Interactions of Cbl with Two Adaptor Proteins, Grb2 and Crk, upon T Cell Activation*

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Several recent studies have demonstrated that Grb2, composed entirely of SH2 and SH3 domains, serves as an adaptor protein in tyrosine kinase signaling pathways. Cbl, the proto-oncogene product of c-bcrproto-oncogene, has been reported to be phosphorylated on tyrosine residues upon T cell receptor (TCR) engagement. Here we show that in unstimulated Jurkat cells Cbl is co-immunoprecipitated with monoclonal antibody against Grb2. However, in lymphocytes activated through the TCR, Cbl loses its ability to bind to Grb2 phosphorylated either with anti-Grb2 antibody or with an immobilized tyrosine phosphopeptide, Y1068-P, derived from the epidermal growth factor receptor. In vitro studies confirm that the ability of Cbl to bind to both SH3 domains of Grb2 is strongly reduced in activated T lymphocytes. Investigation of the time course of Cbl dissociation from Grb2 reveals that it is transient and correlates with the kinetics of tyrosine phosphorylation of Cbl. Moreover, Cbl is co-immunoprecipitated with Crk, another SH2/SH3 domain-containing protein, upon TCR stimulation. Tyrosine-phosphorylated Cbl binds exclusively to the SH2 domain of Crk. These results suggest that different adaptor proteins may have different roles in the regulation of c-bcr proto-oncogene product.

Recent studies by a number of laboratories have characterized the mechanism by which growth factors activate the Ras signaling pathway. This mechanism involves formation of complexes of the Sos guanine nucleotide exchange protein, and Grb2, an SH2 and SH3 domain-containing adaptor protein with autophosphorylated growth factor receptors (1–3). The SH3 domains of Grb2 bind to the carboxyl-terminal proline-rich domain of Sos (2), whereas the SH2 domain binds to tyrosine-phosphorylated sites (1–3). Complex formation of Sos with autophosphorylated growth factor receptors (1–3) results in the translocation of Sos from the cytosol to the plasma membrane, where its substrate Ras is localized (1).

The Crk protein, a homologue of the product of the v-crk oncogene, is an SH2 and SH3 domain-containing adaptor protein related to Grb2 and Nck (4). Two forms of cellular Crk proteins have been found: Crk I has the domain structure SH2-SH3-SH3, while the shorter Crk I consists of one SH2 and one SH3 domains (5, 6). Recently, a third member of the Crk family has been cloned from chronic myelogenous leukemia cells and referred to as Crk-L (7). It has been shown that Crk proteins associate with two guanine nucleotide exchange proteins for Ras, Sos, and C3G (8–10). In addition, they interact via their SH2 domains with both phosphorylated paxillin and p130Cas (11, 12).

Stimulation of T lymphocytes via their TCR results in phosphorylation of multiple intracellular proteins on tyrosine residues (13, 14). We and others have shown that in the lyses of activated T cells Grb2 associates with several phosphotyrosine proteins (15–18), including proteins with molecular masses of 36, 52, 75, and 120 kDa. The 36-kDa membrane-bound phosphoprotein binds to the SH2 domain of Grb2. In UCHT1-stimulated T cells, p36 was shown to be associated with the complex of Sos and Grb2 and thus implicated in Ras activation (15, 16). The 52-kDa tyrosine phosphoprotein is identical with Shc adaptor protein (19). The 75-kDa tyrosine kinase substrate, that has been recently cloned and referred to as SLP-76, interacts with the SH3 domains of Grb2 (17, 20).

It has been shown that a 116-kDa tyrosine phosphoprotein implicated in the TCR signaling pathway binds to the amino-terminal SH3 domain of Grb2 (18). Recently, a 120-kDa tyrosine phosphoprotein which in vitro binds to SH3 domains of Fyn, Lck, and Grb2, has been identified as Cbl (21). The v-cbl oncogene is the transforming gene of the murine Cas NS-1 retrovirus which induces pre-B cell lymphomas and myeloid leukemias (22). The homologue of v-cbl in mammalian cells is the c-bcrproto-oncogene that encodes Cbl, a 120-kDa protein predominantly localized in the cytoplasm (23). Furthermore, in vivo association of Nck, another SH2 and SH3 domain-containing adaptor protein with Cbl, was also demonstrated (24).

In this paper, we characterize the in vivo interaction of Cbl with two adaptor proteins, Grb2 and Crk. We report that, upon T cell activation, Cbl rapidly and transiently dissociates from Grb2. Tyrosine-phosphorylated Cbl then binds to the SH2 domain of Crk. We provide evidence that Grb2 and Crk have a critical role in the regulation of Cbl proto-oncogene product.

MATERIALS AND METHODS

Cbl Culture, Stimulation, and Lysates—Jurkat cells were obtained from the Cell Culture Bank of ICRF and maintained in RPMI 1640 medium containing penicillin (100 units/ml), streptomycin (50 μg/ml), and 1% fetal calf serum. 5 × 10⁶ cells were used per point (1 ml of lysis buffer) stimulated with 10 μg/ml monoclonal anti-CD3 antibody UCHT1. Cells were harvested by centrifugation at 15,000 × g for 20 s and then lysed in 1 ml of ice-cold 50 mM HEPES buffer, pH 7.4, containing 3% Triton X-100, 100 mM NaCl, 20 mM NaF, 1 mM EGTA, 0.1 mM NaVO₃, 1 mM p-nitrophenyl phosphate, 10 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 25 μM each of leupeptin, trypsin inhibitor, aprotinin, and pepstatin A. Lysates were clarified by centrifugation at 15,000 × g for 10 min at 4°C. Antibodies, Fusion Proteins, and Peptides—Anti-CD3 antibody UCHT1 was from D. A. Cantrell, ICRF. Monoclonal anti-phospho-tyrosine monoclonal antibody Y1068-P was from Transduction Laboratories. Anti-CD3 antibody was from D.A. Cantrell, ICRF. 32P-labeled monoclonal antibody Y1068-P was used to detect tyrosine phosphorylated proteins by autoradiography.
immunoblotting with an antibody against the 120 kDa c-cbl proto-oncogene product shows the presence of Cbl in the Y1068-P precipitates from unstimulated cells and a decreased amount of Cbl in the precipitates from UCHT1-stimulated cells (Fig. 1). These data suggest that the 120-kDa tyrosine phosphoprotein observed in our experiment is identical with Cbl or at least Cbl is a component of the 120-kDa tyrosine phosphoprotein band. The Y1068-P phosphopeptide precipitates equal amounts of Grb2 from either unstimulated or activated T lymphocytes (15); thus, the decreased amount of Cbl in the precipitates indicates that the association of Grb2 with Cbl is strongly inhibited in the lysates of activated cells. Identical results were obtained using human peripheral blood T lymphoblasts (data not shown). It is noteworthy that while Cbl disappeared from the Grb2 complex, in the 4G10 blot a relatively large amount of a 120-kDa tyrosine phosphoprotein still bound to Grb2 even in stimulated cells. It is likely that another tyrosine phosphoprotein exists in stimulated T cells with a molecular mass of 115–120 kDa.

Analysis of the partial amino acid sequence of an unidentified 116-kDa phosphoprotein, that can also bind to Grb2, suggests that it is different from Cbl (20).

To prove the interaction of Grb2 with Cbl in another experiment, immunoprecipitations with anti-Grb2 antibody covalently bound to Sepharose beads were performed. J urkat cells were stimulated with UCHT1 antibody for 2 min or left untreated, proteins were immunoprecipitated with anti-Grb2, and then probed with anti-Cbl antibody. Fig. 2 demonstrates that in the lysate of resting cells Cbl co-immunoprecipitates with Grb2. In contrast, in the lysate of activated cells, Cbl is not associated with Grb2. Immunoblotting of whole cell lysates from quiescent or activated T cells with anti-Cbl antibody shows equal quantities of immunoreactive bands (Fig. 2) (26). T cell stimulation results in a rapid and significant phosphorylation of Cbl detected with anti-phosphotyrosine antibody (21). Donovan et al. (21) have demonstrated Cbl phosphorylation only in whole cell lysates of J urkat cells. Therefore, we immunoprecipitated Cbl and then performed immunoblotting with 4G10. Fig. 3 demonstrates that Cbl has a certain level of basal phosphorylation in quiescent cells. However, in response to TCR activation, Cbl is rapidly phosphorylated on tyrosine residues. Maximal phosphorylation is seen after 5 min of stimulation, with a reduction to background levels by 45 min.

We also investigated the kinetics of Cbl dissociation from

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**RESULTS**

We have recently used an immobilized tyrosine phosphopeptide derived from the Y1068 autophosphorylation site of the EGF receptor, to precipitate the complex of Grb2 and Sos from the lysates of T lymphocytes (15). It has been demonstrated that the phosphopeptide, Y1068-P binds to the SH2 domain of Grb2 with high affinity, whereas the SH3 domains of Grb2 are available for protein-protein interaction (1, 15). To test if the phosphopeptide could precipitate proteins other than Sos in complex with Grb2, both the phosphorylated and unphosphorylated forms of Y1068 peptide immobilized on beads were incubated with lysates from untreated and TCR cross-linked J urkat cells. Probing of Y1068-P precipitates with anti-phosphotyrosine antibody, 4G10, demonstrates that a 120-kDa phosphoprotein is present in the lysate of unstimulated cells (Fig. 1). Interestingly, upon T cell activation, the amount of this phosphoprotein is strongly decreased in the Y1068-P precipitate, while the appearance of a 75-kDa tyrosine phosphoprotein was detected in the same precipitate. Immunoblotting with an antibody against the 120 kDa c-cbl proto-oncogene product shows the presence of Cbl in the Y1068-P precipitates from unstimulated cells and a decreased amount of Cbl in the precipitates from UCHT1-stimulated cells (Fig. 1). These data suggest that the 120-kDa tyrosine phosphoprotein observed in our experiment is identical with Cbl or at least Cbl is a component of the 120-kDa tyrosine phosphoprotein band. The Y1068-P phosphopeptide precipitates equal amounts of Grb2 from either unstimulated or activated T lymphocytes (15); thus, the decreased amount of Cbl in the precipitates indicates that the association of Grb2 with Cbl is strongly inhibited in the lysates of activated cells. Identical results were obtained using human peripheral blood T lymphoblasts (data not shown). It is noteworthy that while Cbl disappeared from the Grb2 complex, in the 4G10 blot a relatively large amount of a 120-kDa tyrosine phosphoprotein still bound to Grb2 even in stimulated cells. It is likely that another tyrosine phosphoprotein exists in stimulated T cells with a molecular mass of 115–120 kDa. Analysis of the partial amino acid sequence of an unidentified 116-kDa phosphoprotein, that can also bind to Grb2, suggests that it is different from Cbl (20).
Grb2 upon T cell activation. Jurkat cells were treated with UCHT1 antibody for 0, 5, 15, and 45 min; then Cbl was immunoprecipitated with anti-Grb2 antibody. Consistent with the expected definitive proof of this hypothesis is not available.

It has been reported recently that Crk adaptor proteins as-
Fig. 6. Cbl associates with Crk in intact Jurkat cells. T cells were stimulated with anti-CD3 antibody UCHT1 for 2 min. Proteins were then immunoprecipitated with anti-Crk antibody. Following SDS-7.5% PAGE and transfer to nitrocellulose, samples were analyzed by anti-Cbl antibody and anti-phosphotyrosine antibody 4G10. For control immunoprecipitation, anti-GRF antibody was used.

Fig. 7. Crk SH2 domain interacts with Cbl from activated T cell lysates. T cells were activated with UCHT1 antibody for 2 min. GST, GST-Crk I, GST-Crk II, GST-Crk SH2, and GST-Crk II SH2 mutant fusion proteins bound to glutathione-agarose beads were added to cell lysates. Proteins bound to the beads were separated by SDS-7.5% PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-Cbl antibody.

Cbl, the protein product of the c-cbl proto-oncogene, has recently been implicated in several tyrosine kinase signaling pathways (21, 28, 29). It has also been reported that Cbl can bind to two adaptor proteins, Grb2 and Nck, via their SH3 domains in T lymphocytes and HL60 cells, respectively (21, 24). Our data are consistent with these reports in that Cbl associates with Grb2 in unstimulated T cells in vitro and in vivo, in an SH3 domain-dependent manner. However, upon CD3 activation of Jurkat cells, Cbl rapidly and transiently dissociates from Grb2 (Figs. 1, 2, and 4A). Argon-induced dissociation of a proline-rich domain-containing protein from Grb2 has not previously been reported for any protein in any cell type. Donovan et al. (21) have shown that Cbl undergoes a rapid tyrosine phosphorylation in response to TCR stimulation. We have demonstrated that the kinetics of Cbl phosphorylation correlate remarkably with the kinetics of Grb2/Cbl dissociation.

The mechanism by which Cbl dissociates from Grb2 upon T cell activation is unknown but may relate to tyrosine phosphorylation of Cbl. Phosphorylation of Sos exchange protein on serine/threonine residues has been reported (30, 31) to result in the disassembly of Sos from Grb2. This finding suggests that conformational changes in the structure of a proline-rich domain-containing protein due to phosphorylation may cause its dissociation from Grb2. In the case of Sos phosphorylation either in fibroblasts or in T cells, this mechanism seems to be involved in the negative feedback regulation of Ras signaling pathway (30–32). By contrast, we show here that a TCR agonist induces a rapid and transient phosphorylation of Cbl and its dissociation from Grb2. Therefore, functionally the dissociation of Cbl from Grb2 is completely different from the disassembly of Sos from Grb2.

While this manuscript was in preparation, association of Cbl with Grb2 and phosphatidylinositol 3'-kinase in J urkat cells has been reported (33). In contrast with our results, Cbl and Grb2 have been shown to form a constitutive complex regardless of the activation state of J urkat cells. One possible explanation is that we stimulated the cells via their TCR, while Meisner et al. (33) used co-stimulation of the TCR and CD4 receptors. Cross-linking of both the TCR and CD4 receptors may result in a more intensive phosphorylation of Cbl, or alternatively, phosphorylation may occur on different sites. Based on in vitro data with SH2 and SH3 domains of Grb2 GST fusion proteins, tyrosine-phosphorylated Cbl can bind to both SH2 and SH3 domains of Grb2 in fully activated cells (33). In CD3-activated J urkat cells, we have not detected binding of Cbl to the SH2 domain of Grb2 (data not shown).

In addition, we show that tyrosine-phosphorylated Cbl binds to another SH2/SH3 domain-containing adaptor protein, Crk, via its SH2 domain. Sawa et al. (27) have reported that in activated T lymphocytes a phosphotyrosine protein with a molecular mass of 116 kDa binds to the SH2 domain of Crk. Therefore, it is highly likely that this 116-kDa phosphotyrosine protein is identical with Cbl. Using SH2 mutant Crk for protein precipitation, we failed to detect the association of Cbl with the Crk SH3 domains. This suggests that the mechanism by which Cbl binds to Crk is completely different from the association of Cbl with Grb2 and Nck. At present, the role of Crk in regulation of Cbl is unclear. Two other phosphotyrosine proteins have been shown to be associated with Crk, in a similar SH2-dependent manner, paxillin and the Crk-associated substrate p130Cas (11, 12). In addition, Crk proteins can be associated, via their SH3 domains, with two Ras-specific guanine nucleotide exchange proteins, Sos and C3G (8–10). Therefore, phosphorylated Cbl in complex with Crk/Sos or Crk/C3G might be involved in Ras signaling pathways in T cells.

It has been suggested that Cbl is a nuclear protein which may function as a transcription factor (23): it contains a possible nuclear localization sequence, a putative leucine zipper at the carboxyl terminus, and a zinc finger-related protein motif (29). Therefore, Cbl or tyrosine-phosphorylated Cbl could be transported into the nucleus. This is supported by the fact that the truncated form of Cbl, the protein product of v-cbl oncogene, can enter the nucleus and bind DNA (23). Moreover, it has been recently suggested that the carboxyl terminus of Cbl is involved in the retention of Cbl in the cytoplasm and the inhibition of DNA binding (23). The truncation in the sequence of v-Cbl occurs at the carboxyl-terminal domain which contains several proline-rich motifs. These motifs are likely to be responsible for the interaction with the SH3 domains of Grb2. Taken together, this suggests that Grb2 might have a role in the regulation of Cbl in quiescent cells by acting as a retention factor. However, every effort so far to detect Cbl in the nucleus has been unsuccessful. Tanaka et al. (28) have very recently reported that in macrophages stimulated via their Fcγ receptor or in HER14 cells activated with EGF, Cbl was found to translocate from the cytoplasm to the trans-Golgi region of the cells. Further studies will therefore elucidate the possible role of Cbl in complexes with different adaptor proteins in tyrosine kinase signaling pathways.
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