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c-Cbl Is Inducibly Tyrosine-phosphorylated by Epidermal Growth Factor Stimulation in Fibroblasts, and Constitutively Tyrosine-phosphorylated and Associated with v-Src in v-*src*-transformed Fibroblasts

Hideharu Odai, Ko Sasaki, Yutaka Hanazono, Hiroo Ueno, Tomoyuki Tanaka, Kiyoshi Miyagawa, Kinuko Mitani, Yoshio Yazaki and Hisamaru Hirai[1]

Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113

The c-*cbl* gene was cloned as the cellular homolog of the v-*cbl* oncogene that is the transforming component of a murine tumorigenic retrovirus, CAS NS-1, though the biological roles of c-Cbl remain to be elucidated. We have previously reported that c-Cbl is implicated in the signal transduction triggered by granulocyte-macrophage colony-stimulating factor or erythropoietin in hematopoietic cells. Here, we observed tyrosine phosphorylation of c-Cbl in cells expressing epidermal growth factor receptor depending on EGF stimulation and in v-*src* transformed cells. Furthermore, c-Cbl was revealed to associate with v-Src *in vivo*. By means of binding experiments using glutathione S-transferase fusion proteins, we have found that the SH2 and SH3 domains of many proteins bind to c-Cbl. These findings strongly suggest that c-Cbl is implicated in a wide variety of signal transduction pathways, including those of EGF receptor and Src protein, as well as in the signaling pathways of hematopoietic cells.

Key words: c-Cbl — EGF — EGFR — v-Src — Tyrosine phosphorylation

Growth factors elicit and control a cascade of biochemical events including activation of Ras, Raf-1, and MAP kinase. The earliest of them is tyrosine phosphorylation of several cellular proteins through the activation of receptor-type or nonreceptor-type tyrosine kinases.[1,2] In the case of epidermal growth factor receptor (EGFR), epidermal growth factor (EGF) induces autophosphorylation of the receptors,[2,3] and subsequently several cytosolic enzymes that regulate intracellular signal transduction, such as phospholipase C-γ (PLC-γ)[4,5] and Ras GTPase-activating protein (GAP),[6] become tyrosine-phosphorylated, and associate with the receptors. It is considered that the primary role of such tyrosine kinases is to generate tyrosine-phosphorylated recognition motifs for binding of Src homology region (SH) 2 domains and to form signal transduction complexes.[7,8] For example, SH2 domain of Grb2/Ash can bind tyrosine-phosphorylated proteins such as EGFR, Shc, IRS-1, and Syp.[9–15] On the other hand, SH3 domains associate with proline-rich regions and are also important for the formation of signal complexes. As for Grb2/Ash adapter protein, it binds to Sos,[16,17] dynamin,[18,19] and C3G,[20] which regulate Ras or Ras-related proteins. Therefore, it is considered that Grb2/Ash couples tyrosine kinases to Ras.[16]

The c-*cbl* gene was cloned as the cellular homolog of the v-*cbl* oncogene, which is the transforming component of a murine tumorigenic retrovirus, CAS NS-1.[21] The v-*cbl* oncogene is transforming in early B-lineage and myeloid cells, and Gag-v-Cbl transforming protein lacks the C-terminal 62% of c-Cbl containing the proline-rich region and leucine zipper motif, but still possesses a high proportion of basic amino acids.[22] Although the biological roles of c-Cbl have remained to be elucidated, it has recently been reported that c-Cbl is involved in the signaling pathway triggered by granulocyte-macrophage colony-stimulating factor (GM-CSF) or erythropoietin (Epo) in hematopoietic cells and by stimulation of T cell receptor in T lymphocytes.[23,24] These results motivated us to assess the significance of c-Cbl in the signal transduction pathway involving the activation of receptor-type or nonreceptor-type tyrosine kinases.

EGFR-overexpressing NIH3T3 (E10) was established as follows. To generate the expression vector of EGFR, *Sac*II-*Xho*I fragment of pCOhEGFR was blunted, linked with *Not*I linker and subcloned into the *Not*I site of an expression vector, pSSRabsr.[25] A v-*src*-transformed NIH3T3 cell line was established as described.[26] Parental NIH3T3, E10, and v-*src*-transformed cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% bovine serum. A human leukemia cell line UT-7 is known to be dependent on GM-CSF for growth, and was maintained in RPMI medium 1640

[1] To whom correspondence should be addressed.

containing 8% bovine serum and 10 ng/ml GM-CSF. Polyclonal rabbit anti-c-Cbl antibody (c-15) purchased from Santa Cruz Biotechnology (Santa Cruz, CA) was used for the immunoprecipitation and the immunoblotting of c-Cbl. Monoclonal antibody to Grb2/Ash, purchased from MBL (Nagoya), was used for the immunoblotting of Grb2/Ash. Polyclonal rabbit antibody to EGFR was purchased from Oncogene Science (Uniondale, NY). Monoclonal antibody to Src (a gift from Dr. J. S. Brugge) was used for the immunoprecipitation. Mouse monoclonal anti-phosphotyrosine antibody (anti-PTyr) 4G10 purchased from UBI (Lake Placid, NY) was used for the immunoblotting of phosphotyrosine-containing proteins. The bacterial expression plasmids coding glutathione S-transferase (GST) fusion proteins containing Grb2/Ash SH3, Tec SH3 or SH2, Lyn SH3 or SH2, Fyn SH3 or SH2 and Abl SH3 or SH2 were generously provided by Dr. T. Takenawa, Dr. H. Mano, Dr. T. Yamamoto, and Dr. B. Mayer, respectively. The bacterial expression plasmids coding GST fusion proteins containing SH3 or SH2 of Crk, Nck, Src, and Shc, and Cas SH3 were from Dr. R. Sakai and T. Nakamoto. These plasmids were transfected into XL I-Blue strain of *Escherichia coli* and GST fusion proteins were obtained as described before.[23]

For preparation of NIH3T3 and E10 cell lysates, cells were incubated in DMEM medium containing 0.1% bovine serum albumin (BSA) without serum for 8–15 h prior to stimulation with EGF and then resuspended in DMEM medium containing 100 m*M* Na_3VO_4. The cells were treated with 100 ng/ml EGF for 5 min at 37°C and then lysed at 4°C in the lysis buffer containing 20 m*M* Tris/HCl (pH 8.0), 1% Nonidet P-40 (NP-40), 1 m*M* PMSF, 500 units/ml aprotinin, 2 m*M* EDTA, 50 m*M* NaF, and 1 m*M* Na_3VO_4. NIH3T3 or v-*src*-transformed cells were washed with PBS, then lysed with the lysis buffer. Unsolubilized materials were removed by centrifugation at 15,000*g* at 4°C for 10 min. Binding of cellular proteins to GST fusion proteins, immunoprecipitation, and immunoblotting were done as described before.[23]

To assess whether c-Cbl is involved in signaling pathways employed in fibroblasts, we first studied the EGF/

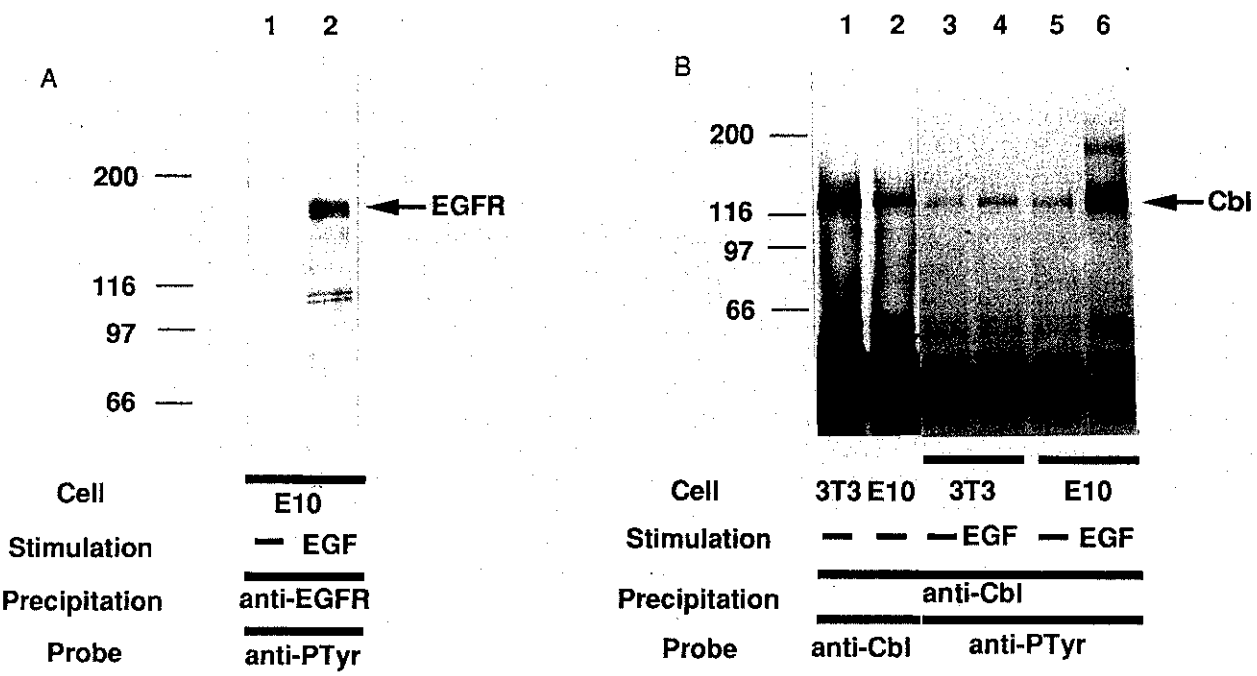

Fig. 1. (A) Autophosphorylation of EGFR induced by EGF stimulation. The lysates from E10 cells unstimulated (lane 1) or stimulated with EGF (lane 2) were mixed with anti-EGFR antibody. The resulting precipitates were subjected to SDS-PAGE and immunoblotted with anti-Ptyr (4G10). Molecular weight markers are indicated at the left (in kDa). The arrow indicates the position of EGFR immunoprecipitated with anti-EGFR antibody. (B) EGF induces tyrosine phosphorylation of c-Cbl in E10. The lysates from E10 cells unstimulated (lanes 2 and 5) or stimulated with EGF (lane 6) or parental NIH3T3 cells unstimulated (lanes 1 and 3) or stimulated with EGF (lane 4) were mixed with anti-c-Cbl. The resulting precipitates were resuspended in Laemmli's sample buffer, subjected to SDS-PAGE and immunoblotted with anti-c-Cbl (lanes 1 and 2) or anti-Ptyr (4G10) (lanes 3–6). Molecular weight markers are indicated at the left (in kDa). The arrow indicates the position of c-Cbl immunoprecipitated with anti-c-Cbl antibody.

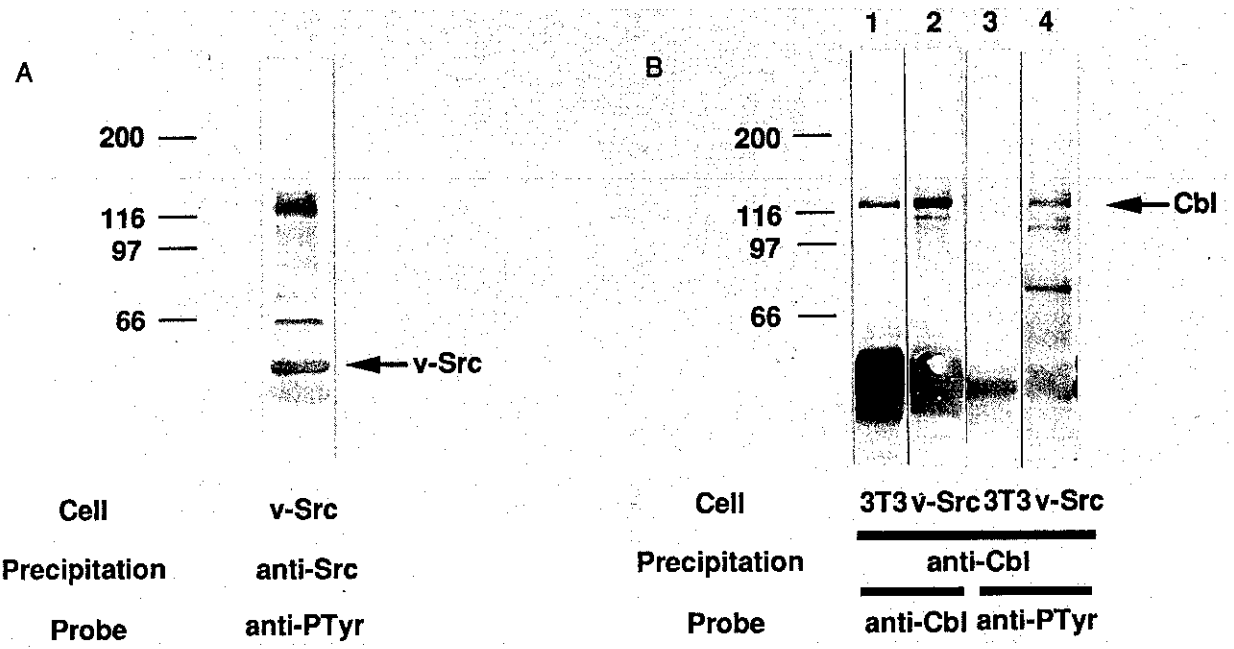

Fig. 2. (A) Autophosphorylation of v-Src. The lysates from v-*src*-transformed NIH3T3 cells were mixed with anti-Src antibody. The resulting precipitates were subjected to SDS-PAGE and immunoblotted with anti-PTyr (4G10). Molecular weight markers are indicated at the left (in kDa). The arrow indicates the position of v-Src immunoprecipitated with anti-Src antibody. (B) v-*src* transformation induces tyrosine phosphorylation of c-Cbl in NIH3T3 cells. The lysates from v-*src*-transformed NIH3T3 cells (lanes 2 and 4) or parental NIH3T3 cells (lanes 1 and 3) were mixed with anti-c-Cbl. The resulting precipitates were resuspended in Laemmli's sample buffer, subjected to SDS-PAGE and immunoblotted with anti-c-Cbl (lanes 1 and 2) or anti-PTyr (4G10) (lanes 3 and 4). Molecular weight markers are indicated at the left (in kDa). The arrow indicates the position of c-Cbl immunoprecipitated with anti-c-Cbl antibody.

EGFR signaling pathway for tyrosine phosphorylation of c-Cbl. For this analysis, we used EGFR-overexpressing NIH3T3 cells, designated E10, as well as parental NIH3T3 cells, since EGF highly promoted *in vitro* growth of EGFR-overexpressing NIH3T3 cells in an EGF-dependent manner (data not shown). Tyrosine phosphorylation of EGFR and other cellular proteins was induced by EGF stimulation of E10 (Fig. 1A), supporting the hypothesis that these events were triggered by binding of EGF to EGFR, autophosphorylation of EGFR, and phosphorylation of cellular proteins.[2] Next, E10 and parental NIH3T3 were treated with EGF and the cell lysates from these cells were mixed with the anti-c-Cbl antibody. The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotted with anti-phosphotyrosine antibody. As shown in Fig. 1B, tyrosine phosphorylation of c-Cbl was also induced by the stimulation of EGF, highly in E10 but only slightly in the parental cells. The expression level of c-Cbl was almost the same in both cell lines. These results demonstrate that the tyrosine phosphorylation of c-Cbl occurs in the signal transduction pathway of the tyrosine-kinase receptor for the epithelial cell growth factor, as well as in those of the receptors for hematopoietic growth factors.

To test the possibility that c-Cbl could also be implicated in the transformation of fibroblasts, we studied NIH3T3 cells transformed by v-*src*.[26] v-Src protein has a high level of tyrosine kinase activity owing to mutations of c-*src* and undergoes autophosphorylation. The high kinase activity of v-Src is thought to be essential for the transformation of cells by v-*src*.[27] To assess whether c-Cbl is tyrosine-phosphorylated in v-*src*-transformed cells, the cell lysates were immunoprecipitated with the anti-c-Cbl antibody and immunoblotted with anti-c-Cbl or anti-phosphotyrosine antibody. A high level of tyrosine-phosphorylation of v-Src was observed, as shown in Fig. 2A. The increase in the tyrosine phosphorylation level of c-Cbl in v-*src*-transformed cells was greater than the difference of the expressed level of c-Cbl between normal 3T3 cells and v-*src*-transformed cells (Fig. 2B), suggesting that v-Src tyrosine-phosphorylates c-Cbl owing to its elevated tyrosine kinase activity.

As previously reported, c-Cbl becomes tyrosine-phosphorylated in the signaling pathways of GM-CSF, Epo, and the T-cell receptor.[23,24] The role of tyrosine-phos-

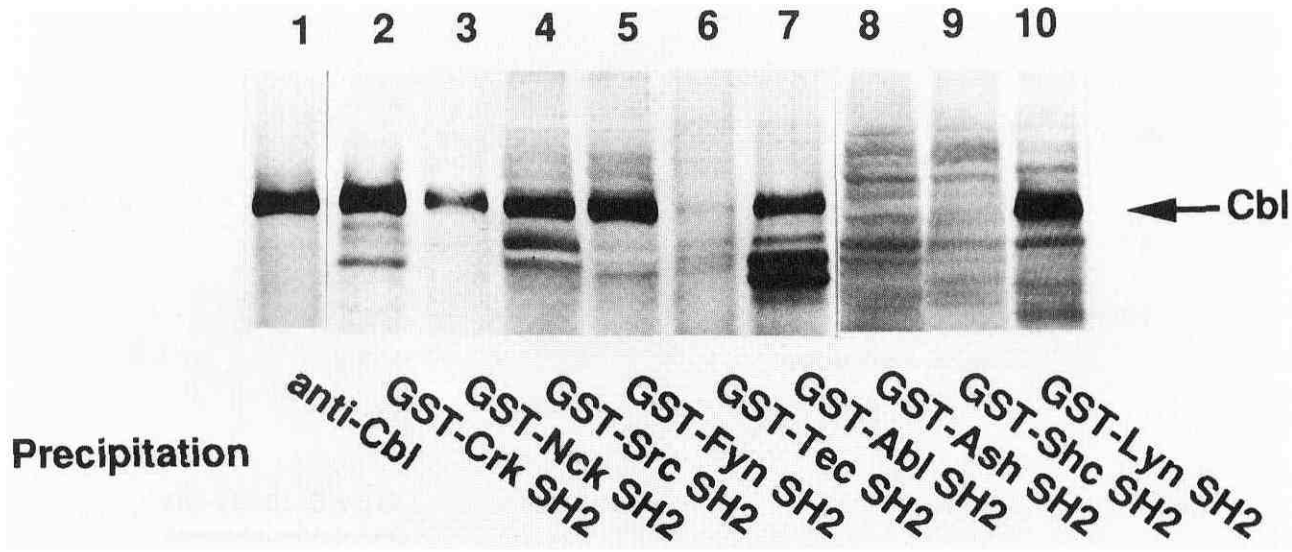

Fig. 3. Tyrosine-phosphorylated c-Cbl associates with the SH2 domains of different proteins. The lysates from K562 cells containing tyrosine-phosphorylated c-Cbl were mixed with each GST-SH2 fusion protein noncovalently coupled to glutathione-agarose beads. The resulting precipitates were resuspended in Laemmli's sample buffer, subjected to SDS-PAGE and immunoblotted with anti-c-Cbl. The arrow indicates the position of c-Cbl immunoprecipitated and bound with GST-SH2 fusion proteins.

phorylated cellular proteins is considered to be generation of the recognition site for binding to the SH2 domains, leading to formation of the signal transduction complexes.[7,8] Therefore, we examined whether the tyrosine-phosphorylated c-Cbl also complexes with other proteins *via* their SH2 region. We used K562 cells because c-Cbl was reported to be tyrosine-phosphorylated in the cells. The lysates were mixed with the GST-SH2 domain fusion proteins noncovalently coupled to glutathione-agarose beads and immunoblotted with the anti-c-Cbl antibody. Tyrosine-phosphorylated c-Cbl was revealed to associate with GST fusion proteins with SH2 domains of Nck and Crk adapter proteins and Src, Fyn, Lyn, Abl, and Tec tyrosine kinases, but not with those of the Ash/Grb2 and Shc proteins (Fig. 3). It has also been reported that tyrosine-phosphorylated c-Cbl can bind to the SH2 domains of Fyn, Lck, Blk, GAP, and PLC-γ.[24] These results suggest that tyrosine phosphorylation of c-Cbl leads to association of c-Cbl with various SH2-containing adapter proteins and tyrosine kinases, forming potentially functional complexes in those signaling pathways.

As reported previously, we demonstrated that c-Cbl constitutively associates with Grb2/Ash.[23] This association was shown to be between the SH3 domain of Grb2/Ash and the proline-rich region of c-Cbl. Moreover, it was reported that c-Cbl also binds to the SH3 domain of Nck.[28] Therefore, we examined whether c-Cbl binds to SH3 domains of other proteins. The cell lysates from UT-7 cells unstimulated or stimulated with GM-CSF were mixed with GST-SH3 fusion proteins noncovalently coupled to glutathione-agarose beads. The resulting precipitates were subjected to SDS-PAGE and immunoblotted with the anti-c-Cbl antibody. As shown in Fig. 4, among the GST fusion proteins with SH3 domains of adapter proteins, GST-Nck SH3 associated with c-Cbl as well as GST-Grb2/Ash SH3, but GST-Crk SH3 did not. GST fusion proteins with SH3 domains of Src, Fyn, Lyn, and Tec tyrosine kinases, but not GST-Abl SH3, constitutively bound to c-Cbl. The fusion protein of GST with the SH3 domain of Cas (Crk-associated substrate)[29] did not associate with c-Cbl. The association of GST-SH3 and c-Cbl was independent of GM-CSF stimulation of UT-7 cells. These findings indicate that the proline-rich region of c-Cbl has some specificity for the association with the SH3 domains.

The identification of binding molecules to c-Cbl *in vivo* is informative as regards the signaling mechanism of c-Cbl. To examine the *in vivo* association of c-Cbl with Grb2/Ash in E10 and v-*src*-transformed NIH3T3 cells, the lysates from v-*src*-transformed NIH3T3 cells and E10 cells unstimulated or stimulated with EGF were immunoprecipitated with the anti-c-Cbl antibody and immunoblotted with the anti-Grb2/Ash monoclonal antibody. As shown in Fig. 5A, Grb2/Ash was detected in anti-Cbl immunoprecipitates in both cell lines. We next assessed

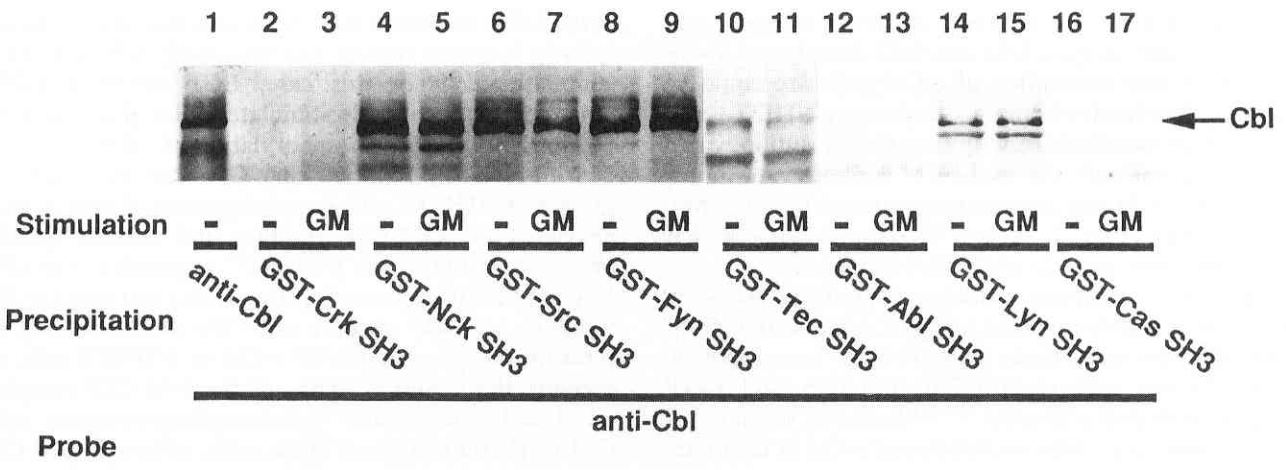

Fig. 4. c-Cbl associates with the SH3 domains of different proteins. The lysates from UT-7 cells unstimulated (lanes 1, 2, 4, 6, 8, 10, 12, 14, and 16) or stimulated with GM-CSF (lanes 3, 5, 7, 9, 11, 13, 15, and 17) were mixed with anti-c-Cbl (lane 1) or each GST-SH3 fusion protein noncovalently coupled to glutathione-agarose beads. The resulting precipitates were resuspended in Laemmli's sample buffer, subjected to SDS-PAGE and immunoblotted with anti-c-Cbl. The arrow indicates the position of c-Cbl immunoprecipitated and bound with GST-SH3 fusion proteins. The details of this experiment were as described before.[23] Cas is a Crk-associated substrate.

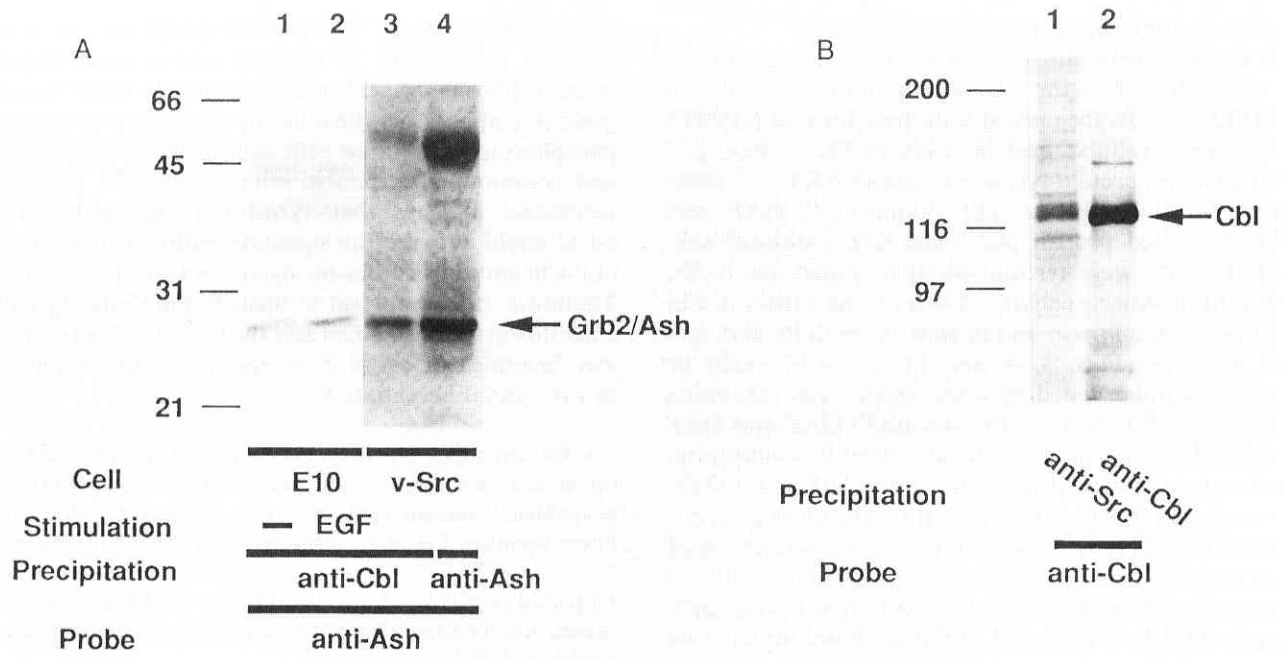

Fig. 5. (A) c-Cbl associates with Grb2/Ash *in vivo*. The lysates from E10 cells unstimulated (lane 1) or stimulated with EGF (lane 2) and v-*src*-transformed NIH3T3 cells (lanes 3, 4) were mixed with anti-c-Cbl (lanes 1–3) or anti-Grb2/Ash antibody (lane 4). Immunoprecipitates were subjected to SDS-PAGE and immunoblotted with anti-Grb2/Ash. Molecular weight markers are indicated at the left (in kDa). The arrow indicates the position of Grb2/Ash. (B) c-Cbl associates with v-Src *in vivo*. The lysates from 3×10^7 v-*src*-transformed NIH3T3 cells were mixed with anti-Src (lane 1) or anti-c-Cbl (lane 2). Immunoprecipitates were subjected to SDS-PAGE and immunoblotted with anti-c-Cbl. Molecular weight markers are indicated at the left (in kDa). The arrow indicates the position of c-Cbl.

the association of c-Cbl with Src. As shown in Figs. 3 and 4, c-Cbl bound to both SH3 and SH2 domains of Src *in vitro*, so *in vivo* association of c-Cbl with Src could be expected. The lysates from v-*src*-expressing NIH3T3 cells were immunoprecipitated with the anti-Src antibody and immunoblotted with the anti-c-Cbl antibody. As shown in Fig. 5B, c-Cbl was co-immunoprecipitated with v-Src. These results (Figs. 3, 4, and 5) strongly suggest that c-Cbl associates directly with v-Src *in vivo*.

There is considerable evidence supporting the view that c-Cbl is widely involved in signal transduction pathways, for instance, those triggered by hematopoietic growth factors such as GM-CSF and Epo, and T-cell receptor-mediated activation.[23,24] Moreover, we have recently demonstrated the implication of c-Cbl in thrombopoietin-mediated signaling (Sasaki *et al.*, submitted). The v-*cbl* oncogene is transforming for early B-lineage and myeloid cells in mice, but the expression of c-*cbl* gene was ubiquitous, although the level of c-Cbl expression was variable.[22] In this report, we have demonstrated that tyrosine phosphorylation of c-Cbl was inducibly increased by activation of EGF receptor depending on EGF stimulation and was constitutively enhanced in v-*src*-transformed NIH3T3 cells (Figs. 1 and 2). This suggests the possible implication of c-Cbl in the other signaling pathways utilizing receptor-type tyrosine kinases or nonreceptor-type tyrosine kinases.

It would be interesting to know which tyrosine kinase is responsible for the phosphorylation of c-Cbl in NIH3T3 cells. In the case of v-*src*-transformed NIH3T3 cells, several cellular proteins such as Shc,[30] PLC-γ,[31] focal adhesion protein-tyrosine kinase (FAK),[32,33] phosphatidylinositol 3-kinase (PI 3-kinase),[34] GAP and GAP-associated protein p62,[6] and Crk-associated substrate (Cas)[29] were tyrosine-phosphorylated due to the high tyrosine-kinase activity of v-Src.[27] Moreover, c-Cbl can bind to Src *in vivo* and *in vitro* through its SH2 and SH3 domains (Figs. 3, 4, and 5), so c-Cbl might be tyrosine-phosphorylated by v-Src. In the cells expressing EGFR, Shc,[35] PLC-γ,[4,5] PI 3-kinase,[36] GAP and GAP associated protein p62[6] were also tyrosine-phosphorylated, as seen in v-*src*-transformed cells. Although EGFR has intrinsic tyrosine-kinase activity,[3] the identity of the tyrosine kinase(s) responsible for the phosphorylation of these proteins and c-Cbl is not clear. It is reasonable to suppose that the responsible kinase is EGFR kinase itself, because EGFR is activated and autophosphorylated by EGF stimulation and tyrosine-kinase activity of EGFR is essential for the cell growth and mitosis induced by EGF.[27] Another candidate is c-Src. It has been reported that EGFR associates with c-Src and that the Src-kinase activity increases rapidly and transiently following EGF stimulation.[37,38] In this case, EGF activates EGFR kinase that subsequently stimulates c-Src kinase activity, resulting in tyrosine phosphorylation of c-Cbl.

We previously reported that GM-CSF stimulates the growth of NIH3T3 cells expressing reconstituted α and β chains of the GM-CSF receptor and induces tyrosine phosphorylation of the β chain,[39] suggesting that GM-CSF could activate some tyrosine kinase and thus the Ras signaling pathway in those cells. We then examined the tyrosine phosphorylation of c-Cbl in NIH3T3 cells expressing the α and β chains of the GM-CSF receptor. c-Cbl did not become tyrosine-phosphorylated upon GM-CSF stimulation of these cells, although GM-CSF stimulation of hematopoietic cells induced tyrosine phosphorylation of c-Cbl. These results indicate that the tyrosine kinase responsible for the tyrosine phosphorylation of c-Cbl is different from the tyrosine kinase responsible for the activation of the Ras signaling pathway in the NIH3T3 cells in which the GM-CSF receptor is reconstituted, and that c-Cbl could be involved in another signaling pathway different from the Ras pathway.

The significance of tyrosine phosphorylation of c-Cbl is not clear either, but several reports have shown the importance of tyrosine phosphorylation of c-Cbl for tumorigenesis. In the 70Z/3 pre-B lymphoma, a mutant form of c-Cbl with 17 deleted amino acids became tyrosine-phosphorylated and activated to show tumorigenic potential.[40] On the other hand, c-Cbl was tyrosine-phosphorylated in tumor cells expressing v-*abl* or *Bcr-abl* and co-immunoprecipitated with anti-Abl.[40] These observations indicate that tyrosine phosphorylation of c-Cbl could activate the signaling pathway downstream of c-Cbl and thus confer oncogenic potential on the cells. Therefore, it is important to identify the signaling molecules downstream of c-Cbl and the biological or biochemical functions of c-Cbl in signal transduction and cell growth and differentiation.

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