85-SH3 domain binding motifs. Consequently, these data support the idea that CIN85 allows scaffolding of signaling proteins vital for the response to extracellular stimuli in B cells.

Putative Roles for a CIN85/SHIP-1/PI3K Complex—Among our CIN85 binding proteins are two inositol-5’-phosphatases of the SHIP family. SHIP-1 is restricted to hematopoietic cells whereas SHIP-2 is more ubiquitously expressed (62). By degrading the second messenger PIP3 both enzymes play pivotal roles in negatively regulating the PI3K pathway (43). The membrane localization of SHIP proteins is crucial for their phosphatase effect (63), and both SHIP-1 and SHIP-2 provide multiple prerequisites for their translocation to activated receptors, but little is known about the mechanism(s) of their recruitment. In this report we show that CIN85 and SHIP-1 are closely associated in primary and immortalized B cells (Fig. 7, Table I). Therefore, the adaptor protein CIN85 might be an important link in transporting SHIP-1 to the plasma membrane. At the same time, binding of SHIP-1 to SH3 domains of CIN85 could expose other domains by disrupting CIN85’s dimeric formation and thereby convert CIN85 to a more active state. Such a synergistic action seems to be plausible, because it could potentiate the inhibitory effect on the PI3K pathway, which has been verified for each of both proteins (4, 27). Moreover, the interaction with CIN85 could help distinguish the functions of SHIP-1 and SHIP-2 from another PIP3 degrading key phosphatase, PTEN (phosphatase and tensin homolog deleted on chromosome 10) (64, 65). It is assumed that PTEN and SHIP-1/2 have fundamentally different biological functions. Although PTEN appears to be active in unstimulated cells, SHIP-1 and SHIP-2 are not and only become active after extracellular stimulation (66). These profound functional differences could be explained, at least partially, by differences in their binding partners. Related to this, PTEN does not contain any known binding motifs for CIN85 and, consistent with this, PTEN was not detectable in complexes pulled down with full-length CIN85 or with its separate SH3 domains.

Given that SHIP-1 can bind to any of the 3 SH3 domains of CIN85 (Fig. 6), and that PI3K binds to the amino-terminal SH3 domain of CIN85 (Table I), the formation of a ternary complex of these proteins is conceivable. In the activated (monomeric) state, the adaptor CIN85 may act as a scaffold protein to bring SHIP-1 into close proximity to PI3K. In this way, CIN85 could direct SHIP-1 to the site of the formation, and thereby the highest concentration, of its substrate PIP3, effectively increasing SHIP’s reaction rate. We propose this novel mechanism to explain, at least in part, the previously observed inhibitory effect of CIN85 on the PI3K pathway (4). This hypothesis is supported by previous studies showing that reintroduction of full-length SHIP-1 into SHIP-1−/− mast cells reduces the level of Steel Factor (SF)-induced PIP3 and blocks SF-induced degranulation, as expected, but a C-terminal truncation mutant of SHIP-1 (Fig. 8, “T3”), which lacks the structural prerequisites to bind CIN85 (Fig. 9), could not (27). Thus, it is possible that SHIP-1 requires CIN85 to take it to the plasma membrane to hydrolyze PIP3 and thus prevent mast cell degranulation.

Our data also suggest a role for this putative CIN85/SHIP-1/PI3K complex in vesicular trafficking and cytoskeleton organization. PI3K has been shown to contribute, independently of its catalytic activity, to cytoskeletal changes (67). This effect is apparently mediated via association with the small Rho GTPase, Cdc42, and septin 2 (68), a function classically assigned to CIN85. In this way, translocation of SHIP-2 to membrane ruffles has been shown to have a major impact on ligand-induced EGFR endocytosis (68), a function classically assigned to CIN85. In this way, binding of CIN85 to PI3K and/or SHIP1/2 might be important to link PI3K-mediated signaling to vesicular trafficking and cytoskeletal rearrangement.

In summary, through our systematic proteomic approach we have identified known and novel proteins that interact with the cytosolic adaptor protein CIN85 in B cells. Because our data suggest an important role for CIN85 in regulating PIP3 levels in B cells, this study may provide a useful resource to...
CIN85 Interacts with SHIP-1 in B Cells

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CIN85 Interacts with SHIP-1 in B Cells

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