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# Characterization of the CIN85 Adaptor Protein and Identification of Components Involved in CIN85 Complexes

Shinji Watanabe,* Hitoshi Take,* Kazuyo Takeda,† Zu-Xi Yu,†
Nobuhisa Iwata,* and Sachiko Kajigaya*,[1]

**Hematology Branch and †Pathology Section, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892*

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**CIN85 is an 85-kDa adaptor protein whose functions in signaling pathways are presently unknown. Using the yeast two-hybrid screen, the B cell linker protein (BLNK) was identified as a binding partner of CIN85. Coimmunoprecipitation experiments using mammalian cells revealed that CIN85 directly bound to BLNK through its SH3 domains. Immunostaining analysis showed that CIN85 and BLNK were colocalized in the cytoplasm. These results indicate a potential role of CIN85 in the B cell receptor-mediated signaling pathway. It was also found that Crk-I, Crk-II, p130$^{Cas}$, p85-PI3K, Grb2, and Sos1 were components of CIN85 complexes. CIN85 interacted with itself through its coiled-coil region, resulting in formation of a tetramer. Both the coiled-coil region and SH3 domains of CIN85 were responsible for its subcellular localization. Our data suggest that CIN85 may serve for regulation of various signaling events through formation of its diverse complexes.**

***Key Words:*** **BLNK; c-Cbl; CMS; CD2AP; coiled-coils.**

CIN85 is composed of three Src homology 3 (SH3) domains in an $NH_2$-terminal half, a proline-rich region in a central part and a putative $\alpha$-helical coiled-coil region in an extremely COOH-terminal region (1). CIN85 displays sequence and structural similarity to adaptor proteins, Cas ligand with SH3 domains (CMS) and CD2-associated protein (CD2AP) (2, 3). CIN85 was originally identified as a c-Cbl-binding protein which was basally associated with c-Cbl. The association level closely coincides with the phosphorylation levels of specific tyrosine residues (Y700, Y731 and Y774) of c-Cbl following stimulation of epidermal growth factor (EGF).

[1] To whom correspondence should be addressed. Fax: 301-496-8396. E-mail: kajigays@nhlbi.nih.gov.

The B cell linker protein (BLNK) functions as an essential component of the B cell receptor (BCR)-mediated signaling events (4–10). BLNK is expressed throughout the B cell ontogeny, with the highest expression in early development (9). Upon BCR engagement, BLNK is phosphorylated by Syk and interacts with specific molecules (4, 6, 11, 12). BLNK is strikingly homologous to the SLP-76 adaptor protein expressed in T cells (4, 6), sharing common structural features such as a basic region, several tyrosine phosphorylation sites, proline-rich regions and a single SH2 domain.

The c-Cbl protein is a 120-kDa product of the c-*Cbl* proto-oncogene (13). c-Cbl binds the SH2 or SH3 domain-containing proteins (reviewed in Refs. 14–17). c-Cbl is a prominent substrate of protein tyrosine kinases following activation of a wide range of receptors. c-Cbl functions as a negative regulator by interacting with receptor or nonreceptor tyrosine kinases (18–23).

The presence of multiple protein binding sites in CIN85 provides the notion that CIN85 belongs to the growing family of scaffold proteins. To assess the possibility, we here sought to identify other molecules bound to CIN85.

## MATERIALS AND METHODS

*Plasmid construction.* For the two-hybrid screen, pGBT9-CIN85 was constructed using a pGBT9 yeast expression vector (Clontech, Palo Alto, CA) and a full-length open reading frame (ORF) of human CIN85 cDNA which was excised from a pcDNA3-Flag-CIN85 plasmid (1).

For mammalian expression, pcDNA3 (Invitrogen, San Diego, CA) was used as a basic vector. pcDNA3-HA was created to express a protein fused to an HA-tag at its $NH_2$ terminus using two complementary oligonucleotides encoding the Kozak sequence and a HA peptide composed of nine amino acids (aas) derived from hemagglutinin of influenza virus. pcDNA3-HA-CIN85 was constructed by insertion of the full-length ORF of CIN85 cDNA into pcDNA3-HA. pcDNA3-Flag, pcDNA3-c-Cbl and six different plasmids carrying SH3-domain mutants of CIN85 (pcDNA3-Flag-SH3ABC to pcDNA3-



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Flag-SH3C) were described previously (1). pcDNA3-Flag-δCC or pcDNA3-Flag-δSH3 was generated by deletion of a CIN85 DNA fragment encoding aas 595 to 665 (a coiled-coil region) or aas 1 to 321 (SH3 domains) from pcDNA3-Flag-CIN85, respectively. To construct pcDNA3-BLNK or pcDNA3-HA-BLNK, cloning of BLNK cDNA was carried out using a human lymphoma (Raji cells) 5′-stretch plus cDNA library in λgt11 (Clontech) as instructed by the manufacturer. A full-length ORF of BLNK cDNA was inserted into pcDNA3 or pcDNA3-HA.

*Yeast two-hybrid system.* The MATCHMAKER two-hybrid screen (Clontech) was performed to investigate proteins bound to CIN85 using pGBT9-CIN85 and a human B-lymphocyte cDNA library (Clontech) according to the manufacturer's protocols. Nucleotide and amino acid sequence alignments were carried out by screening the databases with the BLAST program.

*Cell culture and transfection.* 293, HeLa and COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), L-glutamine and antibiotics. Transient transfection into these cell lines was performed by use of the SuperFect reagent (QIAGEN, Valencia, CA) according to the manufacturer's instructions. Raji cells were maintained in RPMI 1640 with 10% FBS, L-glutamine and antibiotics. Plasmid transfection into Raji cells was carried out by electroporation.

*Immunoprecipitation and immunoblotting.* At 48 h posttransfection, 293 or HeLa cells were serum-starved in DMEM for 16 h and then treated with or without recombinant human EGF (100 ng/ml; Genzyme, Cambridge, MA) at 37°C for 2 min. Transfected Raji cells were cultured for 48 h, serum-starved and stimulated with 50 $\mu$M pervanadate/$H_2O_2$ at 37°C for 2 min. Cells were treated with a lysis buffer (0.5% Nonidet P-40, 50 mM Tris, pH 7.5, and 150 mM NaCl) containing protease inhibitors.

For immunoprecipitation, cleared cell lysates containing an equal amount of proteins were incubated with specific antibody, or normal mouse immunoglobulin (Ig) or rabbit Ig. Immune complexes were subjected to immunoblot analysis as described previously (1).

*Immunofluorescence microscopy.* COS-7 or HeLa cells grown on 2-well chamber slides (Nunc, Naperville, IL) were transiently transfected with individual plasmids. Cells were fixed, permeabilized and incubated with antibody against Flag, HA or Cbl. The primary antibody was detected with FITC- or rhodamine-conjugated secondary antibody, followed by a confocal laser scanning fluorescence microscope.

*Cross-linking.* 293 cells were transfected with pcDNA3-Flag-CIN85 and cultured for 48 h. Cell lysates were incubated at 4°C for 1 h with or without freshly prepared 3 mM bis(sulfosuccinimidyl)-suberate ($BS^3$; Pierce, Rockford, IL). The reaction was stopped by addition of a final concentration of 50 mM Tris–HCl, pH 7.5, and then incubation for an additional 10 min. Cross-linked products were detected by immunoblot analysis.

*Isolation of Triton X-100-soluble and -insoluble fractions of HeLa cells.* 293 cells were transfected with pcDNA3-Flag-CIN85, cultured for 48 h and subjected to treatment with a lysis buffer (0.5% Triton X-100, 0.3 M sucrose, 10 mM Pipes, pH 6.8, 100 mM KCl, 1 mM $CaCl_2$, 2.5 mM $MgCl_2$ and protease inhibitors). Pellets were washed once more with the same volume of the lysis buffer. The supernatants and pellets were used as Triton X-100-soluble and -insoluble fractions, respectively.

## RESULTS

*Isolation and identification of BLNK as a CIN85-binding protein.* The yeast two-hybrid screen was employed to identify cellular proteins bound to CIN85. Sequential transfection and then screening of cotransformants gave rise to 18 double-positive colonies, four

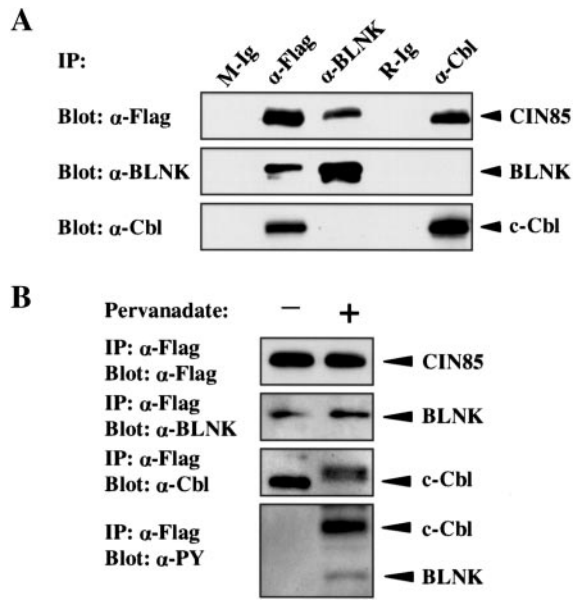


**FIG. 1.** Complex formation of CIN85 with BLNK. Lysates from transfected cells were subjected to immunoprecipitation and immunoblotting with the indicated antibodies. (A) Interaction of CIN85 with BLNK in 293 cells. pcDNA3-BLNK was cotransfected with both pcDNA3-Flag-CIN85 and pcDNA3-c-Cbl. As a control, normal mouse Ig (M-Ig) or rabbit Ig (R-Ig) was used for immunoprecipitation. (B) Interaction of CIN85 with BLNK in Raji cells. After transfection with pcDNA3-Flag-CIN85 and culture for 48 h, cells were stimulated with (+) or without (−) 50 $\mu$M pervanadate/$H_2O_2$. The same immunoblot was stripped and reblotted with anti-phosphotyrosine antibody.

out of which contained inserts (~1.0 kb) showing a 100% match to a COOH-terminal cDNA of human BLNK. Specificity of the binding in the yeast system was verified by control experiments. A full-length ORF of BLNK was isolated by screen of the Raji cDNA library.

To confirm specificity of the interaction in mammalian cells, pcDNA3-BLNK was cotransfected into 293 cells with pcDNA3-Flag-CIN85 or pcDNA3-c-Cbl, subjected to immunoprecipitation and then immunoblot analysis using adequate antibodies (data not shown). From the results, it was revealed that CIN85 interacted with BLNK and that no detectable association between BLNK and c-Cbl was observed, thereby strongly supporting that CIN85 directly bound to BLNK, not via c-Cbl. Further, it was shown that tyrosine phosphorylation of BLNK was undetectable in 293 cells. We next examined whether CIN85 had ability to concurrently interact with both BLNK and c-Cbl since CIN85 had been originally isolated as a c-Cbl-binding protein. pcDNA3-Flag-CIN85 was cotransfected into 293 cells together with pcDNA3-BLNK and pcDNA3-c-Cbl. After the appropriate coexpression of the three proteins was confirmed, aliquots of the same cell lysates were subjected to immunoprecipitation and then immunoblotting (Fig. 1A). When anti-Flag immunoprecipitates were examined by immunoblotting with

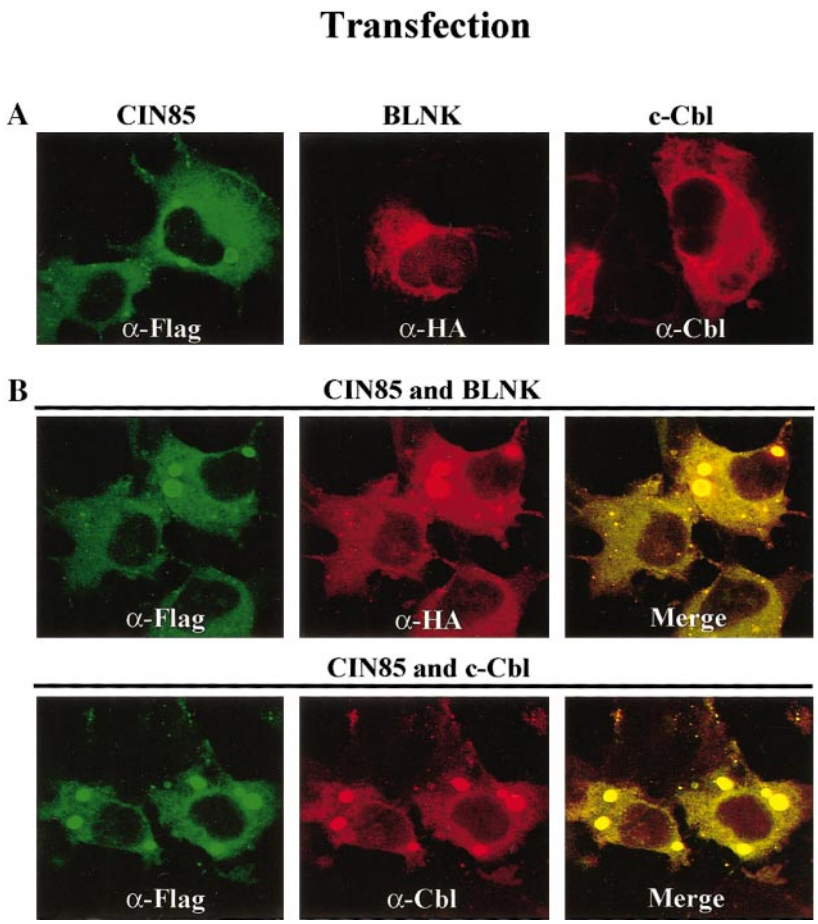


**FIG. 2.** Colocalization study by immunofluorescence microscopy. (A) COS-7 cells were transfected with pcDNA3-Flag-CIN85, pcDNA3-HA-BLNK, or pcDNA3-c-Cbl. (B) pcDNA3-Flag-CIN85 was cotransfected into COS-7 cells with pcDNA3-HA-BLNK (top) or pcDNA3-c-Cbl (bottom). Cells stained with the indicated primary antibodies were visualized with the following secondary antibodies: anti-Flag antibody, FITC-conjugated antibody; anti-Cbl or anti-HA antibody, rhodamine-conjugated secondary antibody. Yellow-orange color in merged images represents colocalization of proteins.

antibodies against BLNK and c-Cbl, these two proteins were readily detected on the blot, with an almost equal level. In reciprocal experiments, CIN85 was clearly detected in both anti-BLNK and anti-Cbl immunoprecipitates. In contrast, c-Cbl and BLNK were rarely detectable in anti-BLNK and anti-Cbl immunoprecipitates, respectively.

It should be important to ensure the association of CIN85 with BLNK in B cells since BLNK is assumed to be expressed specifically in B cells among human cells so far. For this purpose, Raji cells were transfected with pcDNA3-Flag-CIN85 by electroporation. To examine whether the CIN85-BLNK association is affected by BCR activation, cells were incubated with or without pervanadate prior to immunoprecipitation with anti-Flag antibody. Immunoblotting was carried out with antibody against Flag, BLNK, Cbl or phosphotyrosine (Fig. 1B). Both BLNK and c-Cbl were detected in anti-Flag immunoprecipitates, regardless of stimulation. Pervanadate stimulation induced tyrosine phosphorylation of BLNK and simultaneously facilitated the CIN85-BLNK association. The c-Cbl immunoreactive band became broader upon stimulation, with a slightly higher range of molecular sizes, probably due to phosphorylation. This caused difficulty to assess a relative level of the CIN85-c-Cbl association before and after stimulation.

*Colocalization of CIN85 with BLNK or c-Cbl.* Immunofluorescence microscopy was employed to examine whether CIN85 was colocalized with BLNK. When CIN85, BLNK or c-Cbl alone was expressed in COS-7 cells, CIN85 was present diffusely, but with various numbers and sizes of punctate structures, in the cytoplasm, while BLNK or c-Cbl was uniformly distributed throughout the cytoplasm (Fig. 2A). Coexpression of either BLNK or c-Cbl with CIN85 was accompanied by massive redistribution and accumulation of BLNK or c-Cbl into the punctate structures of CIN85, respectively, supporting its colocalization with CIN85 (Fig. 2B, top and bottom). On the other hand, when BLNK was coexpressed with c-Cbl, the two proteins were

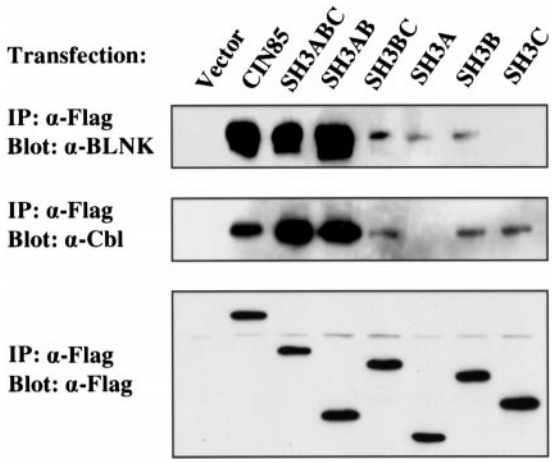


**FIG. 3.** Association of the SH3 domain-containing CIN85 mutants with BLNK. pcDNA3-BLNK was cotransfected into 293 cells with individual CIN85 mutant (SH3ABC to SH3C) plasmids, pcDNA3-Flag-CIN85, or pcDNA3-Flag (vector). After immunoprecipitation with anti-Flag antibody, immunoblot analysis was performed with antibody against BLNK or Cbl (an 8% gel), or Flag (a 15% gel). The mutant carrying the first CIN85 SH3 domain was termed SH3A and other mutants were designated in a similar manner as mentioned earlier (1).

evenly localized in the cytoplasm, without any punctate structures (data not shown). Similar results were obtained using HeLa and 293 cells (data not shown).

*Interaction of BLNK with the CIN85 SH3 domains.* To delineate a CIN85 region responsible for interaction with BLNK, coimmunoprecipitation experiments were carried out using a set of truncated CIN85 mutants, indicating that the CIN85 SH3 domains alone were sufficient for the interaction (data not shown). Next, to evaluate the contribution of individual CIN85 SH3 domains to the CIN85-BLNK association, pcDNA3-BLNK was cotransfected into 293 cells in combination with pcDNA3-Flag-CIN85, or each of the plasmids expressing CIN85 mutants which encompassed individual or combined SH3 domains (Fig. 3). Anti-Flag immunoprecipitates prepared from these transfectants were resolved by immunoblotting with anti-BLNK antibody. SH3ABC and SH3AB mutants displayed similar or higher levels of abilities for binding to BLNK compared to the wild-type CIN85, whereas binding abilities of SH3BC, SH3A and SH3B were much lower, with the lowest ability of SH3A. Of note, no appreciable binding was observed between SH3C and BLNK in three separate experiments. The results were consistent in the data obtained by the yeast two-hybrid assay (data not shown). To assess a relative amount of endogenous c-Cbl bound to CIN85 in the presence of BLNK, the same membrane was immunoblotted with anti-Cbl antibody, resulting in a binding pattern of c-Cbl distinct from that of BLNK. Thus, BLNK and c-Cbl interacted with CIN85 in a slightly different fashion. As judged by immunoblotting of the membrane with anti-Flag antibody, CIN85 SH3 mutants were present in individual lanes, with almost equivalent levels. The concurrent presence of SH3A and SH3B appeared to be essential for an optimal association between CIN85 and BLNK. It is yet unclear, however, whether the binding manner of BLNK in 293 cells reflects that in B cells since neither natural expression nor tyrosine-phosphorylation of BLNK was observed in 293 cells.

*Various c-Cbl-related proteins involved in formation of the CIN85 complexes.* We sought to identify additional constituents of the CIN85 complexes. Cellular factors related to the c-Cbl signaling and Sos1 were screened by coimmunoprecipitation experiments using specific antibodies. After transfection of pcDNA3-Flag-CIN85, unstimulated or EGF-stimulated cell lysates were subjected to immunoprecipitation and then serial immunoblot analyses with the indicated antibodies. Shown are the results obtained by use of 293 cells, with one exception mentioned below, which are representative of one of three separate experiments using 293 and HeLa cells (Fig. 4). Crk-I, Crk-II, $p130^{Cas}$, p85-PI3K and Sos1 were apparently detected in anti-Flag immunoprecipitates prepared from the EGF-stimulated cells, and to a lesser extent in those from the unstimulated cells. Immunoreactive Sos1 bands were barely detectable in anti-Flag immunoprecipitates derived from 293 cells, even after EGF stimulation. Therefore, the Sos1 immunoblot obtained from HeLa cells, instead of 293 cells, was shown in Fig. 4. An increased amount of c-Cbl was also detected in anti-Flag immunoprecipitates upon EGF stimulation, in agreement with previous our results (1). Supportive results were obtained in reciprocal experiments. In the case of Grb2, we were unable to verify its existence in anti-Flag immunoprecipitates under our experimental conditions, due to its comigration with mouse Ig light-chain on polyacrylamide gels. However, when anti-Grb2 immunoprecipitates were immunoblotted with anti-Flag antibody, Grb2 were poorly observed, regardless of stimulation. Taken together, these results suggest that Crk-I, Crk-II, $p130^{Cas}$, p85-PI3K, Grb2 and Sos1 in addition to c-Cbl and BLNK are involved in formation of the CIN85 complexes.

*The coiled-coil region responsible for the tetramer formation of CIN85.* The substantial structural similarity between CIN85 and CMS implicated that CIN85 might form a homodimer, as was the case with CMS. To explore this possibility, pcDNA3-HA-CIN85 was cotransfected into HeLa cells with each of the plasmids expressing the Flag-tagged wild-type or mutant CIN85 proteins (Fig. 5A). Anti-HA immunoprecipitates were prepared from individual lysates of unstimulated and EGF-stimulated cells, and subjected to immunoblotting with anti-Flag antibody, resulting in appearance of immunoreactive bands of Flag-CIN85 and Flag-

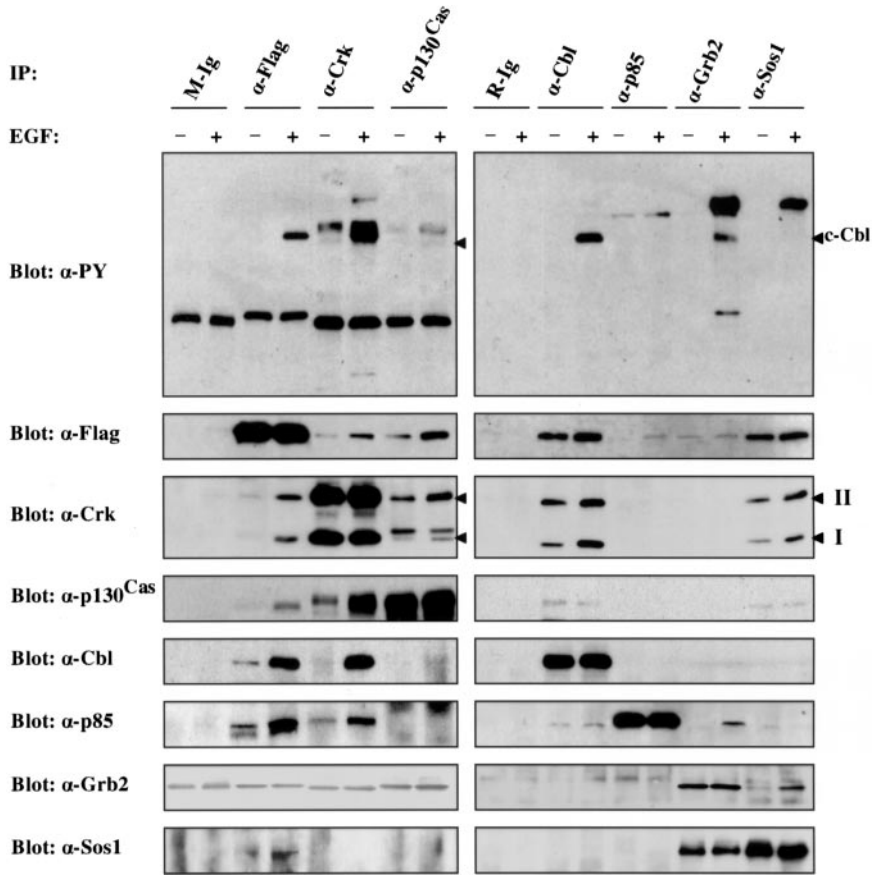


**FIG. 4.** Various c-Cbl-related proteins involved in the CIN85 complexes. 293 cells were transfected with pcDNA3-Flag-CIN85 and cultured for 48 h, followed by stimulation with (+) or without (−) EGF (100 ng/ml). Immunoprecipitates with the indicated antibodies were separated by SDS–PAGE (8 or 10%) and analyzed by immunoblotting with individual antibodies. As a control, normal mouse Ig (M-Ig) or rabbit Ig (R-Ig) was used for immunoprecipitation. The data presented here are representative of one of three separate experiments with similar results.

δSH3, but not Flag-δCC and Flag-SH3ABC (Fig. 5B). The results were confirmed by reciprocal experiments in which anti-Flag immunoprecipitates were immunoblotted with anti-HA antibody. These data demonstrated that CIN85 interacted with itself via its coiled-coil region, regardless of EGF stimulation. Moreover, to examine the oligomeric state of CIN85, chemical cross-linking was performed using HeLa cell lysates. Cross-linked products were analyzed by immunoblotting with anti-Flag antibody, resulting in a high molecular weight species of 320–340 kDa which corresponded to a tetrameric form of CIN85 (Fig. 6).

*Subcellular localization of CIN85 directed with the coiled-coil region and SH3 domains.* To elucidate the CIN85 regions responsible for the subcellular localization, HeLa cells were transfected with each of the plasmids encoding the CIN85 mutants (δCC, SH3ABC and δSH3) and the wild type as a control (Fig. 7A). δCC and SH3ABC, which carried three SH3 domains but lacked a coiled-coil region, were exclusively localized in the nucleus. δSH3 carrying the coiled-coil region, but not SH3 domains, was distributed uniformly in the cytoplasm, without punctate structures. Similar results were obtained by use of 293 and COS-7 cells (data not shown). These results suggest that the coiled-coil region serves to retain CIN85 in the cytoplasm, in addition to playing a crucial role for its tetramer formation, and that the CIN85 SH3 domains are required for formation of punctate structures. To obtain convincing evidence that CIN85 binds to the cytoskeletal components, HeLa cells were transfected with pcDNA3-Flag-CIN85 or pcDNA3-Flag-CIN85δCC, and subjected to isolation of Triton X-100-soluble and -insoluble fractions. Immunoprecipitation experiments revealed that the wild-type CIN85 was detected in not only the soluble but also insoluble fractions, while δCC was present only in the soluble fraction (Fig. 7B). These results indicate that the coiled-coil region is a key player in attachment of CIN85 to the cytoskeleton. To explore whether EGF stimulation affects the distribution of CIN85 between soluble and insoluble fractions, transfected cells were stimulated with or without EGF

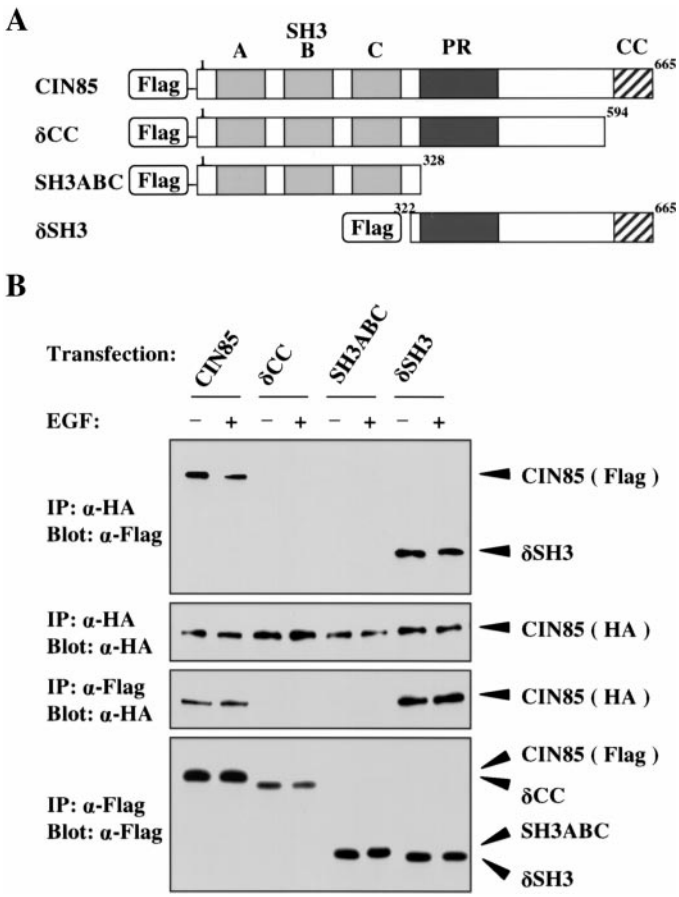


**FIG. 5.** The coiled-coil region required for the tetramer formation of CIN85. (A) Schematic representation of the wild-type and truncated CIN85 proteins. Names of individual proteins are indicated on the left. Amino acid positions are represented above the diagrams. SH3, SH3 domains; PR, the proline-rich region; CC, the coiled-coil region. (B) Coimmunoprecipitation experiments. pcDNA3-HA-CIN85 was cotransfected into HeLa cells with each of the plasmids described above. After treatment with (+) or without (−) EGF, cell lysates were subjected to immunoprecipitation and immunoblotting using the indicated antibodies.

prior to the Triton X-100 fractionation. EGF stimulation induced no significant alteration in the distribution manner of CIN85 (Fig. 7C).

## DISCUSSION

We have identified BLNK as a new binding partner of CIN85 using the yeast two-hybrid screen. Cotransfection experiments with mammalian cells revealed that CIN85 considerably associated with the non tyrosine-phosphorylated form of BLNK. As expected, the association was facilitated by BCR activation of Raji cells, with concomitant tyrosine phosphorylation of BLNK, indicating that the association was partly dependent on the tyrosine phosphorylation state. As was the case with c-Cbl (1), the physical association of CIN85 with BLNK was synergistically mediated by the three CIN85 SH3 domains, with the overall binding manner different from that of c-Cbl. From our results, it would be obvious that both the CIN85-BLNK and CIN85-c-Cbl complexes are present in parallel in the B cell signaling, a natural cellular context, and that the CIN85 SH3 domains play a crucial role in formation of these two complexes. On the other hand, there is the potential that the ternary complex composed of CIN85, BLNK and c-Cbl is present in B cells, albeit to a much lesser extent relative to the CIN85-BLNK and CIN85-c-Cbl complexes. Together, our results provide the notion that there is a novel mechanism in regulation of the B cell signaling events in which CIN85 plays a specific role by association with BLNK or c-Cbl.

Accumulating evidence has demonstrated that BLNK plays a nonredundant role in B cell development and activation (4–10). A recent report has documented that c-Cbl directly binds to the phosphorylated BLNK through its SH2 domain and competitively inhibits the association of phospholipase C (PLC)-γ2 with BLNK, resulting in negative regulation of the PLC-γ2 pathway (24). c-Cbl thus cooperates with BLNK in regulation of the PCL-γ2 signaling in B cells. Considering the fact, it is postulated that CIN85 may play a regulatory role to modulate the PLC-γ2 pathway. It is also likelihood that CIN85 functions as an intermediate in connecting the PLC-γ2 pathway to another pathway by binding to BLNK or c-Cbl.

By analogy with the cases of other adaptor proteins, it is anticipated that CIN85 may contribute to formation of a diverse array of signaling complexes. In fact, we could identify Crk-I, Crk-II, $p130^{Cas}$, p85-PI3K, Grb2 and Sos1 as additional components involved in the CIN85 complex formation. The involvements of these molecules were substantially enhanced by EGF stimulation. We further investigated the association manner of CIN85 with p85-PI3K or Sos1, and found that the CIN85 SH3 domains were required for the respective association (data not shown). To date, direct

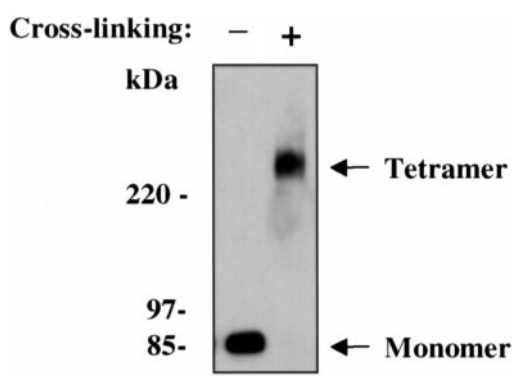


**FIG. 6.** Chemical cross-linking. 293 cells were transfected with pcDNA3-Flag-CIN85. Cell lysates were treated with or without 3 mM $BS^3$, and subjected to SDS–8% PAGE and then immunoblotting with anti-Flag antibody.

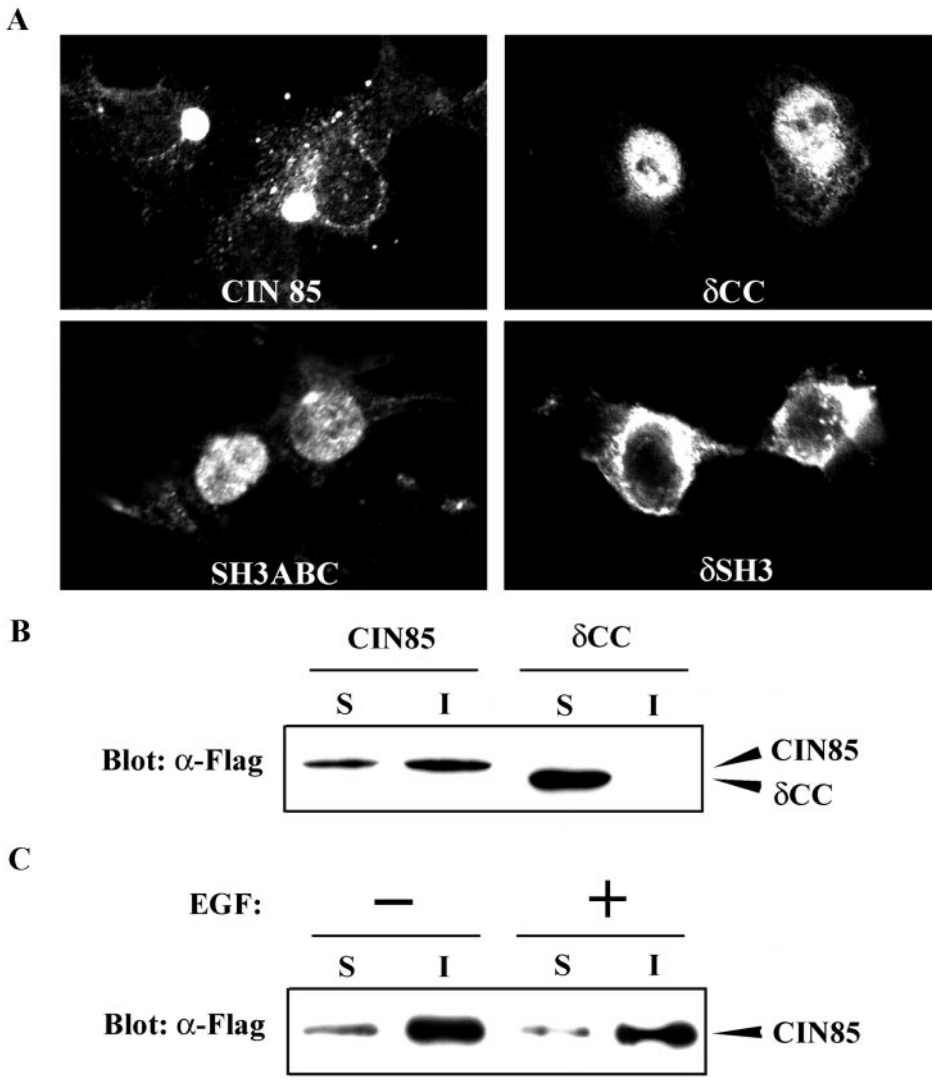


**FIG. 7.** Identification of the regions responsible for the subcellular localization of CIN85. (A) Subcellular localization of the CIN85 mutants. HeLa cells transfected with pcDNA3-Flag-CIN85 or individual CIN85 mutant plasmids were stained with anti-Flag antibody and then FITC-conjugated antibody. (B) Attachment of CIN85 to the insoluble cellular components via its coiled-coil region. 293 cells were transfected with pcDNA3-Flag-CIN85 or pcDNA3-Flag-δCC, followed by the Triton X-100 fractionation. The Triton X-100-soluble (S) or -insoluble (I) fraction was resolved by SDS–8% PAGE and analyzed by immunoblotting with anti-Flag antibody. (C) No obvious effect of EGF stimulation on attachment of CIN85 to the cytoskeleton. 293 cells transfected with pcDNA3-Flag-CIN85 were stimulated with (+) or without (−) EGF, subjected to the Triton X-100 fractionation and then treated in a similar manner described above.

association of Sos1 with c-Cbl has not been reported. Accordingly, it is likely that CIN85 directly interacts with Sos1 in a CIN85 SH3 domain-dependent manner, although it cannot be completely ruled out that the association was mediated through another molecule. Crk-I, Crk-II, $p130^{Cas}$, p85-PI3K and Grb2 have been reported to directly interact with c-Cbl and also known to associate directly or indirectly with one another (reviewed in Ref. 14–17). The complexity of these multimolecular associations led to difficulty to determine whether CIN85 bound to these factors via a direct physical contact, or through other molecules including c-Cbl. Despite the facts, our data clearly described that at least part of the CIN85-p85-PI3K association was mediated by direct physical connection via the CIN85 SH3 domains. Another line of evidence that CMS directly binds to $p130^{Cas}$, p85-PI3K or Grb2 (2) also supports the direct interaction of CIN85 with p85-PI3K. Altogether, it is highly plausible that CIN85 acts as a mediator to couple the c-Cbl signaling pathway with other pathways through its multiple protein complexes, thereby leading to their interplay.

A coiled-coil exists in a large variety of gene products (reviewed in Refs. 25, 26). In certain regulatory elements such as transcriptional activators, the coiled-coil structures lead to the selective dimerization, resulting

in combinatorial rearrangement of specific molecules. The coiled-coil region of CMS is also known to mediate homodimerization of CMS (2). As predicted from CMS, CIN85 could assemble with itself through the coiled-coil region, leading to formation of the tetramer. Removal of the coiled-coil region from CIN85 caused the translocation of CIN85 from the cytoplasm to the nucleus, indicating that the coiled-coil region served for retention of CIN85 to the cytoplasm. SH3 domains-missing CIN85 mutant was completely retained and diffusely localized in the cytoplasm, without any punctate structures. This suggests a potential role of the SH3 domains in assembling multiple CIN85 proteins into large complexes, probably by cooperation of a certain SH3 domain-binding molecule. The subcellular localization patterns of the CIN85 truncated mutants were also quite similar to those of the comparable CMS mutants (2). It still remains, however, to be determined whether the subcellular localization pattern of CIN85 with large punctate structures, which was observed by our transfection experiments, reflects that of endogenous CIN85. Taken together, it would be apparent that both the coiled-coil region and SH3 domains of CIN85 are involved in intermolecular interactions. Further, it is assumed that CIN85 plays a critical role in localization of its binding partners into specific intracellular compartments by attaching to the cytoskeletal structure via its coiled-coil region.

Our data provide an insight into cellular signaling events involving CIN85. CIN85 appears to form complexes composed of homomeric and heteromeric multi-subunits proteins which have a strong tendency to assemble to higher order multimeric complexes. CIN85 may acquire versatile signaling properties via its homo- and hetero-molecular complexes.

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