Serine Phosphorylation of Cbl Induced by Phoroptol Ester Enhances Its Association with 14-3-3 Proteins in T Cells via a Novel Serine-rich 14-3-3-binding Motif

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Stimulation of the T cell antigen receptor (TCR)-CD3 complex induces rapid tyrosine phosphorylation of Cbl, a protooncogene product which has been implicated in intracellular signaling pathways via its interaction with several signaling molecules. We found recently that Cbl associates directly with a member of the 14-3-3 protein family (14-3-3z) in T cells and that the association is increased as a consequence of anti-CD3-mediated T cell activation. We report here that phorbol 12-myristate 13-acetate stimulation of T cells also enhanced the interaction between Cbl and two 14-3-3 isoforms (z and δ). Tyrosine phosphorylation of Cbl was not sufficient or required for this increased interaction. Thus, cotransfection of COS cells with Cbl plus Lck and/or Syk family protein-tyrosine kinases caused a marked increase in the phosphotyrosine content of Cbl without a concomitant enhancement of its association with 14-3-3. Phorbol 12-myristate 13-acetate stimulation induced serine phosphorylation of Cbl, and dephosphorylation of immunoprecipitated Cbl by a Ser/Thr phosphatase disrupted its interaction with 14-3-3. By using successive carboxy-terminal deletion mutants of Cbl, the 14-3-3-binding domain was mapped to a serine-rich 30-amino acid region (residues 615–644) of Cbl. Mutation of serine residues in this region further defined a binding motif distinct from the consensus sequence RXSXP, which was recently identified as a 14-3-3-binding motif. These results suggest that TCR stimulation induces both tyrosine and serine phosphorylation of Cbl. These phosphorylation events allow Cbl to recruit distinct signaling elements that participate in TCR-mediated signal transduction pathways.

of protein-tyrosine kinases (PTKs) of the Src and Syk families (1), which in turn function to propagate activation signals by phosphorylating multiple intracellular proteins in T lymphocytes, eventually leading to T cell activation, lymphokine production, and proliferation. One of the major PTK substrates in TCR/CD3-activated T cells is Cbl (2, 3). The corresponding protooncogene, c-cbl, is the cellular homologue of a transforming gene of Cos NS-1 retrovirus, which induces pro-B cell lymphomas and myeloid leukemias in mice (4). The 120-kDa product of c-cbl consists of a highly basic amino-terminal region, a Ring zinc finger motif, multiple proline-rich stretches, and contains several potential tyrosine phosphorylation sites (6, 7). Cbl associates with the Fyn (3, 5) and Zap-70 (8) kinases both in vitro and following T cell activation in vivo, and Zap-70 causes tyrosine phosphorylation of Cbl in an Lck- and Fyn-dependent manner indicating that Cbl may couple Zap-70 to downstream biochemical events during T cell activation (8). This idea is supported by the findings that the proline-rich domain of Cbl mediates constitutive associations with Src homology 3-containing signaling proteins, including the adaptor protein Grb2 (2, 9–11), and phosphorylated tyrosine residues in Cbl associate with the Src homology 2 domains of other signaling proteins, e.g. phosphotyrosinol 3-kinase (PI3-K) (2, 9, 11–13) and Crk (14–17) in an activation-dependent manner.

Recently, we found that Cbl interacts directly with 14-3-3z in T cells (18). The 14-3-3 protein family, which is expressed in many organisms and tissues, consists of highly conserved ~30-kDa isoforms possessing a variety of biological activities (19–21). 14-3-3 proteins were recently found to bind oncogene and protooncogene products such as polyoma virus middle-T antigen (22), Raf-1 (23–25), Ber-Ab1 (26), PI3-K (27), protein kinase C (PKC, Ref. 28), and the cdc25 phosphatase (29), implicating this family of proteins as regulators of intracellular signaling pathways. More recently, it was reported that a phosphorylated consensus sequence, RXSXSXP, in Raf and other proteins, represents a 14-3-3-binding motif (30). Interestingly, Cbl does not contain this consensus sequence. Moreover, in contrast to Raf-1, interaction of 14-3-3z with Cbl is markedly enhanced by T cell stimulation (18), suggesting that the interaction of 14-3-3 with Cbl or Raf is differentially regulated in T cells. However, the molecular mechanism underlying this TCR-stimulated interaction between 14-3-3 and Cbl remains unclear.

In the present study, we demonstrate that PMA stimulation also enhances the association of Cbl with 14-3-3, and that tyrosine phosphorylation of Cbl is dispensable for this interaction. Furthermore, we show that PMA induces serine phosphorylation of Cbl in Jurkat cells which, in turn, function to propagate activation signals by phosphorylating multiple intracellular proteins in T lymphocytes, eventually leading to T cell activation, lymphokine production, and proliferation. One of the major PTK substrates in TCR/CD3-activated T cells is Cbl (2, 3). The corresponding protooncogene, c-cbl, is the cellular homologue of a transforming gene of Cos NS-1 retrovirus, which induces pro-B cell lymphomas and myeloid leukemias in mice (4). The 120-kDa product of c-cbl consists of a highly basic amino-terminal region, a Ring zinc finger motif, multiple proline-rich stretches, and contains several potential tyrosine phosphorylation sites (6, 7). Cbl associates with the Fyn (3, 5) and Zap-70 (8) kinases both in vitro and following T cell activation in vivo, and Zap-70 causes tyrosine phosphorylation of Cbl in an Lck- and Fyn-dependent manner indicating that Cbl may couple Zap-70 to downstream biochemical events during T cell activation (8). This idea is supported by the findings that the proline-rich domain of Cbl mediates constitutive associations with Src homology 3-containing signaling proteins, including the adaptor protein Grb2 (2, 9–11), and phosphorylated tyrosine residues in Cbl associate with the Src homology 2 domains of other signaling proteins, e.g. phosphotyrosinol 3-kinase (PI3-K) (2, 9, 11–13) and Crk (14–17) in an activation-dependent manner.

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In the present study, we demonstrate that PMA stimulation also enhances the association of Cbl with 14-3-3, and that tyrosine phosphorylation of Cbl is dispensable for this interaction. Furthermore, we show that PMA induces serine phosphorylation of Cbl in Jurkat cells, a simian virus 40 T antigen-transfected human leukemic Jurkat T cells; PP, protein phosphatase.

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Serine Phosphorylation-dependent Binding of Cbl to 14-3-3

rlation of Cbl and that Ser/Thr dephosphorylation of Cbl abolishes its association with 14-3-3. Finally, our experiments define a novel 14-3-3-binding serine-rich motif in Cbl. Our findings suggest that TCR stimulation activates both PTKs and Ser/Thr kinases which can phosphorylate Cbl, leading to recruitment of distinct proteins that serve to propagate TCR-mediated signals.

MATERIALS AND METHODS

Antibodies—Polyclonal rabbit anti-Cbl (c-15), -Lck, -Zap-70, -Syk, or -Raf-1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine (Tyr(P)) monoclonal antibody (mAb) 4G10 and polyclonal anti-P13-K (p85) or -GST antibodies were from Upstate Biotechnology (Lake Placid, NY). The anti-14-3-3 mAb was described previously (27). Anti-hemagglutinin (HA) mAb (12CA5) was from Boehringer Mannheim. Anti-Grb2 was from Transduction Laboratories (Lexington, KY). Anti-human IgG was from Dako (Denmark). An anti-CD3e mAb, OKT3, was purified from hybridoma culture supernatants by using protein A-Sepharose affinity chromatography. Horseradish peroxidase-conjugated F(ab)2 fragments of donkey anti-rabbit IgG or sheep anti-mouse IgG was from Amersham Corp.

Cell Culture and Stimulation—Simian virus 40 T antigen-transfected Simian Jurkat T cell line (17) was obtained from the American Type Culture Collection (Manassas, VA). The RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and antibiotics. Cells were resuspended (2 × 10^6/ml) in 0.5 ml of medium, equilibrated at 37 °C for 5 min, and activated with either OKT3 (4 µg/ml) for 5 min or with PMA (50 ng/ml) for 15 min. Stimulation was terminated by adding 0.5 ml of 2 × Nonidet P-40 lysis buffer (2% Nonidet P-40, 40 mM Tris-HCl, pH 7.5, 300 mM NaCl, 10 mM EDTA, 10 mM NaF, 4 mM Na3VO4, 20 µg/ml each aprotinin and leupeptin). Cells were lysed for 10 min at 4 °C, and insoluble material was removed by centrifugation at 15,000 × g (4 °C for 10 min).

COS-1 cells were isolated in Isscove’s modified Dulbecco’s medium (Life Technologies, Inc.) containing 10% heat-inactivated fetal bovine serum at 37 °C in 5% CO2. Jurkat-TAg or COS-1 cells were transiently transfected with an optimal amount of plasmid DNA (usually 10 µg) by electroporation as described previously (31). To stimulate COS-1 cells, PMA (100 ng/ml) was added at 37 °C for 30 min.

Plasmids—The human Cbl, v-cb1, 70Z/3 (a 17-amino acid deletion of Cbl; Ref. 32) cDNAs, and several carboxyl-terminal deletion mutants of Cbl were subcloned into the pEFneo (33) mammalian expression vector (as described in Ref. 34). To construct cDNAs encoding Cbl proteins with additional carboxyl-terminal deletions, 200–300-base pair fragments corresponding to defined Cbl sequences were generated by PCR amplification. The PCR products were cloned into the TA cloning vector (Invitrogen). The CDNA were then ligated back to the original pEFneo 70Z/3 cDNA which has been digested with BglII and XbaI to remove a 3'-coding sequence. The residue numbers of the different truncation mutants correspond to the sequence of wild-type Cbl. A fragment encoding Cbl residues 15–644 or mutants thereof was expressed by PCR amplification of the corresponding cDNA fragment and ligated in-frame to a sequence encoding the Fc fragment of human IgG1 (33) in pEFneo.

In Vitro Binding Assay—Cell lysates were incubated with 10 µg of GST, GST-14-3-3, or GST-14-3-3 fusion proteins (27) for 2 h at 4 °C, followed by the addition of 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 further analysis.

Far Western Blots—Membranes were denatured in 6 M guanidine-HCl, dissolved in 50 mM Tris-HCl, pH 8.0, 5 mM 2-mercaptoethanol, 2 mM EDTA for 1 h at room temperature, and treated 0.25% SDS at 90 °C for 30 min, followed by incubation 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 enhanced chemiluminescence (ECL) detection system (Amersham Corp.).

Immunoprecipitation and Immunoblotting—Lysates (1 × 10^6) cells were mixed with antibodies for 2 h, followed by the addition of 40 µl of protein A/G Plus-Sepharose beads (Santa Cruz Biotechnology) for an additional hour at 4 °C. Immunoprecipitates (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 polyvinylidine difluoride membranes (Millipore). Membranes were immunoblotted with the indicated primary antibodies (1 µg/ml), followed by horseradish peroxidase-conjugated secondary antibodies. Membranes were washed and visualized by ECL. A minigel (9 × 8 cm) was used for the separation of Cbl, Raf-1, and PI3-K, and a 13.8 × 13-cm gel was used for the separation of 14-3-3 and Grb2. When necessary, membranes were stripped by incubation in 62.5 mM Tris-HCl, pH 6.7, 100 mM 2-mercaptoethanol, 2% SDS for 1 h at 70 °C with constant agitation, washed, and then reprobed with other antibodies as indicated.

32P Labeling and Phosphoamino Acid Analysis—Cells were starved in phosphate-free medium for 2 h and labeled with 32P (0.5 mM/32P/ml) for another 4 h. The cells were then treated with or without PMA (50 ng/ml) for 15 min, lysed, and immunoprecipitated with anti-Cbl antibodies as described above. The membrane containing SDS-PAGE-resolved proteins was subjected to autoradiography, and the protein bands corresponding to Cbl were cut out, hydrolyzed in 6 N HCl, and subjected to two-dimensional thin layer chromatography analysis by electrophoresis using a Hunter thin layer electrophoresis apparatus (C.B.S. Scientific Co., Del Mar, CA) in the first dimension and by ascending chromatography in isobutyric acid, 0.5 at NH4OH, 5.3 (v/v) in the second dimension (37). The blots were exposed to x-ray film at 70 °C. The positions of phosphoamino acids were determined by staining the cold phosphoamino acids included in the mixture with ninhydrin.

Dephosphorylation of Cbl—Beads containing anti-Cbl IPs were washed three times with Nonidet P-40 lysis buffer and twice with protein phosphatase (PP) buffer containing 50 mM Tris-HCl, pH 7.0, 0.1 mM EDTA, 5 mM dithiothreitol. The beads were resuspended in 100 µl of PP buffer, and the reaction was initiated by adding MgCl2 to a final concentration of 1 mM and 1 unit of recombinant PP1 (New England Biolabs Inc.). After 1 h at 30 °C with constant shaking, the beads were spun down and eluted with SDS-PAGE buffer. Proteins were analyzed by SDS-10% PAGE and far Western blotting with GST-14-3-3.

RESULTS

PMA Stimulation Increases the Interaction between 14-3-3 and Cbl in Vitro and in Vivo—To further define the nature of the enhanced Cbl-14-3-3 association mediated by TCR/CD3 ligation (18), we evaluated the ability of PMA, a phorbol ester which is known to activate PKC and other Ser/Thr kinases, to modulate the association of Cbl with 14-3-3. Jurkat-TAg cells were either stimulated with OKT3 or PMA or left unstimulated. Cell lysates were mixed with GST or GST-14-3-3, and bound proteins, which were recovered with glutathione-Sepharose beads, were subjected to SDS-PAGE and immunoblotting with an anti-Cbl antibody. Consistent with our previous report (18), anti-CD3 stimulation of T cells greatly increased the amount of Cbl precipitated by GST-14-3-3 (Fig. 1A, top panel). However, PMA treatment similarly enhanced this interaction. GST alone did not bind Cbl, confirming the specificity of the association. Probing the same membrane with an antibody against Raf, another 14-3-3-binding protein (23–25), revealed that, unlike Cbl, similar amounts of Raf from resting and OKT3- or PMA-activated cells were associated with GST-14-3-3 (Fig. 1A, middle panel). The mobility shift of Raf observed following PMA or anti-CD3 stimulation is consistent with its activation and most likely results from its serine and threonine phosphorylation by PKC (38). These results suggest that different mechanisms which regulate the interaction between 14-3-3 and distinct binding proteins exist in T (and possibly other) cells. To determine whether PMA increases the interaction between 14-3-3 and Cbl by indirectly inducing tyrosine phosphorylation of Cbl, the membrane fractions containing precipitated samples was probed with an anti-Tyr(P) antibody. Although Cbl was readily tyrosine-phosphorylated in OKT3-stimulated cells, no Tyr(P) could be detected in immunoprecipitated Cbl from PMA-stimulated cells (Fig. 1A, bottom panel). Thus, tyrosine phosphorylation of Cbl appears to be dispensable for its interaction with 14-3-3.
FIG. 1. PMA enhances the interaction of 14-3-3 with Cbl both in vitro and in vivo. Jurkat-TAg cells were left unstimulated (Control) or were stimulated for 5 min at 37 °C with OKT3 or for 15 min with PMA. Lysates (1 × 10⁷ cell equivalents) were precipitated with 10 μg of GST or GST-14-3-3 and recovered with glutathione-Sepharose beads (A) or were immunoprecipitated with anti-Cbl antibody and recovered with protein G-Sepharose beads (B). A, the washed beads were subjected to SDS-10% PAGE, transferred onto polyvinylidene difluoride membrane, immunooblotted with antibodies against Cbl, Raf, or Tyr(P) as indicated, and visualized with ECL. The position of Cbl in the anti-Tyr(P) blot is indicated by the arrowhead. B, the membrane was immunoblotted with antibodies against 14-3-3, Grb2, p85 of PI3-K, Tyr(P), or Cbl as indicated. Molecular weight markers are shown on the left.

We then ascertained whether PMA could also enhance the interaction between 14-3-3 and Cbl in intact T cells by probing Cbl IPs from resting or OKT3- or PMA-stimulated cells, with an anti-14-3-3 mAb. As shown in Fig. 1B, a very small amount of 14-3-3 could be detected in the Cbl IPs from unstimulated cells (Fig. 1B, top panel); both anti-CD3 and PMA treatment increased the interaction between 14-3-3 and Cbl to a similar degree. However, Cbl was tyrosine-phosphorylated only in OKT3-stimulated cells as revealed by anti-Tyr(P) immunoblotting of the same IPs. We further assessed the effect of the same activating stimuli on the association of Cbl with two other proteins, i.e. Grb2 and P13-K. When the membrane was probed with an antibody specific for Grb2, which is known to associate constitutively with Cbl (2, 9–11), similar amounts of Grb2 coimmunoprecipitated with Cbl from resting and stimulated cells. In contrast, probing the membrane with an anti-p85 antibody demonstrated that p85 was only present in Cbl IPs from OKT3-stimulated cells, consistent with the results obtained in T cells (Fig. 1), cotransfection with PTKs and, consequentially, the level of Cbl tyrosine phosphorylation in intact T cells and that this association is independent of tyrosine phosphorylation of Cbl.

Tyrosine Phosphorylation of Cbl Is Dispensable for Its Interaction with 14-3-3.—Physiological activation of T lymphocytes via their TCR-CD3 complex activates both PTKs and Src/Thr kinases. To determine the effects of Cbl tyrosine phosphorylation in isolation on its association with 14-3-3, we cotransfected COS-1 cells with a Cbl expression vector plus plasmids encoding each of the PTKs potentially involved in regulating Cbl, i.e. Lck and/or Syk family kinases (Zap-70 or Syk). As shown in Fig. 2A, transfection of the Cbl expression plasmid resulted in overexpression of a 120-kDa protein which was recognized by the anti-Cbl antibody (top panel). Similarly, anti-PTK immunoblotting confirmed the expression of Syk, Zap-70, and/or Lck in cells transfected with the corresponding vectors (three bottom panels). When the membrane containing the resolved cell lysates was immunoblotted with anti-Tyr(P) antibody, it was found that the Tyr(P) content of Cbl was very low in cells transfected with the Cbl cDNA alone. Cotransfection with Zap-70 or, to a larger extent, with Syk alone increased the tyrosine phosphorylation of Cbl, and this level was further increased when the cells were triple-transfected with Cbl, Lck, and Zap-70 or Syk. These results agree with the previous observation that Cbl is probably a downstream target of Zap-70 and Src family kinases such as Lck or Fyn in T cells (8), as well as with the recent report demonstrating that Syk is more active than Zap-70 in terms of its ability to phosphorylate Cbl in COS-1 cells (39).

Cell lysates from the Cbl- plus PTK-transfected COS-1 cells, which were either unstimulated or treated with PMA, were then precipitated with GST-14-3-3, and the binding of Cbl or Raf was determined by immunoblotting with the corresponding antibodies (Fig. 2B). GST-14-3-3 precipitated a small amount of Cbl from unstimulated cells, and this level was markedly increased when the cells were stimulated with PMA. However, consistent with the results obtained in T cells (Fig. 1), cotransfection with PTKs and, consequentially, the level of Cbl tyrosine phosphorylation, did not have a significant effect on its association with GST-14-3-3 (Fig. 2B, top panel). These results reinforce the conclusion that tyrosine phosphorylation of Cbl is not required for its interaction with 14-3-3. As noted before, PMA stimulation did not increase the amount of Raf associated with GST-14-3-3 (Fig. 2B, bottom panel), although it caused a mobility shift (Fig. 2B, bottom panel) consistent with its...
phosphorylation.

**Specificity of the Interactions between Different 14-3-3 and Cbl Proteins**—To establish the specificity of the interaction between Cbl and 14-3-3, we extended our analysis to examine another 14-3-3 isoform (ζ), as well as two additional Cbl-related proteins: 70Z/3, a transforming mutant of Cbl with a 17-amino acid deletion near the Ring zinc finger motif (32); and Cbl-b, a recently identified Cbl homolog expressed in different tissues (18), GST-14-3-3ζ with our previous observations in anti-CD3-stimulated T cells (18), GST-14-3-3ζ bound directly to a 120-kDa protein that comigrated with Cbl in the untreated samples, and binding was markedly enhanced by PMA stimulation (Fig. 5, top panel). PP1 treatment completely abolished the direct interaction of 14-3-3ζ with Cbl. Stripping and reprobing the membrane with an anti-Cbl antibody demonstrated that similar amounts of Cbl were present in all the samples (Fig. 5, bottom panel). These results indicated that phosphoserine residues in Cbl were indispensible for its direct interaction with 14-3-3.

**Mapping of the 14-3-3-Binding Site in Cbl**—It was recently reported that the binding of proteins to 14-3-3 is mediated by the phosphorylated consensus motif RSXXYP (30). Inspection of the sequence of Cbl did not reveal such a motif. Therefore, experiments were conducted to map the 14-3-3-binding domain in Cbl using HA-tagged 70Z/3 proteins containing successive truncations at their carboxyl terminus (34). As shown earlier (Fig. 3A), 70Z/3 binds 14-3-3 to the same extent as wild-type Cbl. Jurkat-TAg cells transiently transfected with 70Z/3 expression plasmids, and the binding of the transfected gene products from untreated or PMA-stimulated cells to GST-14-3-3ζ in vitro was analyzed with an anti-HA antibody. GST-14-3-3ζ bound the full-length 70Z/3 protein and two deletion mutants, Δ1–792, and Δ1–730, from which 114 and 176 amino acids were removed, respectively (Fig. 6, top panel). Additional carboxyl-terminal deletions that removed 346, 366, and 456 carboxyl-terminal residues (Δ1–560, Δ1–540, and Δ1–450, respectively) eliminated the binding to GST-14-3-3ζ. The product of v-cbl, which encodes the 361 amino-terminal residues of Cbl, did not associate with GST-14-3-3ζ. As a control, the membrane

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**Fig. 3. Association between 14-3-3 and Cbl isoforms.** A, cells were transfected with cDNA constructs encoding HA-tagged-70Z/3, -Cbl, or -Cbl-b, and the expression of the corresponding proteins in cell lysates was analyzed by anti-HA immunoblotting (B). A and C, lysates from untreated (lane 1) or PMA-stimulated (lanes 2–4) cells were incubated with 10 µg of GST-14-3-3ζ (A) or GST-14-3-3ζ (C), and bound proteins were recovered with glutathione-Sepharose beads. The precipitates were then analyzed with an anti-HA antibody (top panels). The membrane was stripped and immunoblotted with an anti-Raf antibody (bottom panels).

**Fig. 4. PMA stimulation induces serine phosphorylation of Cbl.** A, Jurkat-TAg cells were metabolically labeled with [32P] orthophosphoric acid and then left untreated (lane 1) or stimulated with PMA (+ lane 2). Cell lysates were immunoprecipitated with an anti-Cbl antibody. The IPs were separated by SDS-10% PAGE, electroblotted onto polyvinyldene difluoride membrane, and subjected to autoradiography. The position of Cbl is indicated by the arrowhead. Molecular weight markers are shown on the left. B, the bands corresponding to Cbl from untreated (lane 1) or PMA-treated (lane 2) samples were excised and processed for phosphoamino acid analysis. The positions of phosphoserine (Ser(P)), phosphothreonine (Thr(P)), and phosphotyrosine (Tyr(P)) standards are indicated within dotted lines.

Tyr(P) was detectable. These results strongly suggest that the PMA-enhanced interaction of Cbl with 14-3-3 results from serine phosphorylation of the former.
was stripped and reprobed with an anti-Raf antibody. Comparable amounts of Raf could be detected in all the samples (Fig. 6, middle panel). To confirm that the differential binding to GST-14-3-3 does not reflect different expression levels of the 70Z/3 proteins, cell lysates of the transfected cells were immunoblotted with the anti-HA antibody. Similar amounts of HA-tagged proteins were present in the different groups (Fig. 6, bottom panel). These results demonstrate that a region of 70Z/3 between amino acid residues 560 and 730 most likely contains the 14-3-3-binding motif.

The analysis was refined by creating additional but more limited truncations in the region encompassing residues 560–730 of 70Z/3. The respective proteins from transfected Jurkat-TAg cells were analyzed by anti-HA immunoblotting of the cell lysates showed the presence of comparable amounts of the mutant proteins in all the transfected samples (Fig. 7A, bottom panel). Identical results were obtained when binding to 14-3-3 was assessed (Fig. 7B). Based on these results, we conclude that residues 615–644 of Cbl contain the binding site for 14-3-3 proteins.

Identification of Serine Residues Critical for 14-3-3 Binding—Although Cbl does not contain an exact RSXSXP which has been implicated in 14-3-3 binding (30), residues 615–644 of Cbl contain five serine residues. We focused our attention on two di-serine-containing stretches in this region, i.e. \( R^\text{S4} \)HSLPP\(^\text{S} \) and \( R^\text{S2}LGSTFS \) as plausible sites involved in 14-3-3 binding. To determine the importance of these residues, we replaced the two tandem serine residues in the first, second, or both motif(s) with alanine by site-directed mutagenesis to generate the constructs A2S2, S4A, S2A2, or A4, respectively. These, as well as the non-mutated Cbl construct (S4), were fused in-frame to a pEF vector encoding the Fc fragment of human IgG (33), and the plasmids were then transfected into Jurkat-TAg cells to determine their ability to interact with GST-14-3-3 in vitro.

The protein products of the four expression vectors were expressed at a similar level as revealed by immunoblotting with an anti-human IgG antibody (Fig. 8A). When lysates from the transfected, untreated, or PMA-stimulated cells were incubated with GST-14-3-3, it was found that only the wild-type Cbl construct (S4) bound to the fusion protein; mutation in either or both of the di-serine motifs eliminated the binding (Fig. 8C). Consistent with the earlier findings, the association with 14-3-3 was markedly increased by PMA stimulation.
were incubated with GST-14-3-3 proteins in the 14-3-3-binding domain. Sequence alignment of Cbl-b (Fig. 3) prompted us to compare the sequences of the two proteins. This was consistent with the findings that: first, Ser\(^{259}\) in Raf, which is a known phosphorylation site (40), is critical for 14-3-3 binding (41); second, 14-3-3 proteins associate with phosphorylated, but not unphosphorylated, tyrosine hydroxylase (42) or keratin (43); and third, 14-3-3 interacts with its ligands via a conserved phosphoserine-containing consensus motif, RSXSXP (30).

PMA treatment that induced a mobility shift of Raf consistent with its PKC-mediated phosphorylation (38) did not increase the interaction between Raf and 14-3-3. PKC\(_{\alpha}\) was found to phosphorylate Raf at serine residues 259 and 499 (38). One of these (Ser\(^{259}\)), as well as Ser\(^{621}\), is required for 14-3-3 binding (30, 41). The use of Raf-based phosphoserine-containing synthetic peptides demonstrated that both R\(^{259}\)S\(^{499}\)STTP and R\(^{621}\)S\(^{644}\)SASEP derived from Raf-1 bind to 14-3-3 and, hence, disrupt the interaction between these two proteins (30). Since PMA treatment did not affect the interaction between Raf and 14-3-3 in our experiments, PKC-dependent phosphorylation of Raf at Ser\(^{259}\) seems not to be critical for 14-3-3 binding, in agreement with the recent suggestion that Ser\(^{621}\) is the primary 14-3-3-binding site (30). The observation that the latter residue is constitutively phosphorylated in cells (40) is also compatible with our finding that PMA does not enhance the association between Raf and 14-3-3.

Interestingly, Cbl does not contain the exact consensus sequence RSXSXP that was recently identified as a 14-3-3-binding motif (30). Instead, our analysis of Cbl deletion or point mutants defined a serine-rich 30-amino acid region (residues 615–644) that contains two putative 14-3-3-binding sites encompassed in the sequences R\(^{617}\)HSLPFS and R\(^{626}\)LGSTFS. The second site is not present in Cbl-b. In this difference in 14-3-3 binding between Cbl and Cbl-b predicts some distinctions in their biological functions. Taken together, our findings define a novel serine-based putative 14-3-3-binding motif represented by the consensus sequence RX\(_{1,2}\)S\(_{2,4}\)X\(_{5}\)P (30). Thus, the previously defined 14-3-3-binding consensus motif, RSXSXP (30), does not account for all interactions between 14-3-3 proteins and their ligands.

Our studies do not identify the Ser/Thr kinase(s) that phosphorylates Cbl to increase its association with 14-3-3 proteins. The 14-3-3-binding motif defined by our experiments contains a basic residue (arginine) in position –2 or –3 relative to the first serine residue. This motif could represent a consensus substrate site for several Ser/Thr kinases, including cyclic AMP-dependent protein kinase, (protein kinase A) calmodulin-dependent protein kinase II, or PKC (44). However, treatment of Jurkat T cells with a protein kinase A agonist ( forskolin) did not increase the association between 14-3-3 and Cbl (data not shown). On the other hand, the finding that PMA, a known PKC activator, induced this effect both in T and in COS-1 cells implicates a direct or indirect ubiquitous role for member(s) of the PKC family in this modification. In this regard, the presence of different 14-3-3-binding motifs in Cbl and Raf and the differential effects of anti-CD3 or PMA stimulation on the binding of 14-3-3 proteins to Cbl versus Raf could mean that distinct Ser/Thr kinases, which respond differentially to anti-CD3 or PMA stimulation, phosphorylate these two targets to facilitate their interaction with 14-3-3.

In summary, our findings strongly suggest that TCR/CD3 ligation transduces two distinct signals to Cbl: one delivered by receptor-coupled PTKs, most likely Src and/or Syk family kinases (8), which induce the tyrosine phosphorylation of Cbl; and another, mediated by a Ser/Thr kinase, which phosphorylates Cbl on serine residues. Each of these post-translational modifications leads to a distinct outcome. Whereas tyrosine phosphorylation of Cbl causes it to associate with the Src homology 2 domain of signaling proteins such as Src or Syk family...
Serine Phosphorylation-dependent Binding of Cbl to 14-3-3-3

Phospholipase C (PLC) tyrosine kinases in a manner similar to the regulation of phosphorylation of Cbl may regulate its phosphorylation by the TCR-CD3 complex to downstream targets. In addition, serine phosphorylation of Cbl may regulate its phosphorylation by tyrosine kinases in a manner similar to the regulation of phospholipase Cγ (45, 46). The results reported here, as well as our recent finding that Cbl (70Z/3) interacts with Ras-dependent signaling pathways leading to the nuclear factor of activated T cells activation in T cells (34), may open the way to additional studies aimed at delineating the function of Cbl in signal transduction pathways.

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