Activation-modulated Association of 14-3-3 Proteins with Cbl in T Cells*

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14-3-3 proteins have recently been implicated in the regulation of intracellular signaling pathways via their interaction with several oncogene and protooncogene products. We found recently that 14-3-3 associates with several tyrosine-phosphorylated proteins and phosphatidylinositol 3-kinase (PI3-K) in T cells. We report here the identification of the 120-kDa 14-3-3-binding phosphoprotein present in activated T cell lysates as Cbl, a protooncogene product of unknown function which was found recently to be a major protein-tyrosine kinase (PTK) substrate, and to interact with several signaling molecules including PI3-K, in T lymphocytes. The association between 14-3-3 and Cbl was detected both in vitro and in intact T cells and, in contrast to Raf-1, was markedly increased following T cell activation. The use of truncated 14-3-3 fusion proteins demonstrated that the 15 C-terminal residues are required for the association between 14-3-3 and three of its target proteins, namely, Cbl, Raf-1, and PI3-K. The findings that 14-3-3 binds both PI3-K and Cbl, together with recent reports of an association between Cbl and PI3-K, suggest that 14-3-3 dimers play a critical role in signal transduction processes by promoting and coordinating protein-protein interactions of signaling proteins.

1 The abbreviations used are: TCR, T cell antigen receptor; PI3-K, phosphatidylinositol 3-kinase; mAb, monoclonal antibody; PTK, protein-tyrosine kinase (PTK) substrate, and to interact with several signaling molecules including PI3-K, in T lymphocytes. The association between 14-3-3 and Cbl was detected both in vitro and in intact T cells and, in contrast to Raf-1, was markedly increased following T cell activation. The use of truncated 14-3-3 fusion proteins demonstrated that the 15 C-terminal residues are required for the association between 14-3-3 and three of its target proteins, namely, Cbl, Raf-1, and PI3-K. The findings that 14-3-3 binds both PI3-K and Cbl, together with recent reports of an association between Cbl and PI3-K, suggest that 14-3-3 dimers play a critical role in signal transduction processes by promoting and coordinating protein-protein interactions of signaling proteins.

Binding of antigenic peptides presented by major histocompatibility complex molecules to the T cell antigen receptor (TCR)-CD3 complex induces a rapid increase in protein-tyrosine kinase (PTK) activity, which is the earliest identifiable event in the signaling cascade leading to T cell activation and proliferation. This involves activation of PTKs of the Src and Syk families (1, 2). Recently, a major 120-kDa PTK substrate in TCR-CD3-activated T cells was identified as Cbl (3, 4), which was originally isolated as an oncogene associated with myeloid and lymphoid leukemias in mice (5). This cytoplasmic protein contains a potential RING zinc finger motif, a C-terminal proline-rich region that binds several Src homology 3 (SH3)-containing proteins (6–8), and potential SH2-binding motifs (9). In T cells, Cbl is constitutively associated with Grb2 and interacts with the regulatory subunit (p85) of phosphatidylinositol 3-kinase (PI3-K) upon T cell activation (3, 4).

The 14-3-3 protein family consists of highly conserved ~30 kDa isoforms possessing a variety of biological activities, which are expressed in many organisms and tissues (10–12). 14-3-3 proteins were found recently to bind oncogene and protooncogene products such as polyomavirus middle-T antigen (13), Raf-1 (14–17), Bcr-Abl (18), and the Cdc25 phosphatase (19). This implicates this family of proteins as regulators of intracellular signaling pathways. However, the physiological role of protein-protein interactions mediated by 14-3-3 proteins remains unclear.

We reported recently that in Jurkat T cells, PI3-K kinase is associated with 14-3-3, a T cell-expressed 14-3-3 isoform (20). Additionally, 14-3-3 bound several Tyr(P)-containing proteins in activated T cells, including a major ~120-kDa phosphoprotein. We have now identified this 14-3-3-binding PTK substrate as Cbl. We also demonstrate that the in vitro and in vivo association of Cbl with 14-3-3 is markedly increased following T cell activation. Since PI3-K and Cbl associate with each other (4), our findings raise the possibility that 14-3-3 proteins play a critical role as modulators of protein-protein interactions during signal transduction.

MATERIALS AND METHODS

Antibodies—Polyclonal rabbit anti-c-Cbl (c-15) or -c-Raf-1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Tyr(P) monoclonal antibody (mAb) 4G10 and polyclonal anti-PI3-K (p85) or -GST antibodies were from Upstate Biotechnology (Lake Placid, NY). The anti-14-3-3 mAb was described previously (20). An anti-CD3e mAb, OKT3, was purified from cell culture supernatants by using protein A-Sepharose affinity column. Horseradish peroxidase-conjugated F(ab')2 fragments of donkey anti-rabbit IgG or sheep anti-mouse IgG were from Amersham.

Cell Culture and Stimulation—Human leukemic Jurkat T cells were grown in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and antibiotics. Cells were resuspended (2 × 10^7/ml) in 0.5 ml of medium, equilibrated at 37°C for 5 min, and activated with OKT3 (4 μg/ml) for 5 min. Stimulation was terminated by adding 0.5 ml of 2 × Nonidet P-40 lysis buffer (2% Nonidet P-40, 40 mM Tris-Cl, pH 7.5, 300 mM NaCl, 10 mM EDTA, 10 mM NaF, 10 mM Na3VO4, 20 μg/ml aprotinin and leupeptin). Cells were lysed for 10 min at 4°C, and insoluble materials were removed by centrifugation at 15,000 × g (4°C for 10 min). In some experiments, the cells were transfected 48 h before stimulation with a 14-3-3-3 mammalian expression vector (pEFneo14-3-3; Ref. 20) or with a control empty pEFneo vector (21), by electroporation as described previously (22).

14-3-3 Fusion Proteins and Binding Assays—Deletion mutants of human 14-3-3 were generated by polymerase chain reaction using the corresponding cDNA as a template. Two sense primers, P1 (5'-ATG GAG CAG GGCC GCC GAG CTG T-3') and P2 (5'-ATG GCC AAG CGG GCC GAG-3'), and one antisense primer, P3 (5'-TTA GTC TTC AGC CCC TCC TCC-3'), were used to amplify two cDNA fragments with deletions.
of 30 (Δ30N) or 10 (Δ10N) N-terminal residues, respectively. Similarly, a sense primer, P4 (5'-ATG GAG AAG ACT GAG CTG-3') was used with each of four antisense primers, P5 (5'-TTA CAC CAG GGT GCA GAT-3'), P6 (5'-TTA GTG TGA TGT CCA AAG TGT TAG-3'), P7 (5'-TTA TGC CGC ACA TTC TCC TTC TCC-3'), or P8 (5'-TTA AGC CCC TGC GCG ACTAC-3'), to generate four cDNAs encoding 14-3-3 proteins having deletions of their 95 (Δ95C), 15 (Δ15C), 5 (Δ5C), and 2 (Δ2C) C-terminal residues, respectively. These cDNA fragments were subcloned into a TA cloning vector (Invitrogen) for sequencing and then transformed into E. Lysates from 1 × 10^7 cells were incubated with 10 μg of fusion protein for 2 h at 4°C, followed by adding 40 μl of glutathione-Sepharose beads. After 1 h at 4°C, the binding mixtures were washed extensively in 1 × Nonidet P-40 lysis buffer and used for immunoblotting or P13-K assay.

Immunoprecipitation and Immunoblotting—Lysates (1 × 10^7 cells) were mixed with antibodies for 2 h, followed by addition of 40 μl of protein A/G Plus-Sepharose beads (Santa Cruz Biotechnology) for an additional hour at 4°C. IPs were washed four times with 1 × Nonidet P-40 lysis buffer and boiled in 30 μl of 2 × Laemmli's buffer. Samples were subjected to SDS-10% PAGE analysis and electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). Membranes were immobilized with the indicated primary antibodies (1 μg/ml), followed by horseradish peroxidase-conjugated secondary antibodies. Membranes were washed and visualized with enhanced chemiluminescence (ECL) detection system (Amersham). A minigel (9 × 8 cm) was used for the separation of Cbl, Raf-1, and P13-K, and a 13.8 × 13 cm gel was used for the separation of 14-3-3 proteins. When necessary, membranes were stripped by incubation in 62.5 mM Tris-HCl, pH 6.7, 100 mM 2-mercaptoethanol, 2% SDS for 2 h at 70°C with constant agitation, washed, and then reprobed with other antibodies as indicated.

Far Western Blots—Membranes were denatured in 6 M guanidine, dissolved in 50 mM Tris-HCl, pH 8.0, 5 mM 2-mercaptoethanol, 2 mM EDTA for 1 h at room temperature, and renatured in the same buffer overnight at 4°C. Membranes were then incubated with GST-14-3-3 or GST alone (10 μg/ml) for 2 h at 4°C, followed by anti-GST antibody and ECL detection.

Gel Filtration—Purified GST fusion proteins (10 μg each) were loaded onto a Superose 6 column (1 × 30 cm, Pharmacia) pre-equilibrated in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and analyzed by high performance liquid chromatography (HPLC; System Gold, Beckman). Protein samples were eluted in the same buffer at a flow rate of 0.5 ml/min. The eluates were analyzed by SDS-10% PAGE, blotted against anti-GST antibody, and detected with ECL. The column was calibrated with the following molecular mass standards: catalase, 232 kDa; aldolase, 158 kDa, albumin, 67 kDa; ovalbumin, 43 kDa.

P13-K Assay—P13-K activity was determined as described previously (20). Briefly, the reaction mixture contained 20 μg of phosphatidylinositol, 20 mM MgC\textsubscript{6}H\textsubscript{5}O\textsubscript{4}, 50 μM cold ATP, and 10 μCi of [γ-\textsuperscript{32}P]ATP (7000 Ci/mmol). After 10 min at room temperature, the reaction products were analyzed by thin layer chromatography on silica gel plate followed by autoradiography.

**RESULTS**

Association of 14-3-3 with Cbl in Vitro and in Vivo—Consistent with our previous observation (20), GST-14-3-3 bound several Tyr(P)-containing proteins in lysates of anti-CD3-stimulated J urkat T cells (Fig. 1A). Prominent among these was an ~120-kDa phosphoprotein. To ascertain whether this phosphoprotein corresponds to Cbl, we reprobed the same membrane with anti-Cbl. Cbl comigrated with the Tyr(P)-containing 120 kDa band (Fig. 1B). More Cbl was associated with GST-14-3-3 in lysates from anti-CD3-stimulated cells than in unstimulated samples. Binding was specific since Cbl did not associate detectably with a control GST protein.

To determine whether the interaction between 14-3-3 and Cbl is direct, membranes containing SDS-PAGE-resolved IPs from resting or anti-CD3-stimulated J urkat T cells were incubated with GST-14-3-3 or GST alone, and binding was detected by an anti-GST antibody. Raf-1, which associates directly with Cbl (14-17), was used as a positive control. As expected, GST-14-3-3 bound immunoprecipitated Raf-1 from both unstimulated or activated cells (Fig. 2, A and B). GST-14-3-3 (but not GST) also bound a 120-kDa protein present in the anti-Cbl IPs (Fig. 2, A and B). This protein comigrated with authentic Cbl (Fig. 2D). However, in contrast to Raf-1 preparations from resting versus activated cells, which bound 14-3-3 to a similar degree, 14-3-3 bound more effectively to Cbl from activated Jurkat cells (Fig. 2A). Immunoblotting of the same membranes with anti-Raf-1 (Fig. 2C) or -Cbl (Fig. 2D) antibodies indicated that similar amounts of each protein were present in IPs from resting and anti-CD3-stimulated cells. Thus, 14-3-3 most likely associates directly with Cbl.

We next ascertained whether 14-3-3 associates with Cbl in intact T cells. Immunoblotting of Raf-1 or Cbl IPs with anti-14-3-3 revealed coimmunoprecipitation of 14-3-3 with both

![Image](http://www.jbc.org/)

**Fig. 1. Interactions of 14-3-3 with Cbl and other Tyr(P)-containing proteins in activated T cells.** Jurkat T cells were left unstimulated (lanes −) or were stimulated for 5 min at 37°C with OKT3 (lanes +). Cells (1 × 10^7 cell eq) were precipitated with 10 μg of GST or GST-14-3-3 and recovered with glutathione-Sepharose beads. A, the washed beads were subjected to SDS-10% PAGE, transferred onto PVDF membrane, immunoblotted with anti-Tyr(P) mAb, and visualized with ECL. B, the membrane was then stripped and reprobed with anti-Cbl antibody. Molecular mass markers are indicated.

**Fig. 2. Association of 14-3-3 with Cbl in vitro and in vivo.** Cell lysates from 1 × 10^7 unstimulated (lanes −) or OKT3-stimulated (lanes +) Jurkat cells were immunoprecipitated (IP) with either anti-Raf-1 or anti-Cbl antibody, and the IPs were resolved by SDS-10% PAGE and transferred onto PVDF membrane. Membranes were incubated with either GST-14-3-3 (A) or GST alone (B), and binding was detected with an anti-GST antibody and ECL. The membranes were then stripped and reprobed with anti-Raf-1 (C) or -Cbl (D) antibodies. Another membrane containing resolved Raf-1 or Cbl IPs was immunoblotted with an anti-14-3-3 mAb (E).

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proteins (Fig. 2E). Thus, like Raf-1, Cbl interacts with 14-3-3 under physiological conditions. Raf-1 IPs from resting versus activated T cells (Fig. 2E, lanes 1 and 2) contained similar amounts of 14-3-3. However, consistent with the results of the far-Western blot (Fig. 2A), more 14-3-3 coimmunoprecipitated with Cbl in anti-CD3-stimulated Jurkat cells than in resting cells (Fig. 2E, lanes 3 and 4).

Mapping of 14-3-3 Ligand-binding Domains—Biochemical and structural studies have shown that 14-3-3 proteins form a dimer through the interactions of four N-terminal α helices (23-25). To assess the role of dimer formation in associations between 14-3-3 and its target proteins, two 14-3-3 proteins from which 30 or 12 N-terminal amino acid residues have been deleted (Δ30N and Δ12N, respectively), were expressed as GST fusion proteins (Fig. 3A). Consistent with the three-dimensional structure (24, 25), both Δ30N and Δ12N were monomeric by HPLC gel filtration analysis, whereas the wild-type protein was present primarily as a dimer (Fig. 3B). We then assessed the ability of these fusion proteins to bind ligands in lysates of anti-CD3-stimulated T cells. When cellular proteins bound to 14-3-3 were analyzed by immunoblotting with anti-Cbl, -Raf-1, or -PI3-K (p85) antibodies (Fig. 4A, B, and C, respectively), or by a PI3-K enzymatic assay (Fig. 4D), the corresponding proteins were associated with wild-type 14-3-3, but not with Δ30N or Δ12N. These results extend previous findings by demonstrating that 14-3-3 dimer formation correlates with its ability to associate with target proteins.

To further dissect ligand-binding sites in 14-3-3, additional 14-3-3 proteins with deletions of their 95, 15, 5, or 2 C-terminal residues (Fig. 3A; Δ95C, Δ15C, Δ5C, and Δ2C, respectively) were generated, purified, and analyzed by gel filtration. Unexpectedly, removal of 95 C-terminal residues, which were not formerly implicated in dimer formation, resulted in a failure to form dimers; in addition, Δ15C was also expressed, for the most part, as a monomer (Fig. 3B). In contrast, the Δ5C and Δ2C mutants preferentially formed dimers although a significant fraction was expressed as monomers (Fig. 3B). Thus, the C-terminal region of 14-3-3 is also important for dimer formation. In correlation with the failure to form dimers, Δ95C failed to bind Cbl (Fig. 4A), Raf-1 (Fig. 4B), and immunoreactive (Fig. 4C) or enzymatically active (Fig. 4D) PI3-K in Jurkat cell lysates. Δ15C bound these targets to a low, but detectable, degree, and Δ5C and Δ2C bound them effectively, Δ2C being somewhat more effective than Δ5C. We conclude that residues 231–240 of 14-3-3 are required for efficient ligand binding. Moreover, similar, if not identical, domain(s) of 14-3-3 are apparently involved in binding to Cbl, Raf-1, and PI3-K.

Effects of 14-3-3 Overexpression—To explore potential functional consequences of the interaction between 14-3-3 and Cbl, we analyzed Cbl IPs from 14-3-3-overexpressing Jurkat cells (Fig. 5). Consistent with previous reports (3, 4), anti-CD3-induced phosphorylation of Cbl on tyrosine (Fig. 5A, lanes 2 and 4 versus lanes 1 and 3). The Tyr(P) content of Cbl was similar.
in control- or 14-3-3-transfected cells. Similar amounts of Cbl were immunoprecipitated in all cases as demonstrated by anti-Cbl immunoblotting (Fig. 5B). Reprobing the membrane with anti-14-3-3α revealed that overexpression of 14-3-3α increased the amount of 14-3-3α associated with Cbl by ~3-fold in both resting and activated Jurkat cells (Fig. 5C, lanes 3 and 4 versus lanes 1 and 2, respectively). Consistent with our previous results, anti-CD3 stimulation resulted in an increased amount of 14-3-3α-associated with Cbl (Fig. 5C).

We found that 14-3-3α overexpression inhibits PI3-K activity recovered in Tyr(P) IPs from activated T cells (20), and PI3-K associates with Cbl upon T cell activation (4). To investigate the possible contribution of 14-3-3α to complex formation between Cbl and PI3-K, the Cbl IPs were analyzed by anti-phospho-PtdIns(3)P and PI3-K enzymatic assay. Consistent with the earlier findings (4), the p85 subunit of PI3-K (Fig. 5D) as well as PI3-K activity (Fig. 5E) communoprecipitated with Cbl in an activation-dependent manner. Although similar amounts of p85 were detected in both control and 14-3-3α-overexpressing stimulated cells (Fig. 5D, lanes 2 and 4), the amount of Cbl-associated PI3-K activity was consistently reduced (by ~50%) in the 14-3-3α-overexpressing T cells (Fig. 5E, lanes 4 versus 2). These results are compatible with our previous findings (20) and suggest that 14-3-3 proteins can regulate PI3-K enzymatic activity in the context of a cellular Cbl-PI3-K complex.

**DISCUSSION**

Cbl is expressed abundantly in B, T, and myeloid cells (5) and is phosphorylated rapidly on tyrosine upon T cell activation (3, 4). The present study identifies Cbl as the major 120-kDa 14-3-3-binding PTK substrate in activated T cells. This association occurs in intact cells and is shown to be direct. A cysteine-rich RING zinc-binding motif present near the C terminus of Cbl (6) may mediate this association. This is based on the findings that three 14-3-3-binding proteins, i.e. Raf-1 (14, 26), Bcr-Abl (18), and polyomavirus middle T antigen (13), display similar cysteine-rich regions which, in the case of the former two proteins, are required for the interaction with 14-3-3. Thus, interaction of 14-3-3 proteins with this zinc finger motif may represent a common mechanism for ligand binding.

Phosphorylated amino acids in target proteins may represent another important determinant in 14-3-3 binding. 14-3-3 binds phosphorylated, but not unphosphorylated, forms of Raf-1 (26) and tryptophan hydroxylase (27). A significant finding of the present study is that, unlike Raf-1, the Cbl-14-3-3α association was markedly increased upon T cell activation (Figs. 1, 2, 3, and 5). Enhanced association correlated with increased tyrosine phosphorylation of Cbl, raising the possibility that Tyr(P) residues in Cbl contribute, directly or indirectly, to its association with 14-3-3α. Alternatively, it is possible that serine/threonine residues in Cbl, which may also become phosphorylated upon activation, are involved in the association.

Recent studies showed that 14-3-3 proteins consist of 9 antiparallel α helices. The four N-terminal helices participate in dimer formation, which then creates a ligand-binding groove (24, 25). Our analysis of N-terminally deleted 14-3-3α proteins is consistent with the crystal structure since mutants lacking 12 or 30 N-terminal residues failed to form dimers and, in parallel, did not bind Cbl, Raf-1, and PI3-K. Unexpectedly, C-terminal deletions also had an adverse effect on dimer formation and target protein binding. Specifically, the last 10–15 residues appear to be required for these events. This implies that, although C-terminal residues may not be directly involved in the interaction between two 14-3-3α monomers, they may stabilize a proper conformation of the dimer which is necessary for ligand binding. In the crystal structure of 14-3-3, these residues form a poorly ordered loop (25).

Several signaling proteins (10–12) and phospholipids (28) associate with 14-3-3 proteins. Of interest, several pairs of interacting proteins, e.g. Raf-1 and Cdc25 phosphatases (29); middle T antigen and PI3-K (30); Cbl and PI3-K (4); and Bcr and Raf (31), can all bind 14-3-3 (Refs. 13–20 and the present study). In the crystal structure of the 14-3-3 dimer, the ligand-binding groove is sufficiently large to accommodate two ligands (25). Although the functional consequences of the interaction between 14-3-3 proteins and their targets are still unknown, it is tempting to speculate that by binding two distinct ligands, the 14-3-3 homo- or heterodimer (32) may facilitate and coordinate formation of protein-protein or even protein-lipid complexes that are essential for signal transduction. Depending on the nature of the interacting partners, the consequences of this trimerolecular complex formation may be translated into a positive or a negative signal. This model is consistent with findings that 14-3-3 overexpression increases the stoichiometry of complex formation between Raf-1 and Cdc25 (19). Similarly, 14-3-3 may enhance the association between PI3-K and Cbl, resulting in inhibition of PI3-K activity. Our failure to observe increased association between Cbl and PI3-K in 14-3-3α-overexpressing cells may simply reflect insufficient sensitivity, since 14-3-3α was transiently expressed in only a portion of the cells.

Our findings have potential implications concerning the biological function of Cbl. Cbl binds constitutively, most likely via its C-terminal proline-rich domain (7, 8), SH3-containing proteins such as Grb2 (3, 4, 33), Nck (34), or Bruton’s tyrosine kinase (35). Following T cell activation, tyrosine-phosphorylated Cbl also binds the SH2 domains of p56lck (35), p59fyn, phospholipase Cy1 (3), and p85 (4). Association with p85 may involve interaction with a Y733XXM motif in Cbl (4). SL1-1, a putative Cbl homologue, was recently found to negatively regulate vulval development in Caenorhabditis elegans (36), a process mediated by LET-23 which is a homologue of the epidermal growth factor receptor. In NIH 3T3 cells, ligation of this receptor leads to tyrosine phosphorylation of Cbl (37), suggesting that Cbl is also involved in receptor PTK-mediated signaling pathways. Our findings demonstrate that transient 14-3-3α overexpression in Jurkat T cells resulted in reduced Cbl-asso-
ciated PI3-K enzymatic activity, which correlated with increased complex formation between 14-3-3 and Cbl. We suggest that 14-3-3 proteins could modulate PI3-K either by direct association with the latter or by an indirect mechanism which involves 14-3-3-facilitated association between PI3-K and Cbl, which then inhibits PI3-K or some of its upstream regulators.

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