Protooncogenic protein c-Cbl undergoes tyrosine phosphorylation in response to stimulation through the receptors for antigens, immunoglobulins, cytokines, and growth factors as well as through the integrins. Tyrosine phosphorylation of c-Cbl may play a functional role in signal transduction, since c-Cbl interacts with many crucial signaling molecules including protein-tyrosine kinases, adaptor proteins, and phosphatidylinositol 3'-kinase. Therefore, it is essential for our understanding of the functions of c-Cbl in signal transduction to identify its tyrosine phosphorylation sites, to determine the protein-tyrosine kinases that phosphorylate these sites, and to elucidate the role of these sites in the interactions of c-Cbl with other signaling proteins. In this report, we demonstrate that tyrosines 700, 731, and 774 are the major tyrosine phosphorylation sites of c-Cbl in T cells in response to pervanadate treatment, as well as in response to TcR/CD3 ligation. Coexpression experiments in COS cells demonstrate that among T cell-expressed Src- and Syk-related protein-tyrosine kinases, Fyn, Yes, and Syk appear to play a major role in phosphorylation of c-Cbl, whereas Lck and Zap phosphorylate c-Cbl ineffectively. Fyn, Yes, and Syk phosphorylate the same sites of c-Cbl that become phosphorylated in stimulated T cells. Among these kinases, Fyn and Yes demonstrate strong binding to c-Cbl, which involves both phosphotyrosine-dependent and phosphotyrosine-independent mechanisms.

Fyn, Yes, and Syk Phosphorylation Sites in c-Cbl Map to the Same Tyrosine Residues That Become Phosphorylated in Activated T Cells

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C-Cbl is a protooncogenic protein initially identified as the cellular homologue of a transforming protein expressed by the murine Cas NS-1 retrovirus. The retroviral Gag-v-Cbl protein contains the 355 amino acid-long N-terminal domain of murine c-Cbl, which is 98.5% homologous to the N-terminal 357 amino acid residues of human c-Cbl. The sequence of c-Cbl is extended beyond the stop codon of v-Cbl and, unlike v-Cbl, features a long C-terminal domain containing a RING finger, a leucine zipper, and numerous proline-rich motifs. This C-terminal domain accounts for more than half the total length of c-Cbl, which equals 913 or 906 amino acids for murine and human proteins, respectively (1). Gag-v-Cbl and the truncated form of c-Cbl corresponding to its sequence in Gag-v-Cbl are present in both the nucleus and the cytoplasm, whereas c-Cbl is exclusively cytoplasmic (2). It has been demonstrated that c-Cbl undergoes tyrosine phosphorylation in response to the antigen receptor-mediated stimulation of T cells (3–5) and B cells (6–9), as well as in response to stimulation through other multichain immune recognition receptors (10, 11), cytokine and growth factor receptors (10, 12–19), and integrins (20, 21). Considering that Abl, the protein-tyrosine kinase (PTK)1 known to phosphorylate c-Cbl, fails to phosphorylate its viral form, it is likely that v-Cbl lacks tyrosine phosphorylation sites (22, 23). Tyrosine phosphorylation of c-Cbl may play a functional role in signal transduction in a variety of cell types, since c-Cbl interacts in an activation- and/or tyrosine phosphorylation-dependent manner with several signaling molecules including receptor and nonreceptor PTKs (4, 9, 11, 13, 15, 16, 24–26), adaptor proteins (9, 16, 19, 23, 27–35), phosphatidylinositol 3'-kinase (4, 5, 7–9, 16, 21, 32), and 14-3-3 proteins (36, 37). Because of the large number of interactions involving c-Cbl, it is crucial for our understanding of its functions to locate the tyrosine phosphorylation sites of this protein, to identify the PTKs phosphorylating these sites, and to determine the role of individual sites in the interactions of c-Cbl with other signaling proteins. These questions were earlier addressed in a study focusing on tyrosine phosphorylation of c-Cbl in Abl-transformed cells (23), in which two sites that are essential for the tyrosine phosphorylation of c-Cbl, Tyr700 and Tyr774, were identified. However, no information is available regarding the location of tyrosine phosphorylation sites in c-Cbl following external stimulation of T cells, the nature of the PTKs phosphorylating these sites, or the interactions of these phosphotyrosines with proteins that are involved in signal transduction in T cells.

In this report, we demonstrate that tyrosines 700, 731, and 774 are the major tyrosine phosphorylation sites of c-Cbl in T cells in response to pervanadate, a potent inhibitor of protein-tyrosine phosphatases, as well as to the TcR/CD3-induced stimulation. We also analyzed tyrosine phosphorylation of c-Cbl in COS cells coexpressing various Src- and Syk-related PTKs with either wild-type or mutant c-Cbl. This analysis demonstrates that Fyn, Yes, Src, and Syk are capable of phosphorylating c-Cbl. Expression of Fyn, Yes, and Syk is typical for T cells, although not restricted to them. Furthermore, Fyn and Syk are thought to play a major role in the TcR/CD3-mediated signal transduction (38–42). Fyn, Yes, and Syk phosphorylate the same tyrosine residues that are phosphorylated in stimulated T cells. Phosphorylation of c-Cbl by Lck was substantially less profound than that by other Src family PTKs. Zap, another PTK crucial for T cell activation, was unable to phosphorylate c-Cbl in the absence of other PTKs but phosphorylated it in the presence of Lck, albeit to a lower extent than Syk, Src, Fyn, or Yes. Furthermore, we have demonstrated that binding of c-Cbl to

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1 The abbreviations used are: PTK, protein-tyrosine kinase; HA, hemagglutinin tag; mAb, monoclonal antibody; Tyr(P), phosphotyrosine; PAGE, polyacrylamide gel electrophoresis; TcR, T cell antigen receptor; SH2, Src homology 2; MOPS, 4-morpholinepropanesulfonic acid.
Fyn and Yes is higher than its binding to other T cell-expressed Src- and Syk-related PTKs. Physical interactions between Fyn and c-Cbl involve phosphotyrosine-dependent binding that is mediated by the Fyn SH2 domain. However, phosphotyrosine-independent mechanisms also contribute substantially to the overall binding of Fyn to c-Cbl. In contrast, binding of Yes and Src to c-Cbl is primarily phosphotyrosine-dependent.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The cDNA of human wild-type c-Cbl and that of its C-terminal truncations containing 357 (v-Cbl), 480, and 655 (HUT) amino acids were obtained from Dr. T. Moran (Mt. Sinai Hospital, New York, NY). Glutathione S-transferase fusion proteins containing T cell-specific Fyn SH2 domain was produced in Escherichia coli (Sugen, Redwood City, CA). The cDNA of human wild-type c-Cbl and that of its C-terminal truncations containing 357 (v-Cbl), 480, and 655 (HUT) amino acids were obtained from ATCC. Jurkat and JCaM1.6 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (HyClone, Logan, UT), HEPES, l-glutamine, and antibiotics. COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with the same reagents.

**Antibodies and Fusion Proteins**—Antisera to Src family kinases and Zap have been previously described (49, 50). The anti-Syk antisera was obtained from BioSource (Camarillo, CA). The pSGT/pSG5 vectors expressing Src family protein-tyrosine kinases and their enzymatically inactive forms (47, 48) were kindly provided by Dr. S. Courtneidge (Bristol-Myers Squibb, Princeton, NJ). The pSGT/pSG5 vectors expressing Src family protein-tyrosine kinases and their enzymatically inactive forms (47, 48) were kindly provided by Dr. S. Courtneidge (Bristol-Myers Squibb, Princeton, NJ). The pSGT/pSG5 vectors expressing Src family protein-tyrosine kinases and their enzymatically inactive forms (47, 48) were kindly provided by Dr. S. Courtneidge (Bristol-Myers Squibb, Princeton, NJ).

**Transfection**—Plasmid DNA was transfected into Jurkat, JCaM1.6, and COS-7 cells using DMRIE-C lipid reagent (Life Technologies, Inc.) following the manufacturer's recommendations. 5 μg of cDNA was used per 2 × 10⁷ T cells or 3 × 10⁶ COS cells. pAlterMAX-based plasmids were used unless indicated otherwise. Cells were activated where indicated and lysed 24 h (Jurkat, JCaM) or 48 h (COS) after transfection.

**Activation of Cells**—Jurkat cells were resuspended in RPMI 1640 supplemented with 20 mM HEPES at a density of 2 × 10⁶/ml and activated by either pervanadate or the anti-CD3 mAb OKT3. Sodium vanadate and hydrogen peroxide were mixed in RPMI 1640 at final concentrations of 10 and 30 mM, respectively, preincubated for 10 min, and added to cell suspensions at a 1:100 dilution for an additional 10 min. OKT3 was added to the cells at a concentration of 2 μg/ml. F(ab)², goat-anti-mouse IgG (Cappel, Durham, NC) at a concentration of 8 μg/ml was added 1 min later for an additional 3 min. JCaM1.6 and, where indicated, COS cells were activated with pervanadate as described above for Jurkat cells.

**Antibodies and Fusion Proteins**—Antisera to Src family kinases and Zap have been previously described (49, 50). The anti-Syk antisera was kindly provided by Dr. J. Fargnoli (Bristol-Myers Squibb, Princeton, NJ). The affinity-purified polyclonal antibody against c-Cbl (C-15) and Zap have been previously described (49, 50). The anti-Syk antisera was obtain from ATCC. Jurkat and JCaM1.6 cells were maintained in RPMI 1640 supplemented with the same reagents.

**Immunoprecipitation**—The immunoprecipitates were added to the pCEP4 vector for expression in mammalian cells. The pSV7 vectors expressing wild-type and kinase-inactive Zap (44, 45) were kindly provided by Dr. A. Weiss (University of California, San Francisco). The pSV7cII vectors expressing SykA and SykB as well as the kinase-inactive form of SykB (46) were kindly provided by Dr. B. Rowley (Bristol-Myers Squibb, Princeton, NJ). The pSGT/pSG5 vectors expressing Src family protein-tyrosine kinases and their enzymatically inactive forms (47, 48) were kindly provided by Dr. S. Courtneidge (Sugen, Redwood City, CA).

**Tyrosine Phosphorylation Sites of c-Cbl in T Cells**

*Fig. 1.* c-Cbl mutagenesis. A, sequence of the C-terminal fragment of c-Cbl starting with amino acid residue 631. Tyrosines are shown in boldface type. Mutated tyrosine residues are underlined. B, schematic representation of mutant c-Cbl proteins. Tyrosines (Y) and phenylalanines (P) in the corresponding positions are indicated.

**Immune Complex Kinase Assays**—Immune precipitates were washed five times in TNE buffer and used for either immunoblotting or immune complex kinase assays.

**Protein Concentrations**—Protein concentrations were determined in precleared lysates using Coomassie reagent (Pierce). Aliquots of these lysates containing equal amounts of total cellular protein were incubated with appropriate antisera or specific immunoglobulins for 1 h at 4 °C. Normal rabbit antisera or isotype-matched IgGs (Sigma) were added to some samples as specificity controls. This was followed by incubation with suspension of S. aureus-derived PANSORBIN particles (Calbiochem) for an additional 1 h (25 μl of 10% suspension/I μl of antisera). When mouse antibodies were used for immunoprecipitation, Pansorbin particles were precoated with rabbit anti-mouse IgG. The immunoprecipitates were washed five times in TNE buffer and used for either immunoblotting or immune complex kinase assays.

**Immune Complex Kinase Assays**—Immune precipitates were additionally washed in kinase buffer (20 mM MOPS, pH 7.0, containing 5 mM of each MnCl₂ and MgCl₂) and incubated for 5 min at room temperature in 25 μl of kinase buffer containing 1μM [γ-³²P]ATP (NEN Life Science Products) at a final concentration of 0.5 μCi/μl. The reaction was stopped by boiling samples in 10 mM Tris containing 1% SDS and 1 mM spermidine. PANSORBIN particles were centrifuged at 14,000 × g for 2 min, and supernatants were collected and diluted 1:10 with buffer containing 30 mM Tris, 50 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 10 mM sodium fluoride. Proteins of interest were precipitated with the corresponding antibodies. These immunoprecipitates were incubated with SDS-PAGE sample buffer for 10 min at room temperature and cleared of PANSORBIN by centrifugation. Supernatants were boiled and subjected to SDS-PAGE separation. Phosphoprotein bands were visualized by autoradiography. Reaction time courses were examined for all kinases studied, and the 5-min point was determined to be in the linear range in each case.
Tyrosine Phosphorylation Sites of c-Cbl in T Cells

Stimulation of T Cells Induces Phosphorylation of c-Cbl Tyrosines 700, 731, and 774—Identification of tyrosine phosphorylation sites in c-Cbl is complicated by the fact that 22 tyrosine residues are present in the wild-type human c-Cbl. To focus on the location of tyrosine phosphorylation sites, we expressed wild-type c-Cbl and a C-terminally truncated form, termed HUT-Cbl, in Jurkat cells and determined whether these recombinant proteins became tyrosine-phosphorylated following activation of T cells with pervanadate. The recombinant forms of Cbl were HA-tagged to facilitate their separation from the endogenous c-Cbl. The full-length c-Cbl and HUT-Cbl were immunoprecipitated using anti-HA, separated by SDS-PAGE, and immunoblotted with either anti-Tyr(P) (anti-P-Tyr; left) or anti-HA (right) as described under “Experimental Procedures.” No precipitation of c-Cbl was observed with MOPC195, a nonspecific IgG2b myeloma protein (data not shown). Each combination of anti-Tyr(P) and anti-HA immunoblots represents an independent experiment. Each experiment was repeated at least three times and yielded similar results. The positions of prestained protein standards and their molecular masses in kilodaltons are indicated. Abbreviations are the same as in the legend to Fig. 2. Designations for mutant proteins are shown in Fig. 1.

into Jurkat cells. HA-tagged c-Cbl mutant proteins were immunoprecipitated from lysates of pervanadate-activated Jurkat cells, and their expression and tyrosine phosphorylation were assessed using immunoblotting with anti-HA and anti-Tyr(P), respectively. Anti-HA immunoblotting demonstrated that expression levels for wild-type and mutant c-Cbl proteins were similar in different samples (Fig. 3, A and B), indicating that the anti-Tyr(P) reactivity detected for these proteins correctly reflected their tyrosine phosphorylation levels. Quantitation of the corresponding bands demonstrated that tyrosine phosphorylation of the mutant c-Cbl lacking tyrosines 674–774 did not exceed 5% of that for wild-type c-Cbl (Fig. 3, A and B). In contrast, most of the single Tyr→Phe mutations examined did not show substantially decreased tyrosine phosphorylation of c-Cbl. The only exception among the single mutations was Y774F, which detectably reduced tyrosine phosphorylation of c-Cbl (Fig. 3A).

To avoid the possible interference of secondary phosphate acceptor sites, which may become prime targets for c-Cbl-phosphorylating PTKs when the primary sites are mutated, we

Denoted in the figures as $5Y\rightarrow F$ and in the text as Tyr$_5 \rightarrow$Phe.
further analyzed the role of individual tyrosine residues by reconstituting them in the TγR → Phe c-Cbl using site-directed Phe → Tyr mutagenesis. To examine phosphorylation of reconstituted tyrosine sites, we employed the approach described in the previous paragraph and demonstrated that phosphorylation of tyrosine residues 700, 731, and 774, but not 674 or 735, was increased over the level corresponding to TγR → Phe (Fig. 3B). The intensity of phosphorylation for these Phe → Tyr mutations can be ranked in the order F774Y > F731Y ≥ F700Y, consistent with the fact that Y774F was the only mutant c-Cbl protein demonstrating a detectable, approximately 2-fold, decrease in tyrosine phosphorylation as compared with the wild-type c-Cbl (Fig. 3A). To verify these results, we simultaneously reconstituted two or all three of these tyrosines in the tyrosine phosphorylation-deficient TγR → Phe c-Cbl (mutations F700Y/F774Y and F700Y/F731Y/F774Y, respectively). Both the F700Y/F774Y c-Cbl and the F700Y/F731Y/F774Y c-Cbl were phosphorylated to a substantially higher extent than the c-Cbl proteins containing the single Phe → Tyr mutations (Fig. 3B). These results were consistent with the lack of appreciable tyrosine phosphorylation for the triple Tyr mutations (Fig. 3B). The intensity of phosphorylation for the expressed recombinant proteins. These experiments revealed that Tcr/CD3 stimulation caused tyrosine phosphorylation of the same c-Cbl sites that were phosphorylated following pervanadate treatment (Fig. 3C). This overexpression might result in incomplete immunoprecipitation of the proteins examined, introducing quantitative errors in the experimental results. To address this issue, we subjected the lysates of COS cells transfected with c-Cbl cDNA to three sequential rounds of anti-HA immunoprecipitation. We then analyzed the amounts of c-Cbl in these immunoprecipitates by anti-Cbl immunoblotting. The experiment demonstrated that over 90% of HA-tagged c-Cbl was precipitated by anti-HA in the first round (Fig. 4B). Overexpression of the c-Cbl proteins might also increase their nonspecific binding to immunoprecipitating antibodies. However, we found no precipitation of wild-type or mutant c-Cbl with the isotype-matched nonspecific IgG2b myeloma protein MOPC195 (Fig. 4C). Similar specificity controls were used in all subsequent experiments and consistently demonstrated the lack of detectable nonspecific immunoprecipitation. Taken together, these results indicated that the conditions of immunoprecipitation used in this study permitted comparing the amounts of precipitated proteins in a quantitative fashion.

The results of coexpression experiments demonstrated that wild-type, but not the TγR → Phe, c-Cbl was highly tyrosine-phosphorylated in Fyn-expressing COS cells (Fig. 5). Tyrosine phosphorylation of wild-type c-Cbl in COS cells transfected with the cDNA of kinase-inactive Fyn did not exceed that in the cells transfected with vector alone (Fig. 5), indicating that the observed phosphorylation was caused by Fyn. Coexpression of Fyn with c-Cbl containing individual Phe → Tyr mutations indicated that Fyn phosphorylates tyrosines 700, 731, and 774. Tyrosine phosphorylation of the doubly reconstituted (F700Y/F774Y) or triply reconstituted (F700Y/F731Y/F774Y) TγR → Phe c-Cbl was higher than that of any singly reconstituted TγR → Phe in Fyn-expressing COS cells (Fig. 5). Expression of c-Cbl in COS cells was analyzed using anti-HA immunoprecipitation followed by anti-HA immunoblotting. These experiments revealed that the amounts of c-Cbl proteins were very similar in different samples (Fig. 5), indicating that the detected anti-Tyr(P) reactivity for these proteins truly reflected the levels of their tyrosine phosphorylation. Furthermore, reprobing of anti-Tyr(P) immunoblots with anti-HA indicated no appreciable differences between various samples with regard to the amounts of c-Cbl (data not shown). Likewise, the amounts of Fyn in different samples of COS cells were very similar as indicated by immunoblotting (Fig. 5) and in vitro kinase assays (data not shown).

We next examined tyrosine phosphorylation of c-Cbl by Syk using the same approach as described above for Fyn. In the initial experiments, we analyzed tyrosine phosphorylation of
c-Cbl by two alternatively spliced forms of Syk, A and B (46). These experiments showed no difference between the abilities of SykA and SykB to phosphorylate c-Cbl (Fig. 6). Therefore, only SykB was analyzed in subsequent experiments as the form that is predominantly expressed in many cell types, including T cells (46). Overall, the experiments with Syk yielded results very similar to those for Fyn (Fig. 6). Wild-type Syk actively phosphorylated wild-type c-Cbl, but not Tyr3 → Phe. Kinase-inactive Syk was incapable of phosphorylating wild-type c-Cbl. Tyrosine residues 700, 731, and 774 were phosphorylated in COS cells expressing Syk. The doubly reconstituted Tyr3 → Phe c-Cbl (F700Y/F774Y) was phosphorylated to a higher extent than any singly reconstituted form, whereas the level of tyrosine phosphorylation for triply reconstituted Tyr3 → Phe c-Cbl (F700Y/F731Y/F774Y) was equal to that for the wild-type c-Cbl (Fig. 6). The amounts of c-Cbl and Syk were examined using immunoprecipitation followed by immunoblotting (Fig. 6) and found to be very similar in different samples. These findings were confirmed by reprobing of anti-Tyr(P) immunoblots with anti-HA (data not shown). Likewise, autokinase activities of wild-type Syk in different samples were similar (data not shown).

The approach described above for Fyn and Syk was used to examine phosphorylation of c-Cbl by other PTKs, including Src-family PTKs Yes, Src, and Lck. Similar to Fyn and Syk, Yes appeared to phosphorylate wild-type c-Cbl at tyrosines 700, 731, and 774 (Fig. 7, A and B). Src was also found to efficiently phosphorylate wild-type, and not Tyr3 → Phe, c-Cbl (Fig. 7A). In contrast, phosphorylation of c-Cbl was very low in COS cells coexpressing c-Cbl and Lck (Fig. 7A and data not shown). This was apparently due to different phosphorylation specificity of Lck and not to the lack of Lck expression, which was high in these cells, as evidenced by Lck immune complex kinase assays (Fig. 7A) and anti-Lck immunoblotting (data not shown). We also compared the amount of Lck expressed in COS cells following transfection of the Lck expression vector to the amount of endogenous Lck in T cells and determined that the expression in COS was substantially higher (data not shown). To further analyze the effect of Lck on c-Cbl, we examined tyrosine phosphorylation of c-Cbl in JCaM1.6 cells, which lack Lck and are deficient in TCR/CD3 signaling (52). These experiments showed that c-Cbl was phosphorylated in JCaM1.6 cells in response to pervanadate (data not shown), further indicating that Lck does not play a prominent role in the tyrosine phosphorylation of c-Cbl in T cells. We also examined whether c-Cbl becomes tyrosine-phosphorylated by the Zap PTK. Our experiments demonstrated no substantial phosphorylation of c-Cbl in COS cells coexpressing c-Cbl and Zap (Fig. 7C). To determine whether this phosphorylation can be facilitated by the presence of a Src-family PTK as described in Ref. 25, we coexpressed c-Cbl, Zap, and Lck in COS cells. These experiments demonstrated that tyrosine phosphorylation of c-Cbl in this system was elevated relative to COS cells expressing Lck or Zap alone. However, this increase was modest, and the tyrosine phosphorylation level of c-Cbl in the presence of both Zap and Lck did not reach that corresponding to Syk, Fyn, Src, or Yes (Fig. 7D and data not shown), in agreement with the findings described in Ref. 25.

**Role of Tyrosine Phosphorylation in the Association between**

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**FIG. 5. Tyrosine phosphorylation of c-Cbl by Fyn in COS cells.** cDNA of wild-type c-Cbl and those of its mutant forms were transfected into COS-7 cells as indicated at the top of the figure. c-Cbl was precipitated from COS lysates with anti-HA and immunoblotted with either anti-HA or anti-Tyr(P) as described under “Experimental Procedures.” Fyn was immunoprecipitated and then blotted with anti-Fyn as described under “Experimental Procedures.” No precipitation of c-Cbl or Fyn was observed with MOPC195 or preimmune rabbit serum, respectively (data not shown). Each combination of anti-Tyr(P), anti-HA, and anti-Fyn immunoblots in the left and right parts of the figure shows results of an independent representative experiment from a total of four experiments for each group. The methods of immunoprecipitation and detection are indicated at the right. The bands corresponding to c-Cbl and Fyn are indicated by arrowheads. The band corresponding to IgG heavy chain of anti-Fyn antibody is indicated by an arrow. Abbreviations and designations for mutant proteins are shown in the legends to Figs. 1 and 2, respectively. kin−, kinase-inactive.

**FIG. 6. Tyrosine phosphorylation of c-Cbl by Syk in COS cells.** cDNA of wild-type c-Cbl and those of its mutant forms were transfected into COS-7 cells as indicated at the top of the figure. c-Cbl was precipitated from COS lysates with anti-HA and immunoblotted with either anti-HA or anti-Tyr(P). Syk was immunoprecipitated and then blotted with anti-Syk. No precipitation of c-Cbl or Syk was observed with MOPC195 or preimmune rabbit serum, respectively (data not shown). Each combination of anti-Tyr(P), anti-HA, and anti-Syk immunoblots in the left and right parts of the figure shows results of an independent representative experiment from a total of four experiments for each group. The methods of immunoprecipitation and detection are indicated at the right. The bands corresponding to c-Cbl and Syk are indicated by arrowheads. Abbreviations and designations for mutant proteins are the same as in Fig. 5. A and B indicate alternatively spliced forms of Syk. Their molecular masses differ slightly, and both are different from that of endogenous Syk, which lacks HA.
Tyrosine Phosphorylation Sites of c-Cbl in T Cells

The difference in binding to the wild-type and Tyr5-phosphorylation-deficient c-Cbl was lower with enzymatically inactivated wild-type or kinase-inactive Fyn and Src. In both cases, coprecipitation of wild-type c-Cbl was more profound for Src and Yes than for Fyn (see Fig. 7).

To further analyze the contribution of tyrosine phosphorylation of c-Cbl to its association with Src family PTKs, we compared binding of these PTKs to wild-type and tyrosine phosphorylation-deficient Tyr5-phosphorylation-deficient c-Cbl with Src family PTKs. This comparison demonstrated that Fyn, Yes, and Src were capable of binding to both wild-type and Tyr5-phosphorylation-deficient c-Cbl, although their binding to the Tyr5-phosphorylation-deficient c-Cbl was lower than that to the wild-type c-Cbl. The difference in binding to the wild-type and Tyr5-phosphorylation-deficient c-Cbl was more profound for Src and Yes than for Fyn (see Fig. 8A). To further examine the role of tyrosine phosphorylation in the interactions of c-Cbl with Src-related PTKs, we compared coprecipitation of wild-type c-Cbl with either wild-type or kinase-inactive Fyn and Src. In both cases, coprecipitation of wild-type c-Cbl was lower with enzymatically inactive PTKs (Fig. 8B). Taken together, these findings indicated the involvement of Tyr(P) residues of c-Cbl in the interactions of c-Cbl with Src-family PTKs.

We then evaluated the ability of individual phosphotyrosine residues of c-Cbl to bind to Fyn and to Yes. These PTKs coexpressed with wild-type and various mutant forms of c-Cbl in COS cells, and their expression and association was assessed as described above. These experiments confirmed that binding of Fyn and Yes to wild-type c-Cbl was substantially higher than their binding to Tyr5-phosphorylation-deficient c-Cbl (Fig. 8C and D), indicating, once again, that tyrosine phosphorylation is crucial for the interactions of c-Cbl with Src-family PTKs. Furthermore, these results pointed out differences between Fyn and Yes in regard to their interactions with c-Cbl. Indeed, single Phe → Tyr reconstitution mutations of the Tyr5-phosphorylation-deficient c-Cbl did not appreciably increase binding of Fyn over the level corresponding to the Tyr5-phosphorylation-deficient c-Cbl (Phe form), whereas the double and the triple reconstitution mutations increased binding of Tyr5-phosphorylation-deficient c-Cbl (Phe form) to Yes to the level characteristic for wild-type c-Cbl (Fig. 8C).

In contrast, the results of similar analysis of Yes/c-Cbl interactions demonstrated that any single reconstitution mutation increased, albeit very modestly, the ability of Tyr5-phosphorylation-deficient c-Cbl to interact with Fyn SH2. Unlike the single reconstitution mutations, the double and the triple reconstitution mutations substantially increased binding of Tyr5-phosphorylation-deficient c-Cbl to Yes (Fig. 8D).

We also examined the binding of c-Cbl to PTKs in COS cells by immunoprecipitation of either c-Cbl or the corresponding PTKs followed by immunoblotting with anti-PTKs and anti-HA, respectively. The choice of this experimental system was determined, among other things, by the insufficient coimmunoprecipitation of c-Cbl with PTKs from Jurkat T cells (data not shown). This finding was consistent with our earlier reports revealing only a modest binding between c-Cbl and PTKs—earlier reports revealing only a modest binding between c-Cbl and PTKs.
S was blotted with anti-Cbl. anti-Fyn. precipitated with anti-HA, and then Fyn was immunoblotted with tively. c-Cbl was immunoblotted with anti-HA.

5–7. The lack of unspecific precipitation with isotype-matched IgG or normal rabbit serum was also confirmed as described in those legends.

A, PTKs were immunoprecipitated with the corresponding anti-PTK antibodies, and the associated c-Cbl was blotted with anti-Cbl. B, Fyn and Src were immunoprecipitated with anti-Fyn and anti-Src, respectively. c-Cbl was immunoblotted with anti-HA. C, c-Cbl was immuno-precipitated with anti-HA, and then Fyn was immunoblotted with anti-Fyn. D, Yes was immunoprecipitated with anti-Yes, and then c-Cbl was blotted with anti-Cbl. E, c-Cbl was coprecipitated with the isolated Fyn SH2 domain fused to glutathione S-transferase (GST). 15 μg of glutathione S-transferase-FynSH2 was added to each sample, followed by 2 mg of glutathione-agarose (dry weight, Sigma). c-Cbl was blotted with anti-Cbl. In A, D, and E, bands were visualized by enhanced chemiluminescence. In B and C, bands were visualized by autoradiography. The bands corresponding to c-Cbl and PTKs are indicated by arrowsheads. Abbreviations and designations are the same as in the legend to Fig. 5.

triple reconstitution mutations dramatically increased binding of Tyr3 to Phe to Fyn SH2 (Fig. 8). Therefore, these findings were consistent with the results obtained with full-length Fyn (see Fig. 8C).

DISCUSSION

It has previously been shown that several proteins, including PTKs, interact with c-Cbl in a tyrosine phosphorylation-dependent manner and that several PTKs of T cells are capable of phosphorylating c-Cbl. In this report, we identify the tyrosine phosphorylation sites of c-Cbl in stimulated T cells as tyrosines 700, 731, and 774. This identification is based on the analysis of tyrosine phosphorylation of the c-Cbl molecules containing Tyr → Phe mutations, as well as on the analysis of Phe → Tyr reconstitution mutations of the tyrosine phosphorylation-defective Tyr3 → Phe form of c-Cbl, which lacks tyrosines 674, 700, 731, 735, and 774. Most of our experiments were conducted with reconstitution mutations, because a decrease in tyrosine phosphorylation caused by a single Tyr → Phe mutation was typically more difficult to detect than an increase caused by a single reconstitution Phe → Tyr mutation. This fact can be explained by low partial contributions of some tyrosine phosphorylation sites to the overall phosphorylation of c-Cbl, and/or by an increase in phosphorylation of secondary sites followed by the removal of any major phosphorylation site. Tyrosines of c-Cbl other than 700, 731, and 774 do not appear to be substantially phosphorylated in T cells. First of all, the mutant form of c-Cbl, which lacks tyrosines 700, 731, and 774 is not appreciably phosphorylated. Furthermore, the reconstitution mutations of tyrosines 674 (F674Y) and 735 (F735Y) do not result in a detectable increase in c-Cbl tyrosine phosphorylation. Finally, the level of tyrosine phosphorylation for the c-Cbl proteins containing double and triple reconstitution mutations approaches that of the wild-type c-Cbl. However, the possibility cannot be ruled out entirely that mutations of c-Cbl induce structural changes in this protein, preventing recognition and/or phosphorylation of other sites of c-Cbl by PTKs. In such a case, some of the phosphorylation sites might go undetermined using this approach. Nevertheless, this possibility does not invalidate the identification of tyrosines 700, 731, and 774 as notable tyrosine phosphorylation sites of c-Cbl.

Although the pervanadate-induced tyrosine phosphorylation is caused by the inhibition of protein tyrosine phosphatases and not by the activation of PTKs as in the case of TcR/CD3 ligation (51), the same sites of c-Cbl are phosphorylated following both pervanadate treatment and TcR/CD3 ligation. Moreover, the relative phosphate acceptor potentials of c-Cbl tyrosine phosphorylation sites appear to rank in the same order for both anti-CD3 and pervanadate stimulation of Jurkat cells. These findings are consistent with the fact that pervanadate mimics natural stimuli with respect to both early signal transduction events and biological responses in T cells (51, 53, 54) as well as in other cell types (55–59).

The results obtained by the expression of wild-type and mutant forms of c-Cbl in Jurkat cells do not allow us to determine what PTKs phosphorylate c-Cbl. It was previously demonstrated that Fyn (25, 26, 49, 60), Syk (24, 61, 62), and Zap plus a Src family PTK (25) are capable of phosphorylating c-Cbl. Therefore, we further analyzed the ability of four Src family kinases, Fyn, Src, Yes, and Lck, as well as both Syk-family kinases, Zap and Syk, to phosphorylate c-Cbl in COS cells. Among Src family PTKs, Src, Fyn, and Yes phosphorylate c-Cbl to a high level, whereas Lck phosphorylates c-Cbl rather poorly. It is impossible to explain the observed differences in the ability of various Src family PTKs to phosphorylate c-Cbl by their differential enzymatic activities toward all kinds of substrates. Indeed, the enzymatic activity of Lck in COS cells, as judged by its autophosphorylation, is substantially higher than that of Yes, approaching that of Fyn. Despite the relatively high autokinase activity of Lck, the level of c-Cbl tyrosine phosphorylation in Lck-expressing COS cells barely exceeds such a level in COS cells transduced with an empty vector. This result is consistent with the ability of Fyn and Yes, but not Lck, to coprecipitate with c-Cbl from T cell lysates and to phosphorylate c-Cbl in these immune complexes (25, 26, 49). The ability of Src-related PTKs to phosphorylate c-Cbl correlates with their structure, because Src, Yes, and Fyn belong to the same structural subfamily of Src-related PTKs, whereas Lck is substantially different from the first three kinases (63).

Among Syk family PTKs, only Syk, and not Zap, is capable of phosphorylating c-Cbl in the absence of Src family PTKs. Interestingly, the difference between these two Syk family PTKs...
with regard to their ability to phosphorylate c-Cbl closely mirrors their differences regarding the tyrosine phosphorylation of the ζ chain of the TcR-CD3 complex (64). Taken together, these results suggest that Fyn and Syk play a major role in tyrosine phosphorylation of c-Cbl in stimulated T cells. Although Yes is also capable of phosphorylating c-Cbl when coexpressed in COS cells, its role in the activation-induced tyrosine phosphorylation of c-Cbl in T cells is unclear, because the physiological stimuli that regulate the activity of Yes in T cells remain to be defined.

Therefore, c-Cbl appears to be preferentially phosphorylated by PTKs that are expressed ubiquitously, not in a T cell-specific fashion. In conjunction with the fact that c-Cbl itself is expressed in a wide variety of cell types and becomes tyrosine-phosphorylated in response to many distinct stimuli, this finding suggests that c-Cbl-dependent signaling is not cell type-specific but is, instead, ubiquitous.

Interestingly, all PTKs that have been shown to phosphorylate c-Cbl in this study phosphorylate the same three sites of this protein, namely tyrosines 700, 731, and 774. These are the same residues that are phosphorylated in response to T cell activation. Indeed, each of the phosphorylated tyrosines is surrounded by a sequence containing recognition elements for Src as defined earlier in Ref. 65. Most of these recognition elements are negatively charged amino acid residues (see Fig. 1), which are a characteristic feature of many tyrosine phosphorylation and auto phosphorylation sites (66). Importantly, none of the tyrosine phosphorylation sites of c-Cbl exhibits recognition elements for Lck (65), consistent with the actual ineffectiveness of Lck in tyrosine phosphorylation of c-Cbl in COS cells. It should be noted, however, that the search of the c-Cbl amino acid sequence for potential tyrosine phosphorylation sites as defined in Ref. 65 suggests that tyrosines 141, 368, 371, and 455 also are sound candidates for being phosphorylated by Src. Our results demonstrate no appreciable phosphorylation of these residues, indicating that the actual tyrosine phosphorylation sites of a large protein molecule, such as c-Cbl, might differ from those predicted.

These experiments also indicate that the double and the triple reconstitution mutations of the Tyr→Phe c-Cbl result in higher tyrosine phosphorylation levels for the "reconstituted" c-Cbl proteins than the simple sums of tyrosine phosphorylation for the corresponding single reconstitution mutations (see Figs. 3 and 5–7). It should be noted that all PTKs phosphorylating c-Cbl demonstrate this trait, although it is more evident for Yes and Syk (Figs. 6 and 7). These results argue that there is a certain degree of positive cooperativity between the tyrosine phosphorylation sites of c-Cbl, which may be due to the fact that phosphorylation of the first site generates a "docking spot" for PTKs facilitating further phosphorylation of c-Cbl.

To determine the role of tyrosine phosphorylation in physical interactions of c-Cbl with Src family and Syk family PTKs, we examined the association of wild-type and mutant forms of c-Cbl with these PTKs. The results of these experiments indicate that association of Src family PTKs with c-Cbl is usually stronger than that of Syk family PTKs, although substantial variations are evident within the Src family. Both phosphotyrosine-dependent and -independent mechanisms are involved in these interactions, although their relative contributions to the overall binding appear to be different for various PTKs. Thus, the role of tyrosine phosphorylation is substantial, but not predominant, for Fyn, whereas Yes and Src bind to c-Cbl primarily through the phosphotyrosine-dependent interactions. Consistent with this notion, Yes is capable of binding to any form of c-Cbl containing at least one tyrosine phosphorylation site. In contrast, phosphotyrosine-dependent binding of Fyn and its SH2 domain to the c-Cbl molecules containing only a single reconstitution mutation is very weak and is dramatically increased by reconstitution of additional tyrosine phosphorylation sites. This synergism between tyrosine phosphorylation sites of c-Cbl may be due to the fact that, for some PTKs, several PTK molecules have to be associated with the molecule of c-Cbl to stabilize this complex.

The tyrosine phosphorylation of c-Cbl is likely to have multiple consequences for signal transduction and cell activation. First of all, phosphorylation of one site may facilitate further phosphorylation of c-Cbl as a result of Src family PTK binding to this site. Furthermore, the existence of several tyrosine phosphorylation sites allows simultaneous binding of several signaling proteins to c-Cbl in an activation-dependent manner. In turn, simultaneous binding of PTKs and their substrates to c-Cbl might result in tyrosine phosphorylation of these substrates. It has earlier been proposed that a similar mechanism might promote Fyn-driven phosphorylation of Zap, a small fraction of which was found to be associated with c-Cbl (25). Furthermore, interactions between many other proteins might also be induced by their simultaneous binding to c-Cbl. Indeed, Tyr(P)-dependent binding of c-Cbl to various signal transduction proteins appears to be a hallmark of receptor-mediated activation in many types of cells. Thus, phosphorylation of tyrosines 700 and 774 has been shown to mediate binding of c-Cbl to the SH2 domain of Crkl in Abl-transformed cells (23). The interaction of c-Cbl with CrkL and Crk has earlier been detected in TcR/CD3-stimulated T cells (27, 29, 30), as well as in Bcr/Abl-transformed cells (32–34). The Crk family adaptor proteins are involved in the activation of small GTP-binding proteins through binding to the GTP/GDP exchange factors SOS and C3G (67–71). Indeed, it was recently reported that tyrosine phosphorylation of c-Cbl in T cells is linked to the activation of Rap1, a small GTP-binding protein regulated by C3G, which appears to down-regulate the TcR/CD3-mediated transcription of the interleukin-2 gene (72). Furthermore, tyrosine 731 is part of the c-Cbl sequence (Y731EAM) that has been reported to be responsible for the activation-dependent binding of phosphatidylinositol 3'-kinase to c-Cbl (73). Therefore, phosphorylation of tyrosine 731 is likely to be critical for the formation of the phosphatidylinositol 3'-kinase-c-Cbl complex, which has been detected in a variety of cell types following stimulation (4, 5, 7–9, 16, 21, 74), as well as in transformed cells (32). In addition, it has been reported (75) that tyrosine 700 might mediate binding of c-Cbl to Vav, a protein that plays a very important role in signal transduction in hematopoietic cells (76, 77). In summary, the data presented here demonstrate that TcR/CD3 ligation triggers phosphorylation of c-Cbl tyrosines 700, 731, and 774, all of which appear to be involved in the interactions of c-Cbl with crucial components of T cell signal transduction pathways, such as PTKs, phosphatidylinositol 3'-kinase, CrkL, and Vav. Hence, phosphorylation of these c-Cbl tyrosine residues is likely to play a crucial role in biologic responses of T cells and, possibly, other cell types to external stimuli.

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