Antisense Repression of Proto-oncogene c-Cbl Enhances Activation of the JAK-STAT Pathway but Not the Ras Pathway in Epidermal Growth Factor Receptor Signaling

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Many growth factors including epidermal growth factor (EGF) induce tyrosine phosphorylation of the c-Cbl proto-oncogene product, whose function, however, remains unclear. Recently, Sli-1, a Caenorhabditis elegans homologue of c-Cbl, was found to be a negative regulator of let-23-mediated vulval induction pathway, suggesting that c-Cbl may negatively regulate EGF receptor (EGFR)-mediated signaling. In this study, by an antisense RNA approach, we examined the effects of expression level of c-Cbl on EGFR signaling and showed that overexpression of c-Cbl reduces and antisense repression of c-Cbl enhances autophosphorylation of EGFR receptors and activation of the JAK-STAT pathway. However, in contrast to the Sli-1 protein, the expressed amount of c-Cbl does not affect activation of the Ras pathway, suggesting that the EGFR-mediated signaling pathways are differently regulated by c-Cbl among nematodes and mammals.

The c-Cbl proto-oncogene was originally identified as a cellular homologue of v-Cbl oncogene, which was cloned from the Caenorhabditis elegans (1). The c-Cbl gene product is a 120-kDa protein that contains an NH2-terminal domain with a nuclear localization signal, followed by a RING finger motif (2). The COOH-terminal half of the protein contains a proline-rich domain that has been shown to function as a ligand for the SH3 domains of many signaling molecules including Grb2, Nck, Src, and Fyn (3–10). The product of v-Cbl is a truncated form of c-Cbl, which lacks both the RING finger motif and the proline-rich domain (2). The v-Cbl protein has been shown to localize to the nucleus, to bind DNA, and to transform NIH3T3 cells, whereas the c-Cbl protein localizes in the cytoplasm and cannot transform NIH3T3 cells (11). Although previous reports demonstrated that many growth factors including epidermal growth factor (EGF) induce tyrosine phosphorylation of c-Cbl (8, 10, 12–16), the function of this molecule in the growth factor receptor-mediated signaling pathway remains unclear. Recently, it was reported that c-Cbl is a homologue of Sli-1, a negative regulator of let-23-mediated signal transduction pathway in Caenorhabditis elegans (17, 18). Therefore, it is expected that c-Cbl negatively regulates EGFR receptor (EGFR)-mediated signaling.

To address the possibility, we established a subline of NIH3T3 cells in which the expression of c-Cbl is repressed by the introduction of antisense c-Cbl cDNA. By using this cell line as well as the parental NIH3T3 cells and c-Cbl-overexpressing cells, we analyzed the roles of c-Cbl in regulation of the EGFR signaling.

MATERIALS AND METHODS

Cells and Antibodies—NIH3T3 cells and all cell lines derived from NIH3T3 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% bovine serum. Anti-c-Cbl antibody and anti-STAT3 antibody were purchased from Santa Cruz Biotechnology Inc. Anti-phosphotyrosine antibody 4G10, anti-Shc antibody, and anti-JAK1 antibody were purchased from Upstate Biotechnology Inc. Anti-Grb2 antibody 3F2 and anti-EGF receptor antibody Ab-1 were purchased from MBL Inc. and Oncogene Science Inc., respectively. The mouse monoclonal antibody to a peptide epitope derived from the hemagglutinin protein of human influenza virus (clone 12CA5) was purchased from Boehringer Mannheim. Anti-Sos1 antibody and anti-MAPK antibody C92 were gifted by T. Kadowaki (University of Tokyo, Tokyo, Japan).

Construction of Vectors Expressing the Antisense and Deletion Mutants of c-Cbl and Transfection—The human c-cbl cDNA epitope tagged with a nine-amino-acid hemagglutinin peptide (YPYDVPDYA) from the human influenza virus was a kind from W. Y. Langdon (University of Western Australia). To construct vectors expressing antisense of c-Cbl, a BamHI-EcoRI fragment that corresponds to 1–578 base pairs or a HaelII-NcoI fragment that corresponds to 378–811 base pairs of c-cbl cDNA was blunted, linked with NolI linker, and subcloned into the NolI site of an expression vector, pUC-CAGGS (19), in a reverse orientation. To construct deletion mutants of c-Cbl, the HindIII-HindIII fragments of c-cbl cDNA corresponding to 724–1618 base pairs (ΔRING-Cbl) or 1619–2776 base pairs (ΔPD-Cbl) were cut out and ligated. The antisense constructs were co-transfected with pSV2neo into NIH3T3 cells according to the protocol of Chen and Okayama (20). 12 h after transfection, cells were washed once with DMEM and cultured in fresh medium containing 5% fetal calf serum for 24 h followed by the G418 selection (500 ng/ml), and resistant clones were isolated and expanded.

Retrovirus vector was used to transfect cDNA lines of c-Cbl and deletion mutants into NIH3T3 cells. Replicon-deficient retroviral stocks were prepared by transient hyperexpression in Cos7 cells. These constructs were transfected together with packaging plasmid by the DEAE-dextran method. Viral infections were performed by exposing cells to virus stocks with 8 μg of polybrene/ml at 37 °C for 12 h, and G418-resistant populations were selected in the presence of 500 μg of G418/ml. To establish EGFR stable transfectants, C2/E10 cells, and AS21/E4 cells, the expression vector EGFR-pSRbaxer (21) was transfected according to the protocol of Chen and Okayama, followed by blastidicin selection (5 μg/ml) (Funakoshi, Inc.).

Immunoprecipitation, Immunoblotting, and in Vitro Kinase Assay—Prior to stimulation, cells were starved in DMEM containing 0.5% fetal calf serum for 12 h. Cells were then stimulated with 100 ng/ml of EGF for 5 min at 37 °C, washed twice with ice-cold phosphate-buffered saline, and lysed in Triton lysis buffer (0.5% (w/v) Triton X-100, 50 mM...
Tris-HCl, pH 7.4, 2 mM phenylmethylsulfonyl fluoride, 10 units/ml aprotinin, 1 mM sodium orthovanadate, 1 mM EDTA). Cell lysates were centrifuged, and the supernatant was collected. For analysis of total cellular proteins, SDS sample buffer was added directly to lysate, and the mixture was denatured for 5 min at 95 °C and subjected to SDS-PAGE. Proteins separated on SDS-PAGE were transferred electrophoretically to a polyvinylidene difluoride membrane (Immobilon, MILLIPORE). The filters were pre-washed five times in the wash buffer (0.1% (v/v) Triton X-100, 50 mM NaCl, 0.05% Tween-20), incubated for 2 h at room temperature with the specific rabbit or mouse antibodies coupled to the protein A-Sepharose beads. Immunoprecipitations were performed at 4 °C for 3 h with specific rabbit or mouse antibodies, the cell morphology of AS21 cells, C2 cells, and AS21 cells were immunoprecipitated (IP) with anti-c-Cbl antibody and immunoblotted with anti-c-Cbl antibody. C, the cell morphology of AS21 cells, C2 cells, and NIH3T3 cells. These cell lines were maintained in DMEM containing 5% bovine serum and photographed.

**RESULTS**

**Construction of Antisense Expression Constructs of c-Cbl Proto-oncogene and Establishment of AS21 Cells That Express a Low Level of c-Cbl—**To investigate the roles of c-Cbl in the intracellular signaling pathways in fibroblasts, we tried to establish cell lines that express c-Cbl at a low level or at a high level. First, we generated two antisense constructs of c-Cbl (AS1 and AS2) derived from the NHL-terminal portion of c-Cbl cDNA (Fig. 1A). We then introduced them into NIH3T3 cells, and the cells were subjected to the G418 selection. Resistant colonies were isolated, and the expression levels of c-Cbl in these cell lines were examined by the immunoblot with anti-c-Cbl antibody. No detectable change was observed in AS1-introduced cell lines, but in several AS2-introduced clones, the expression levels of c-Cbl were found to be decreased (data not shown). Among these cell lines, AS21 cells were chosen for further analysis because they expressed c-Cbl at the lowest level (data not shown). We also established C2 cells, an NIH3T3-derived stable line that overexpresses c-Cbl, by using retrovirus vector carrying c-Cbl cDNA. The expression levels of c-Cbl in C2 cells, AS21 cells and mock-transfected cells are shown in Fig. 1B.

To examine the roles of c-Cbl in the signal transduction pathways through receptor tyrosine kinases, we compared the biological activities of C2 cells and AS21 cells with that of parental NIH3T3 cells. Compared with parental NIH3T3 cells, AS21 cells showed an elongated and spindle shape, resembling a transformed phenotype (Fig. 1C), but their morphological change was less prominent than that of NIH3T3 cells transformed by activated oncogenes such as v-Src (data not shown). Furthermore, they grew in culture as monolayers and did not form foci nor produce colonies in soft agar (data not shown). On the other hand, C2 cells that overexpress c-Cbl showed a slightly flatter morphology than but almost indistinguishable from parental NIH3T3 cells (Fig. 1C). We next examined the growth rate of these cell lines by counting the cell number every 2 days, but no apparent difference was detected (data not shown). These results indicate that the expression levels of c-Cbl do not affect the cell growth or transforming phenotype of NIH3T3 cells.

**Overexpression of c-Cbl Suppresses and Antisense Repression of c-Cbl Enhances Autophosphorylation of EGF Receptors—**By using AS21 cells and C2 cells, we thought that we could evaluate the effects of c-Cbl on growth factor receptors signaling, because they express different amounts of c-Cbl. Because recent studies indicate that the Sh-1 protein, which is a C. elegans homologue of c-Cbl, is a negative regulator of the LET-23 tyrosine kinase receptor (17, 18), it is reasonable to expect that c-Cbl negatively regulates EGFR kinase activity. Because it was difficult to compare the difference of autophosphorylation levels of EGFR between them due to relatively low level of endogenous EGFR in NIH3T3 cells, we established stable transfectants from them by introducing EGFR cDNAs. Among these transfectants, AS21/E4 and C2/E10 lines were selected for further analyses because they were found to express approximately the same amount of EGFR as E10 cells, which are human EGFR stable transfectants (10) (Fig. 2A). We stimulated AS21/E4 cells and C2/E10 cells with EGF and examined the autophosphorylation level of EGFR by the immunoblot with anti-phosphotyrosine antibody. Compared with the autophosphorylation level of EGFR in E10 cells, that in C2/E10 cells was suppressed, whereas the autophosphorylation level of EGFR in AS21/E4 cells was apparently enhanced (Fig. 2B). These results indicate that c-Cbl negatively regulates the autophosphorylation activity of EGFR. To determine the region of c-Cbl required for its negative regulatory effects, we constructed deletion mutants of c-Cbl that lack the RING finger domain (ΔRING-Cbl) or the proline-rich domain (ΔPD-Cbl). These constructs were introduced into E10 cells by the retroviral vector. After the G418 selection for 10 days, resistant cells were stimulated with EGF, and the levels of autophosphorylation of EGF receptors were examined. In this experiment, both deletion mutants of c-Cbl could not suppress autophosphorylation of EGF receptors (Fig. 2C).

**The Amount of c-Cbl Does Not Affect Activation of the Ras Pathway—**We next investigated if negative regulation of EGFR
by c-Cbl may affect activation of the Ras pathway. We examined the tyrosine phosphorylation of the Shc adapter protein, which is known to be inducibly phosphorylated by EGF treatment and to regulate activation of the Ras pathway in EGFR signaling (23). AS21/E4 cells, C2/E10 cells and E10 cells were stimulated with EGF, lysed, and immunoprecipitated with anti-

Shc antibody. The immunoprecipitates were subjected to the immunoblotting with anti-phosphotyrosine antibody. In this experiment, contrary to our expectation, tyrosine phosphorylation of Shc in C2/E10 cells was not suppressed but rather modestly increased, whereas that in AS21/E4 cells was slightly decreased compared with that in E10 cells (Fig. 3A). Next, we investigated activation of Sos, which is a guanine nucleotide exchange factor of Ras (24–27), in these cell lines. When activated with EGF, Sos is known to be phosphorylated on serine residues, resulting in electrophoretic mobility shift in SDS-PAGE (28). The total cell lysates from AS21/E4 cells, C2/E10 cells, and E10 cells treated with or without EGF were subjected to the Western blotting with anti-Sos antibody, and the mobility shift of Sos was examined. In this experiment, the degree of mobility shift of Sos was not altered among these cell lines as was the case with Shc (Fig. 3B). By running the sample on the gel for a longer period of time, this shift was made more obvious, although the Sos bands were made slightly blur (Fig. 3B, lower panel). These results suggest that the amount of Cbl has no influence on activation of the Ras pathway downstream of EGFR. To confirm this, the MAP kinase activities in these cell lines, when treated with EGF, were evaluated by the in vitro kinase assay of MAP kinase using myelin basic protein as a substrate. As expected from the results of phosphorylation of Shc and Sos, the MAP kinase activities of AS21/E4 cells, C2/E10 cells, and E10 cells were shown to be approximately equal to each other (Fig. 3C).

Recent studies have indicated that the SH3 domains of Grb2 constitutively bind to the proline-rich region of Sos (26, 27) and Cbl (3, 8). Therefore, it is possible that Cbl interferes with the association between Sos and Grb2. If it is the case, overexpression of c-Cbl will decrease the association between Sos and Grb2, and in contrast, suppression of c-Cbl expression by an antisense RNA will increase it. To test this, the same amount of lysates from AS21/E4 cells, C2/E10 cells, and E10 cells were immunoprecipitated with anti-Cbl or anti-Sos antibodies. The immunoprecipitates were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was cut into two parts, and the upper part was immunoblotted with anti-Cbl or anti-Sos antibodies, whereas the lower part was immunoblotted with anti-Grb2 antibody. In this experiment, c-Cbl overexpression resulted in increase in the amount of Grb2 co-precipitated with c-Cbl. However, the amount of Grb2 associated with Sos did not vary among AS21/E4 cells, C2/E10 cells, and E10 cells (Fig. 3D). These data suggest that c-Cbl and Sos utilize different Grb2 molecule and these two molecules do not compete with each other in binding to Grb2. Taken together, c-Cbl does not affect activation of the Ras pathway in EGFR signaling. We also checked the growth rate of AS21/E4 cells, C2/E10 cells, and E10 cells in the presence of EGF, but no significant differences were detected (data not shown). These results are consistent with our observation that the activity of MAPK of these cell lines stimulated with EGF was equal to each other.

Overexpression of c-Cbl Suppresses and Repressed Expression of c-Cbl Enhances Activation of the JAK-STAT Pathway—Although our data suggest that c-Cbl negatively regulate autophosphorylation of EGFR, it did not affect activation of the Ras pathway. We then examined if other signaling pathways known to exist downstream of EGFR are affected by the amount of c-Cbl expression. For instance, the JAK-STAT pathway is shown to be activated by EGFR (29). EGF induces tyrosine phosphorylation of JAK1, STAT1, and STAT3. STAT1 and STAT3 form heterodimer and homodimer (SIF A, SIF B, and SIF C complexes) and bind to the SIE sequence, which exists in c-Fos promoter (30). Therefore, we examined if c-Cbl
regulates their activation in EGFR signaling. Lysates from AS21/E4 cells, C2/E10 cells, and E10 cells by EGF treatment. AS21/E4 cells, C2/E10 cells, and E10 cells were serum-starved for 18 h, stimulated with or without EGF (100 ng/ml) for 5 min at 37 °C, and lysed in Triton lysis buffer. Lysates were then immunoprecipitated (IP) with anti-Shc antibody and immunoblotted with 4G10 (upper panel) or anti-Shc antibody (lower panel). The arrows indicate the portions of p46Shc, p52Shc, and p66Shc (p46, p52, and p66, respectively). B, mobility shift of Sos in AS21/E4 cells, C2/E10 cells, and E10 cells by EGF treatment. Lysates obtained as described for A were subjected to the immunoblot with anti-Sos antibody. Upper panel, 8% SDS-PAGE. Lower panel, the same samples as the upper panel were electrophoresed on the 5% polyacrylamide gel for a longer period of time. C, the in vitro kinase assay of MAP kinase in AS21/E4 cells, E10 cells, and E10 cells by EGF treatment. Lysates obtained as described for A were immunoprecipitated with anti-MAPK antibody (C92) and subjected to the in vitro kinase reaction using myelin basic protein as a substrate. We confirmed by the anti-MAPK immunoblot that the expression level of MAP kinase are the same among AS21/E4, C2/E10, and E10 cells (data not shown). An arrow indicates myelin basic protein. D, the effect of amount of c-Cbl for association between Sos and Grb2. Lysates from AS21/E4 cells, C2/E10 cells, and E10 cells were immunoprecipitated with anti-Sos antibody (left panel) and anti-c-Cbl antibody (except for AS21/E4 cells) (right panel) and immunoblotted with anti-Sos antibody (left upper panel), anti-c-Cbl antibody (right upper panel), and anti-Grb2 antibody, 3F2 (lower panel).

FIG. 3. The effects of the expression levels of c-Cbl for activation of the Ras pathway.
A, phosphorylation of Shc in AS21/E4 cells, C2/E10 cells, and E10 cells by EGF treatment. AS21/E4 cells, C2/E10 cells, and E10 cells were serum-starved for 18 h, stimulated with or without EGF (100 ng/ml) for 5 min at 37 °C, and lysed in Triton lysis buffer. Lysates were then immunoprecipitated (IP) with anti-JAK1 antibody followed by the immunoblotting with anti-phosphotyrosine antibody. Compared with tyrosine phosphorylation level of JAK1 in E10 cells, that of JAK1 in C2/E10 cells was slightly suppressed, whereas that in AS21/E4 cells was apparently enhanced. The degree of tyrosine phosphorylation of JAK1 in these cell lines approximately correlates with that of EGFR. We also examined tyrosine phosphorylation of STAT3 in AS21/E4 cells, C2/E10 cells, and E10 cells, because it is a substrate for JAK1 kinase. The phosphorylation levels of STAT3 were also changed in proportion to tyrosine phosphorylation levels of EGFR and JAK1 (Fig. 4B). Tyrosine phosphorylation of STAT1 in these cell lines showed a similar result to that of STAT3 (data not shown). These data indicate that activation of the JAK-STAT pathway approximately correlates with EGFR kinase activity and is negatively regulated by c-Cbl.

DISCUSSION

In this study, by an antisense RNA approach, we established NIH3T3 cells in which c-Cbl expression is repressed (AS21 cells). Comparing this cell line with C2 cells that overexpress c-Cbl and with parental NIH3T3 cells, c-Cbl expression level did not affect the growth rate or transforming ability. We introduced EGFR cDNA into these cell lines and established AS21/E4 cells and C2/E10 cells. By using these cell lines and E10 cells, we analyzed the function of c-Cbl in EGFR signaling and found that overexpression of c-Cbl reduces, and antisense repression of c-Cbl enhances, autophosphorylation of EGFR receptors. Yet, the amount of c-Cbl does not affect activation of the JAK-STAT pathway approximately correlates with EGFR kinase activity and is negatively regulated by c-Cbl.
the Ras pathway. However, overexpression of c-Cbl suppresses and antisense repression of c-Cbl enhances activation of the JAK-STAT pathway.

Consistent with our data, Bowtell and Langdon have shown that overexpression of wild-type c-Cbl reduced the amount of tyrosine-phosphorylated EGFR associating with Shc following EGFR stimulation, compared with that in parental Balb 3T3 cells (12). However, the mechanism with which c-Cbl regulates the kinase activity of EGFR receptor is unknown. To determine which domain of c-Cbl is necessary for this regulatory function, we constructed deletion mutants of c-Cbl that lack the RING finger domain or the proline-rich domain. Neither of the mutants were able to suppress autophosphorylation of EGFR receptor. From these results, we could conclude that both domains are required for the negative regulatory effects of c-Cbl. The proline-rich domain of c-Cbl is a target for the SH3 domain of Grb2, enabling c-Cbl to associate with EGFR receptor upon EGFR stimulation. We speculate that the APD-Cbl mutant cannot bind to EGFR as efficiently as the wild-type c-Cbl and thus fails to generate the negative signal. On the other hand, the result showing that the ΔRING-Cbl mutant could not suppress autophosphorylation of EGFR receptor might imply that this region plays a critical role for generating the negative regulatory signal of c-Cbl.

It has been reported that the slt-1 (reduction of function) mutations suppress all known defects of let-23 mutations: (i) viability, (ii) hermaphrodite fertility, (iii) male spicule development, (iv) posterior epidermal development, and (v) vulval differentiation (31) except for sterility. Reduction-of-function mutations in sem-5 and let-60 also display vulvalessa (Vul) phenotype (32, 33) like let-23 (sy97), a severe reduction of function allele (31). The slt-1 (sy143) mutation suppresses the Vul phenotype associated with a weakly hypomorphic sem-5 (n2019) and let-60 (rf) mutation, n2021(18). These observations indicate that Slt-1 negatively regulates the let-23-, sem-5-, and let-60-mediated pathway and suggest that c-Cbl might negatively regulate not only EGFR kinase activity but the Ras signaling pathway. Although our data indicate that c-Cbl suppresses autophosphorylation of EGFR, this has no effect on phosphorylation of Shc and Sos and the MAP kinase activity. In contrast, there is a substantial affect on phosphorylation of JAK1 and STAT3. These data partially contradict the results expected from the role of Slt-1 protein in the let-23-mediated vulval induction pathway.

To address the question, we should consider several studies reporting the relationship between EGFR kinase activity and activation of MAP kinase or JAK kinase. Wright et al. reported that a kinase-defective EGFR receptor mutant (K721M) can induce phosphorylation of Shc and activate MAP kinase, whereas it cannot induce phosphorylation of JAK1 or STAT1. They discussed that kinase-defective EGFRs activate MAP kinase by heterodimerization with and stimulating kinase activity of c-ErbB2, which enhances phosphorylation of and binding with Shc and activates the Ras pathway (34). Furthermore, of c-ErbB family activates, only EGFR binds c-Cbl (35). This may imply that c-Cbl can suppress only kinase activity of EGFR but not that of other c-ErbB kinases. From these observations, we speculate that activation of the Ras pathway is not directly dependent upon the kinase activity of EGFR and that c-Cbl does not affect it in EGFR signaling, because the kinase activity of c-ErbB2 can compensate that of EGFR. We also hypothesize that in nematodes this redundancy may not exist, and therefore Slt-1 can directly regulate the let-23-, sem-5-, and let-60-mediated pathway. On the other hand, activation of the JAK-STAT pathway depends on EGFR kinase activity; therefore, c-Cbl negatively regulate activation of the JAK-STAT pathway in proportion to the autophosphorylation level of EGFR.

In this paper, we investigated the relationship among EGFR kinase activity, activation of the Ras pathway and the JAK-STAT pathway. Our data suggest that c-Cbl negatively regulates EGFR kinase activity but in contrast to the Slt-1 protein did not affect activation of signaling molecules of the Ras pathway. On the other hand, activation of the JAK-STAT pathway correlated with EGFR kinase activity and as a result was negatively regulated by c-Cbl. Our report is the first study describing the regulation of the JAK-STAT pathway by c-Cbl, and we speculate that c-Cbl, by coupling to EGFR, negatively regulates its kinase activity and thus regulates activation of the JAK-STAT pathway. We are currently trying to further characterize the mechanism of these negative regulation by c-Cbl protein.

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