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Cloning and Characterization of a Novel Adaptor Protein, CIN85, That Interacts with c-Cbl

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The c-*Cbl* protooncogene product is a prominent substrate of protein tyrosine kinases and is rapidly tyrosine-phosphorylated upon stimulation of a wide variety of cell-surface receptors. We have identified a novel c-Cbl-interacting protein termed CIN85 with a molecular mass of 85 kDa which shows similarity to adaptor proteins, CMS and CD2AP. CIN85 mRNA is expressed ubiquitously in normal human tissues and cancer cell lines analyzed. CIN85 was basally associated with c-Cbl. For interaction of CIN85 with c-Cbl, the second SH3 domain of CIN85 was shown to serve as a central player. The CIN85–c-Cbl association was enhanced shortly after stimulation of 293 cells with epidermal growth factor (EGF) and gradually diminished to a basal level, which correlated with a tyrosine phosphorylation level of c-Cbl. Our results suggest that CIN85 may play a specific role in the EGF receptor-mediated signaling cascade via its interaction with c-Cbl. © 2000 Academic Press

Key Words: **cloning; c-Cbl-interacting protein; adaptor protein; Src homology 3 domain; epidermal growth factor receptor.**

The c-*Cbl* proto-oncogene is the cellular homologue of the transforming v-*Cbl* oncogene [1]. The 120-kDa product of the c-*Cbl* gene is widely expressed in mammalian cells, with relatively large amounts in thymus, testis and various hemopoietic cell lines [2]. The NH_2-terminal half of c-Cbl is highly conserved among currently known c-Cbl-related proteins [3–7]. The v-Cbl protein composed of the first 355 NH_2-terminal amino acids of c-Cbl can be detected in both cytoplasm and nucleus, whereas c-Cbl is predominantly cytoplasmic and lacks transforming ability [8]. c-Cbl has no obvious catalytic domains but contains several specific sequences (reviewed in Refs. 9–11). Through the proline-rich sequences, c-Cbl constitutively binds the SH3 domain-containing proteins [12–19]. c-Cbl lies downstream of a wide variety of cell-surface receptors [15, 20–34]. c-Cbl is rapidly and differentially phosphorylated in response to activation of different receptors or in distinct cell types. Tyrosine-phosphorylated c-Cbl results in formation of complexes with SH2 domain-containing proteins [13–16, 29, 35–39].

An initial clue about the biological function of c-Cbl was obtained from genetic studies in *Caenorhabditis elegans* [40]. Recently, a *Drosophila* homologue of c-Cbl, D-Cbl, was also identified as a negative regulator of the *Drosophila* epidermal growth factor receptor (EGFR) tyrosine kinase [6]. Consistent with these findings, functional studies of c-Cbl using mammalian cells have provided additional evidence that c-Cbl may negatively regulate the mammalian tyrosine kinase-mediated signaling pathways [41–44]. However, experiments using osteoclast has provided another line of evidence that c-Cbl has a positive regulatory role in the c-Src-mediated signaling pathway responsible for bone resorption [45]. These findings indicate that c-Cbl plays a versatile role in a diverse array of intracellular signaling pathways.

To further elucidate potential roles of c-Cbl, we sought to identify novel proteins that interact with c-Cbl using the yeast two-hybrid system. Here, we report the cDNA cloning and characterization of CIN85, a novel c-Cbl-interacting protein of 85 kDa.

MATERIALS AND METHODS

Plasmid construction. To construct a bait plasmid, pGBT9-c-Cbl, for the two hybrid screen, a full-length open reading frame (ORF) of human c-Cbl cDNA was excised from a pUC19-c-Cbl plasmid and inserted into a pGBT9 yeast expression vector (Clontech, U.S.A.) such that an entire c-Cbl protein was in-frame with a GAL4 DNA-binding domain. For mammalian expression, plasmid construction was carried out using pcDNA3 (Invitrogen, U.S.A.) as a basic vector. To express the entire c-Cbl, the full-length ORF was inserted into pcDNA3 and the resulting construct was denoted pcDNA3-c-Cbl. pcDNA3-Flag was generated to express a protein fused to a Flag-tag at the NH_2-terminus, using two complementary oligonucleotides encoding the Kozak sequence and a Flag peptide (1 kDa). pcDNA3-

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Flag-CIN85 was constructed by insertion of a full-length ORF of CIN85 cDNA into pcDNA3-Flag. To examine binding domains of CIN85 to c-Cbl, seven different plasmids were constructed to express truncated and/or deleted mutants of CIN85 as follows. Fragments encoding amino acids (aas) 1–99 (SH3A), 59–271 (SH3B), 158–328 (SH3C), 1–157 (SH3AB), 103–328 (SH3BC), 1–99/158–328 (SH3AC, deletion of aa 100–157) and 1–328 (SH3ABC) were created by polymerase chain reaction (PCR) with CIN85 cDNA as a template and adequate primers. Individual PCR-fragments were inserted into pcDNA3-Flag to generate in-frame fusion with the Flag peptide. pcDNA3-Flag carrying the SH3A fragment was designated pcDNA3-Flag-SH3A, and other mutant plasmids were termed in a similar fashion. All constructs were verified by DNA sequencing.

Yeast two-hybrid system. The yeast two-hybrid screen was performed using the MATCHMAKER two-hybrid system (Clontech) according to the manufacturer's instruction. Yeast HF7c cells were sequentially transformed with pGBT9-c-Cbl and a human B-lymphocyte cDNA library (Clontech). Library plasmids from positive yeast clones were subjected to DNA sequencing. Nucleotide and amino acid sequence alignments were performed by screening databases with the BLAST program.

cDNA cloning. Three different phage cDNA libraries were used for cloning experiments. A human lymphoma (Raji cells) 5′-stretch plus cDNA library in λgt11 and a human HeLa 5′-stretch plus cDNA library in λgt10 were purchased from Clontech. A human T cell (Jurkat cells) cDNA library in λZAP II was obtained from Stratagene (U.S.A.). All procedures of cDNA cloning were carried out as instructed by the manufacturers. cDNA inserts from positive colonies were cloned into pcDNA3 for sequencing. A nucleotide sequence of CIN85 cDNA was completely determined on both strands with an ABI Prism dye terminator cycle sequencing kit using an automated sequencer (Perkin–Elmer, U.S.A.).

Mutagenesis. Mutations were introduced into the wild-type c-Cbl protein so as to substitute tyrosine residues at the major tyrosine phosphorylation sites [46] to phenylalanine residues by use of the GeneEditor system (Promega, U.S.A.). Site-directed mutagenesis was performed using pcDNA3-c-Cbl as a template, and appropriate mutagenic primers bearing Y700F, Y731F, and/or Y774F mutations where the second nucleotides of the tyrosine-encoding codons were changed from A to T. Mutations of two or three tyrosine residues were accomplished by sequential rounds of mutagenesis and confirmed by DNA sequencing.

Cell culture and transfection. COS-7 and 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine and antibiotics. Transient transfection was carried out using the SuperFect reagent (QIAGEN, U.S.A.) according to the manufacturer's instruction. In brief, cells (1×10^6) were cultured on 100-mm dishes for 16–24 h and transfected with 5 μg of each plasmid DNA (described below), followed by incubation in the complete medium for 48 h.

Cell stimulation and preparation of cell lysates. The transfected and 48-h cultured 293 cells were serum-starved in DMEM without FBS for 12 h and then activated with recombinant human EGF (100 ng · mL^{-1}; Genzyme, U.S.A.) at 37°C for the indicated times. Cells were washed immediately with cold phosphate-buffered saline (PBS) and subsequently treated with a lysis buffer (0.5% Nonidet P-40, 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM NaF, 1 mM Na_3VO_4, 50 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride) at 4°C for 30 min. Crude lysates were cleared by centrifugation and supernatants were subjected to immunoprecipitation. Protein concentration of cleared lysates were determined by the Bradford method. Transfected COS-7 cells were cultured in the complete medium for 48 h and cell lysates were prepared in an identical manner without starvation and stimulation.

Antibodies. Immunoprecipitation or immunoblotting was performed using the following antibodies: anti-Flag M2 mouse monoclonal antibody (Eastman Kodak, U.S.A.), anti-phosphotyrosine PY20 mouse monoclonal antibody (Transduction Laboratories, U.S.A.), anti-Cbl rabbit polyclonal antibody horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody (Santa Cruz Biotechnology, U.S.A.).

Immunoprecipitation and immunoblotting. Prior to immunoprecipitation, Dynabeads M-280 magnetic beads were coated with anti-Flag M2 antibody, anti-Cbl rabbit antibody, normal mouse Ig or normal rabbit Ig as described in the manufacturer's recommendations (DYNAL, U.S.A.). To immunoprecipitate proteins, cell lysates containing equal amounts of proteins were incubated with the antibody-coated beads at 4°C for 2 h. Beads collected with the Dynal MPC magnet were resuspended in a sample buffer for SDS–polyacrylamide gel electrophoresis (PAGE). Bound proteins were boiled, resolved by SDS–PAGE and transferred onto membranes. Immunoblot analysis was performed with specific antibody and horseradish peroxidase-conjugated secondary antibody, followed by detection with the ECL system (Amersham, U.S.A.). To reprobe immunoblots, membranes were stripped, washed and then reblocked prior to incubation with antibody.

Northern blot analysis. Blots of poly $(A)^+$ RNA (2 μg per lane) from normal human tissues and human cancer cell lines were obtained from Clontech. Hybridization was performed following the manufacturer's instructions. The blots were hybridized first with a 570-bp PCR product (nucleotides 634–1203) of CIN85 cDNA and then with a β-actin probe which were labeled with [α-^{32}P]dCTP by a Oligolabeling kit (Pharmacia Biotech, U.S.A.).

RESULTS

Isolation of the CIN85 cDNA. The yeast two-hybrid screen was carried out to identify novel cellular proteins that interacted with c-Cbl. Screen of 1×10^6 HF7c transformants gave rise to 42 double-positive colonies. Six of the 42 plasmids contained cDNA inserts derived from the same gene and had almost similar lengths (~0.9 kb). No exactly matched sequences to the cDNA insert was found in current protein and DNA databases. Computer homology search indicated that the cDNA clone contained two regions homologous to SH3 domains of other known proteins. Specificity of the interaction in the yeast system was confirmed by use of another yeast strain and control plasmids. We referred to the cDNA product as CIN85 due to its characteristic features described below. To isolate its full-length ORF, the Raji cDNA library was screened with probes derived from the CIN85 cDNA, resulting in a clone containing the complete ORF. The entire sequence of CIN85 cDNA was confirmed with cDNA clones obtained from other cDNA libraries (HeLa and Jurkat). A composite nucleotide sequence from these cDNA inserts consists of 3348 bp with ORF of 1995 bp, flanked by 291-bp 5′- and 1062-bp 3′-untranslated sequences (data not shown). Nucleotide and deduced amino acid sequences of the CIN85 ORF are shown in Fig. 1.

Characteristic features of the CIN85 protein. The CIN85 cDNA encodes a predicted 665 amino acid sequence with a calculated molecular mass of 73 kDa (Fig. 1). Homology searches based on the deduced amino acid sequence defined that CIN85 has a distinc-

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   1  ATGGTGGAGGCCATAGTGGAGTTTGACTACCAGGCCCAGCACGATGATGAGCTGACGATCAGCGTGGGTGAAATCATCACCAACATCAGG
   1  M  V  E  A  I  V  E  F  D  Y  Q  A  Q  H  D  D  E  L  T  I  S  V  G  E  I  I  T  N  I  R

  91  AAGGAGGATGGAGGCTGGTGGGAGGGACAGATCAACGGCAGGAGAGGTTTGTTCCCTGACAACTTTGTAAGAGAAATAAAGAAAGAGATG
  31  K  E  D  G  G  W  W  E  G  Q  I  N  G  R  R  G  L  F  P  D  N  F  V  R  E  I  K  K  E  M

 181  AAGAAAGACCCTCTCACCAACAAAGCTCCAGAAAAGCCCCTGCACGAAGTGCCCAGTGGAAACTCTTTGCTGTCTTCTGAAACGATTTTA
  61  K  K  D  P  L  T  N  K  A  P  E  K  P  L  H  E  V  P  S  G  N  S  L  L  S  S  E  T  I  L

 271  AGAACCAATAAGAGAGGCGAGCGACGGAGGCGCCGGTGCCAGGTGGCATTCAGCTACCTGCCCCAGAATGACGATGAACTTGAGCTGAAA
  91  R  T  N  K  R  G  E  R  R  R  R  R  C  Q  V  A  F  S  Y  L  P  Q  N  D  D  E  L  E  L  K

 361  GTTGGCGACATCATAGAGGTGGTAGGAGAGGTAGAGGAAGGATGGTGGGAAGGTGTTCTCAACGGGAAGACTGGAATGTTTCCTTCCAAC
 121  V  G  D  I  I  E  V  V  G  E  V  E  E  G  W  W  E  G  V  L  N  G  K  T  G  M  F  P  S  N

 451  TTCATCAAGGAGCTGTCAGGGGAGTCGGATGAGCTTGGCATTTCCCAGGATGAGCAGCTATCCAAGTCAAGTTTAAGGGAAACCACAGGC
 151  F  I  K  E  L  S  G  E  S  D  E  L  G  I  S  Q  D  E  Q  L  S  K  S  S  L  R  E  T  T  G

 541  TCCGAGAGTGATGGGGGTGACTCAAGCAGCACCAAGTCTGAAGGTGCCAACGGGACAGTGGCAACTGCAGCAATCCAGCCCAAGAAAGTT
 181  S  E  S  D  G  G  D  S  S  S  T  K  S  E  G  A  N  G  T  V  A  T  A  A  I  Q  P  K  K  V

 631  AAGGGAGTGGGCTTTGGAGACATTTTCAAAGACAAGCCAATCAAACTAAGACCAAGGTCAATTGAAGTAGAAAATGACTTTCTGCCGGTA
 211  K  G  V  G  F  G  D  I  F  K  D  K  P  I  K  L  R  P  R  S  I  E  V  E  N  D  F  L  P  V

 721  GAAAAGACTATTGGGAAGAAGTTACCTGCAACTACAGCAACTCCAGACTCATCAAAAACAGAAATGGACAGCAGGACAAAGAGCAAGGAT
 241  E  K  T  I  G  K  K  L  P  A  T  T  A  T  P  D  S  S  K  T  E  M  D  S  R  T  K  S  K  D

 811  TACTGCAAAGTAATATTTCCATATGAGGCACAGAATGATGATGAATTGACAATCAAAGAAGGAGATATAGTCACTCTCATCAATAAGGAC
 271  Y  C  K  V  I  F  P  Y  E  A  Q  N  D  D  E  L  T  I  K  E  G  D  I  V  T  L  I  N  K  D

 901  TGCATCGACGTAGGCTGGTGGGAAGGAGAGCTGAACGGCAGACGAGGCGTGTTCCCCGATAACTTCGTGAAGTTACTTCCACCGGACTTT
 301  C  I  D  V  G  W  W  E  G  E  L  N  G  R  R  G  V  F  P  D  N  F  V  K  L  L  P  P  D  F

 991  GAAAAGGAAGGGAATAGACCCAAGAAGCCACCGCCTCCATCCGCTCCTGTCATCAAACAAGGGGCAGGCACCACTGAGAGAAAACATGAA
 331  E  K  E  G  N  R  P  K  K  P  P  P  P  S  A  P  V  I  K  Q  G  A  G  T  T  E  R  K  H  E

1081  ATTAAAAAGATACCTCCTGAAAGACCAGAAATGCTTCCAAACAGAACAGAAGAAAAAGAAAGACCAGAGAGAGAGCCAAAACTGGATTTA
 361  I  K  K  I  P  P  E  R  P  E  M  L  P  N  R  T  E  E  K  E  R  P  E  R  E  P  K  L  D  L

1171  CAGAAGCCCTCCGTTCCTGCCATACCGCCAAAAAAGCCTCGGCCACCTAAGACCAATTCTCTCAGCAGACCTGGCGCACTGCCCCCGAGA
 391  Q  K  P  S  V  P  A  I  P  P  K  K  P  R  P  P  K  T  N  S  L  S  R  P  G  A  L  P  P  R

1261  AGGCCGGAGAGACCGGTGGGTCCGCTGACACACACCAGGGGTGACAGTCCAAAGATTGACTTGGCCGGCAGTTCGCTATCTGGCATCCTG
 421  R  P  E  R  P  V  G  P  L  T  H  T  R  G  D  S  P  K  I  D  L  A  G  S  S  L  S  G  I  L

1351  GACAAAGATCTCTCGGACCGCAGCAATGACATTGACTTAGAAGGTTTTGACTCCGTGGTATCATCTACTGAGAAACTCAGTCATCCGACC
 451  D  K  D  L  S  D  R  S  N  D  I  D  L  E  G  F  D  S  V  V  S  S  T  E  K  L  S  H  P  T

1441  ACAAGCAGACCAAAAGCTACAGGGAGGCGGCCTCCGTCCCAGTCCCTCACATCTTCATCCCTTTCAAGCCCTGATATCTTCGACTCCCCA
 481  T  S  R  P  K  A  T  G  R  R  P  P  S  Q  S  L  T  S  S  S  L  S  S  P  D  I  F  D  S  P

1531  AGTCCCGAAGAGGATAAGGAGGAACACATTTCACTTGCGCACAGAGGAGTGGACGCGTCAAAGAAAACTTCCAAGACTGTTACCATATCC
 501  S  P  E  E  D  K  E  E  H  I  S  L  A  H  R  G  V  D  A  S  K  K  T  S  K  T  V  T  I  S

1621  CAAGTGTCTGACAACAAAGCATCCCTGCCGCCCAAGCCGGGGACCATGGCAGCAGGTGGCGGTGGGCCAGCCCCTCTGTCCTCAGCGGCG
 541  Q  V  S  D  N  K  A  S  L  P  P  K  P  G  T  M  A  A  G  G  G  G  P  A  P  L  S  S  A  A

1711  CCCTCCCCCCTGTCATCCTCTTTGGGAACAGCTGGACACAGAGCCAACTCCCCGTCTCTGTTCGGCACGGAAGGAAAACCAAAGATGGAG
 561  P  S  P  L  S  S  S  L  G  T  A  G  H  R  A  N  S  P  S  L  F  G  T  E  G  K  P  K  M  E

1801  CCTGCGGCCAGCAGCCAGGCGGCCGTGGAGGAGCTAAGGACACAGGTCCGCGAGCTGAGGAGCATCATCGAGACCATGAAGGACCAGCAG
 601  P  A  A  S  S  Q  A  A  V  E  E  L  R  T  Q  V  R  E  L  R  S  I  I  E  T  M  K  D  Q  Q

1891  AAACGAGAGATTAAACAGTTATTGTCTGAGTTGGATGAAGAGAAGAAAATCCGGCTTCGGTTGCAGATGGAAGTGAACGACATAAAGAAA
 631  K  R  E  I  K  Q  L  L  S  E  L  D  E  E  K  K  I  R  L  R  L  Q  M  E  V  N  D  I  K  K

1981  GCTCTACAATCAAAATGA
 661  A  L  Q  S  K  *
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FIG. 1. Nucleotide and deduced amino acid sequences of the CIN85 ORF. The putative initiator methionine of the ORF and its first nucleotide are represented as an amino acid position 1 and a nucleotide position 1, respectively.

tive primary structure. CIN85 contains three consecutive SH3 domains in the NH_2-terminal half, followed by a proline-rich region at aa 337–428 (27% of proline). Further, two computer programs for coiled-coil prediction [47, 48] showed that the COOH-terminal region (aa 602–661) of CIN85 forms an α-helical coiled-coil structure. The proline-rich region contains multiple PXXP sequences which are possible SH3 domain-binding sites [49]. Further, it was revealed that CIN85 shares significant sequence identity with CMS [50] and CD2AP (a mouse homologue of CMS) [51], with overall 36 and 38% identity in amino acid sequence, respectively. The primary structures of these three proteins are also quite similar to one another.

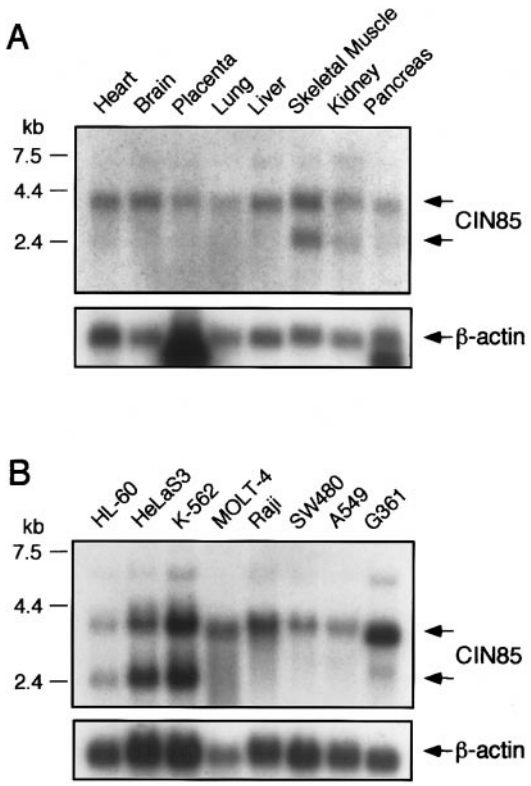

FIG. 2. Tissue distribution of CIN85 transcripts. Northern blot analysis was performed using RNA blots derived from normal human tissues (A) and cancer cell lines (B) according to the manufacturer's instructions. Blots were hybridized with ^{32}P-CIN85 probe (a 570-bp PCR product) and subjected to reprobing with β-actin probe to monitor mRNA loading. Arrows designate positions of CIN85 and β-actin, respectively. RNA size markers (kb) are denoted on the left.

Tissue distribution of CIN85. Using two kinds of RNA blots derived from various normal human tissues and human cancer cell lines, Northern blot analysis was conducted to examine a distribution pattern and a size of a CIN85 transcript. The ^{32}P-labeled CIN85 probe detected two major mRNA species of approximately 3.2 and 2.4 kb on both RNA blots, with a few occasional minor species (Fig. 2). The 3.2-kb species was ubiquitously expressed in all of the tissues analyzed so far, while the 2.4-kb species was restricted to skeletal muscle, kidney and pancreas (Fig. 2A). A comparable distribution pattern of 2.4-kb species was observed even after long exposure. The expression level of the 3.2-kb transcript was highest in skeletal muscle, and low in lung and pancreas. Consistent with its widespread expression in the normal human tissues, the 3.2-kb species was also evident in all of the cancer cell lines examined, but with significant variation in abundance (Fig. 2B). The 2.4-kb species was present only in K562, HeLa S3, and HL-60. Expression of the 3.2-kb species was most predominant in K562 and G361, and lowest in HL-60. A relative ratio of the two transcripts (2.4 kb and 3.2 kb) appeared to be equal among normal tissues and cancer cell lines expressing both transcripts.

Association of CIN85 with c-Cbl in mammalian cells. To validate the association of CIN85 with c-Cbl detected by the two-hybrid system, we investigated whether the c-Cbl protein indeed associates with CIN85 in mammalian cells. First, to verify the proposed ORF of CIN85, either pcDNA3-Flag-CIN85 or pcDNA3-Flag was introduced into COS-7 cells. Anti-Flag M2 antibody clearly recognized a single band migrating at an apparent molecular mass of 85 kDa only in the cells transfected with pcDNA3-Flag-CIN85 (Fig. 3A). Next, COS-7 cells were cotransfected with pcDNA3-Flag-CIN85 and pcDNA3-c-Cbl. A strong immunoreactive band against anti-Cbl antibody was observed in the anti-Flag immunoprecipitates at a position relevant to c-Cbl from the whole lysate (Fig. 3B). In reciprocal experiments, CIN85 was also readily detected in the anti-Cbl immunoprecipitates (Fig. 3C). Subsequently, the same membranes were reblotted with specific antibodies which were used for formation of the immunoprecipitates, confirming that CIN85 and c-Cbl apparently existed on the blots, respectively. No bands were detected in normal mouse or rabbit Ig immunoprecipitates blotted with anti-Cbl or anti-Flag antibody.

Interactions of CIN85 SH3 domains with c-Cbl. The original CIN85 cDNA clones obtained by the two-hybrid screen span an aa 55-331 region of the entire CIN85 protein which includes two consecutive SH3 domains, that is, the second and third SH3 domains. Consequently, it is likely that the two SH3 domains are responsible for the interaction of CIN85 with c-Cbl since SH3 domains are known to mediate protein-protein interaction by binding to specific proline-rich sequences. To address the possibility, seven different plasmids based on pcDNA3-Flag were generated to express CIN85 mutants which encompassed individual or combined SH3 domains (Fig. 4A). pcDNA3-c-Cbl was cotransfected into COS-7 cells in combination with each of the pcDNA3-Flag-based constructs. Following 48-h culture, immunoprecipitates were prepared from cell lysates by incubating with anti-Flag antibody, resolved by SDS–PAGE and then examined by immunoblotting with anti-Cbl antibody (Fig. 4B, top). The double or the triple SH3 domain-containing proteins exhibited predominantly high levels of binding abilities, compared to the single SH3 domain-containing proteins. Especially, SH3AB, SH3BC, and SH3ABC retained the strongest abilities for interaction with c-Cbl, almost equivalent to that of the entire CIN85 protein, followed by SH3AC. By immunoblotting with anti-Flag antibody, comparable levels of the wild-type and mutant CIN85 proteins were detected in individual lanes (Fig. 4B, bottom). These results clearly indicate that the binding efficiency of the first or the third

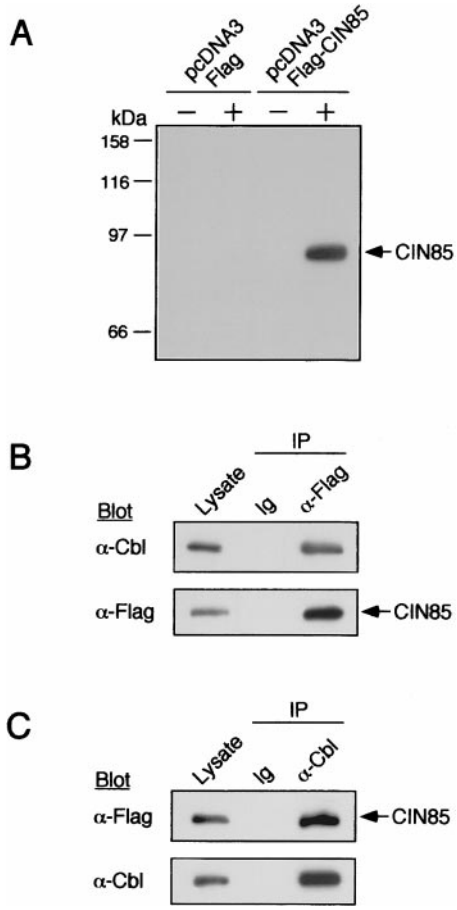

FIG. 3. Expression of the CIN85 protein and its association with c-Cbl. (A) Transient expression of CIN85 in COS-7 cells. COS-7 cells were transfected (+) with either pcDNA3-Flag-CIN85 or pcDNA3-Flag, or untransfected (−). Cells were cultured for 48 h, lysed and subjected to immunoprecipitation with magnetic beads-coated anti-Flag antibody. Following SDS–8% PAGE, proteins were transferred to membranes, blotted with anti-Flag antibody and visualized by ECL. Molecular markers (kDa) are shown on the left. (B), (C) Association of CIN85 with c-Cbl. COS-7 cells were cotransfected with pcDNA3-Flag-CIN85 and pcDNA3-c-Cbl, followed by culture for 48 h. Magnetic beads coated with anti-Flag (B) or anti-Cbl (C) antibody, or with normal mouse Ig (B) or rabbit Ig (C) were used for immunoprecipitation. The immunoprecipitates and whole cell lysates (as controls) were resolved by SDS–PAGE, and detected by immunoblotting with anti-Cbl (B, top) or anti-Flag (C, top) antibody. As a control, whole cell lysate was loaded onto the SDS–polyacrylamide gel. The same membrane was then stripped and reblotted with anti-Flag (B, bottom) or anti-Cbl (C, bottom) antibody. Positions of CIN85 are shown by arrows.

SH3 domain is dramatically enhanced by presence of the second SH3 domain and suggest that CIN85 associates with c-Cbl in a fashion more complex than a simple SH3 domain interaction with a single binding site.

Kinetics of CIN85 and c-Cbl association following EGF stimulation. We next examined the possibility that the interaction between CIN85 and c-Cbl was dependent on activation of the EGFR pathway, by use of human 293 cells expressing a natural EGFR. Cells were cotransfected with pcDNA3-Flag-CIN85 and pcDNA3-c-Cbl, cultured for 48 h and serum-starved for 12 h, followed by stimulation with EGF for the indicated times. Lysates of unstimulated or EGF-stimulated cells were subjected to immunoprecipitation with anti-Flag antibody, separated by SDS–PAGE and analyzed by immunoblotting first with anti-phosphotyrosine antibody (Fig. 5). No tyrosine-phosphorylated bands were seen in unstimulated cells. Upon stimulation with EGF, a relative intensity of the

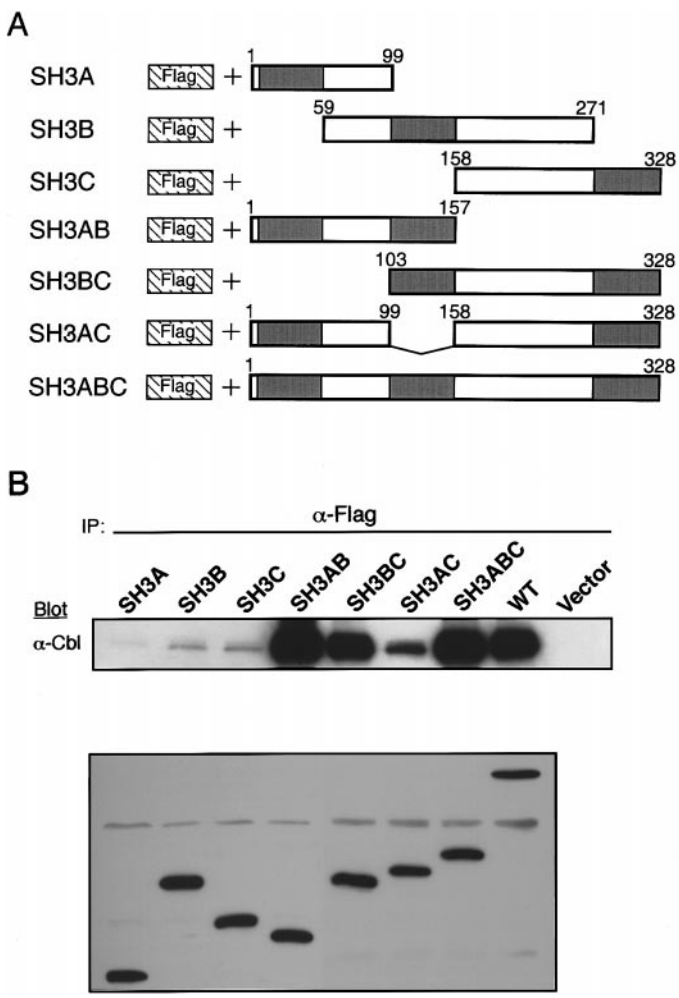

FIG. 4. Association of SH3 domain-containing CIN85 mutants with c-Cbl. (A) Schematic representation of various SH3 domain-containing CIN85 mutants. Names of individual mutants are designated on the left. SH3 domains are shown in shaded boxes. Amino acid positions are represented above the diagrams with numbers corresponding to the amino acid sequence in Fig. 1. (B) Association of individual CIN85 mutants with c-Cbl. pcDNA3-c-Cbl was cotransfected into COS-7 cells with individual CIN85 mutant plasmids (SH3A to SH3ABC) or pcDNA3-Flag-CIN85 (WT). After 48 h, cell lysates were subjected to immunoprecipitation with anti-Flag antibody and thereafter immunoblotting with anti-Cbl antibody (top). The anti-Flag immunoprecipitates were checked by immunoblotting with anti-Flag antibody (bottom).

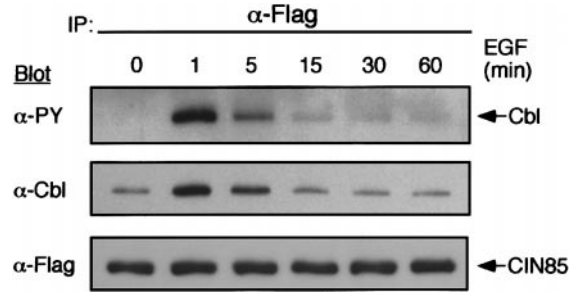

FIG. 5. EGF-stimulated association of c-Cbl with CIN85. 293 cells were cotransfected with pcDNA3-Flag-CIN85 and pcDNA3-c-Cbl. Cells were cultured for 48 h, serum-starved for an additional 12 h and stimulated with EGF (100 ng · mL^{-1}) at 37°C for various times indicated. Immunoprecipitation and then immunoblotting were performed with anti-Flag and anti-phosphotyrosine (top) antibodies, respectively. The blot was stripped and reblotted with anti-Cbl (middle) or anti-Flag (bottom) antibody, respectively.

tyrosine-phosphorylated band reached a peak at 1 min, began to decline by 5 min and thereafter diminished to a near-basal level by 15 min (Fig. 5, top). Subsequently, the blot was stripped and reprobed with anti-Cbl antibody, showing that the phosphorylated bands precisely represented c-Cbl (Fig. 5, middle). In addition, it was defined that a relative amount of c-Cbl changed in a time-dependent manner: a faint-basal level prior to stimulation; marked increase shortly after EGF stimulation; a maximum level at 1 min; thereafter gradual decrease to a basal level by 15 min. Therefore, a level of the c-Cbl-CIN85 association remarkably correlated with that of c-Cbl tyrosine phosphorylation. By immunoblotting with anti-Flag antibody, it was confirmed that equivalent amounts of CIN85 were immunoprecipitated at all time points examined (Fig. 5, bottom). These results indicate that a portion of CIN85 is basally associated with c-Cbl in 293 cells, regardless of EGF stimulation, and that the association is elevated by activation of EGFR.

EGF-induced association of c-Cbl with CIN85 is dependent on phosphorylation of specific tyrosine residues of c-Cbl. To further explore the contribution of tyrosine phosphorylation of c-Cbl to its association with CIN85, three different plasmids were generated to express c-Cbl mutants deficient in tyrosine phosphorylation at aas 700, 731, and 774, according to the previous report [46]. Consistent with reported findings, the tyrosine phosphorylation levels in the mutants exactly reflected the substituted numbers of tyrosine residues, in order of wild type $\geq$ Y731F $>$ Y700F + Y731F $>$ Y700F + Y731F + Y774F (Fig. 6A, top). By reblotting with anti-Cbl antibody, it was verified that amounts of the wild-type and individual mutant c-Cbl proteins were almost equal (Fig. 6A, bottom). These data suggest that EGF stimulation leads to phosphorylation of c-Cbl-tyrosine residues identical to those which are phosphorylated with Fyn, Yes and Syk [46]. We next assessed the binding abilities of the c-Cbl mutants to CIN85 by use of anti-Flag antibody for immunoprecipitation and anti-Cbl antibody for immunoblotting. The single tyrosine mutation (Y731F) caused no appreciable decrease in the interaction of c-Cbl with CIN85, whereas the double (Y700F + Y731F) and the triple (Y700F + Y731F + Y774F) mutations significantly abolished the interaction, with a more severe extent for the triple mutation (Fig. 6B, top). By reblotting with anti-Flag antibody, equivalent amounts of CIN85 were detected in all lanes (Fig. 6B, bottom). Decrease of tyrosine phosphorylation was thus accompanied by an impaired binding ability of c-Cbl to CIN85. The results demonstrate that an association level of CIN85 with c-Cbl closely correlates with a tyrosine-phosphorylation level of c-Cbl following EGFR activation.

DISCUSSION

We have isolated the human cDNA encoding the novel c-Cbl-interacting protein, CIN85, on the basis of

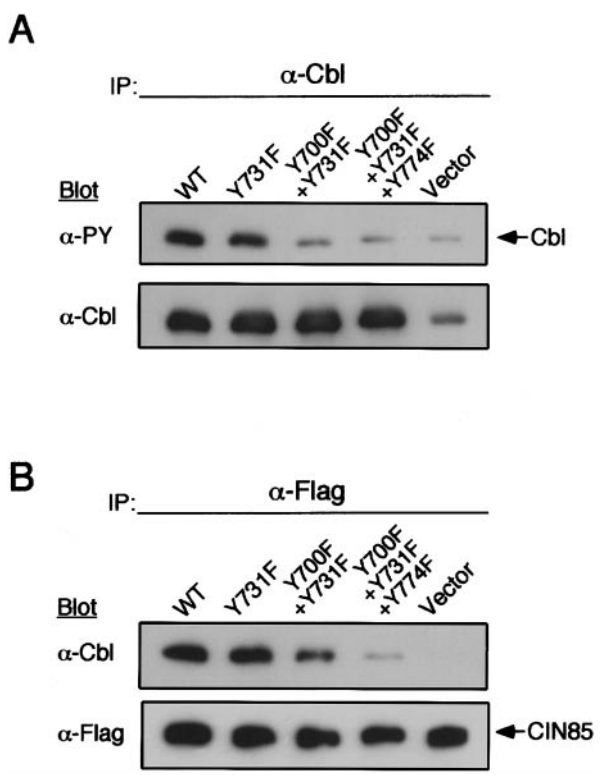

FIG. 6. Tyrosine phosphorylation-dependent interaction of c-Cbl with CIN85 following EGF stimulation. pcDNA3-Flag-CIN85 was cotransfected into 293 cells with individual tyrosine-substituted c-Cbl plasmids (Y731F, Y700F + Y731F, Y700F + Y731F + Y774F), pcDNA3-c-Cbl (WT) or pcDNA3 (Vector). After 48-h incubation, cells were serum-starved for 12 h and stimulated with EGF (100 ng · mL^{-1}) at 37°C for 1 min. Lysates were incubated with anti-Cbl (A) or anti-Flag (B) antibody for immunoprecipitation, and analyzed by immunoblotting with anti-phosphotyrosine (A, top) or anti-Cbl (B, top) antibody. Each blot shown on top was then stripped and reblotted with anti-Cbl (A, bottom) or anti-Flag (B, bottom) antibody.

the protein-protein interaction. By search for homology and analysis of the primary structure using computer-based programs, it was clarified that CIN85 is composed of three SH3 domains, a proline-rich region and a coiled-coil region, indicating that CIN85 is well suited to assign a potential role in assembling intracellular molecules into selective complexes.

Our study has defined that the binding ability of CIN85 to c-Cbl was extremely facilitated when the second SH3 domain of CIN85 was combined with the first and/or the third SH3 domains, greater than the simple sums of the corresponding SH3 domain-binding ability. In the context of the entire CIN85 molecule, the three SH3 domains appear to synergistically mediate the physical association of CIN85 with c-Cbl, in which the second SH3 domain plays a key role. Further, it should be taken into consideration that certain regions adjacent to the core sequences of the three SH3 domains may contribute to the efficient binding, as is the case with phospholipase Cγ-1 [52].

BLAST searches of GenBank revealed that CIN85 is homologous to adaptor proteins, CMS [50] and CD2AP [51]. Besides, these three proteins extremely resemble in their overall-structural compositions. CMS is believed to function as a scaffolding protein involved in cytoskeletal rearrangement, due to its structural features and the colocalization with $p130^{Cas}$ and F-actin. CD2AP, a mouse homologue of CMS, appears to function as a molecular scaffold for clustering of a CD2 membrane protein and cytoskeletal polarization. Substantial similarity in amino acid sequences and well-conserved structural features among CIN85, CMS, and CD2AP implicate that they are members of the same protein family. It is currently unclear whether CIN85 has any relations physiologically with CMS and CD2AP. In general, multiple SH3 domain-containing proteins are assumed to serve as scaffold proteins. It would be evident that CIN85 belongs to a growing family of scaffold proteins.

In an EGF-dependent manner, c-Cbl is tyrosine-phosphorylated, associated directly and indirectly with EGFR, and forms specific complexes with different signaling molecules including Grb2, Src homology and collagen (Shc) protein, p85 PI3K, and Crks [24–27, 36]. Previous studies have demonstrated that c-Cbl basally interacts with Grb2 and the interaction is further increased by EGF stimulation and that the indirect association of c-Cbl with EGFR is mediated through Grb2 [26, 27]. By analogy with this case, we examined the possible participation of CIN85 in the EGFR-c-Cbl signal transduction pathway using 293 cells since CIN85 also has unique structural features as an adaptor protein and the expression of CIN85 in 293 cells could be expected due to the broad spectrum of CIN85 tissue distribution. As expected, the association of CIN85 with c-Cbl was significantly enhanced shortly after EGF stimulation and diminished approximately in parallel to dephosphorylation of c-Cbl, while a relatively low level of basal association was seen prior to stimulation. Thus, in terms of association with c-Cbl, CIN85 displayed a kinetic profile remarkably similar to Grb2. Recently, it has been reported that Y700, Y731, and Y774 of c-Cbl are the major tyrosine phosphorylation sites in T cells upon stimulation with pervanadate and anti-CD3 antibody, in which Fyn, Yes and Syk appear to be major tyrosine kinases responsible for the phosphorylation [46]. The c-Cbl major phosphotyrosine residues are known as binding sites of SH2 domains of p85 PI3K, Crks and Vav [39, 53–56]. We sought to investigate the possible contribution of these tyrosine residues for the interaction between CIN85 and c-Cbl. By generating the tyrosine-substituted c-Cbl mutants, we have clearly demonstrated that the three tyrosine residues of c-Cbl were phosphorylated by EGF stimulation and that the EGF-induced CIN85-c-Cbl association was dependent on the phosphorylation level of c-Cbl. A previous work has demonstrated that stimulation of EGFR induces rapid activation of Src-family kinases including Src, Fyn and Yes [57]. Considering the fact, it is a likelihood that the members of the Src family may play some roles in the EGF-induced interaction between CIN85 and c-Cbl through phosphorylation of c-Cbl tyrosine residues.

Little is known presently about the mechanism whereby tyrosine phosphorylation of c-Cbl causes concomitant increase in the CIN85-c-Cbl association. One possibility is that tyrosine phosphorylation leads to conformational changes of c-Cbl and thereby exposes the otherwise cryptic binding sites, which allows to stabilize or facilitate the interaction between c-Cbl and CIN85. An alternative possibility is that other molecules such as p85 PI3K and Crks, which actually bind to the phosphotyrosine residues of c-Cbl, serve to facilitate the interaction of c-Cbl with CIN85. We found that the EGF-induced CIN85–c-Cbl complex contained p85 PI3K and Crks (data not shown). However, it seems unlikely that p85 PI3K affects the interaction between CIN85 and c-Cbl since the c-Cbl Y731F mutation, which led to the loss of the p85 PI3K-binding site, gave rise to no appreciable change in the association of c-Cbl with CIN85 (Fig. 6). To address whether other c-Cbl binding proteins including Crks modulate the CIN85-c-Cbl association, further study is now in progress.

The functions of CIN85 and the physiological significance of the CIN85-c-Cbl association are only speculative at present. Nonetheless, it should be obvious that CIN85 serves to assemble selective proteins into a specific c-Cbl complex in the EGFR signaling cascade and that the association of CIN85 with c-Cbl brings functionally distinct classes of molecules together to form complexes which fulfill a diverse range of functions.

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