

Association of Tyrosine Protein Kinase Zap-70 with the Protooncogene Product p120^{c-cbl} in T Lymphocytes

By Marielle Fournel,* Dominique Davidson,* Robert Weil,*
and André Veillette*†§||**

From the *McGill Cancer Centre and Departments of †Medicine, §Biochemistry, and ||Oncology, McGill University, Montréal, Canada H3G 1Y6; and the Departments of †Medicine and **Oncology, Montreal General Hospital, Montréal, Canada H3G 1A4

Summary

Accumulating data show that the tyrosine protein kinase Zap-70 plays an essential role in T cell receptor-mediated signal transduction. However, the mode of action, as well as the physiologically relevant substrates of Zap-70, have not been determined. We have attempted to identify a 120-kD tyrosine-phosphorylated protein (p120) that associates with Zap-70 in activated T lymphocytes. The results of our analyses showed that p120 is largely encoded by the *c-cbl* protooncogene. Furthermore, the association of Zap-70 with c-Cbl was shown to be induced by T cell receptor stimulation, implying that it required posttranslational modification of one or both of these products. FynT, but not Lck, also associated with c-Cbl in activated T cells. Finally, using a heterologous system, it was demonstrated that the ability of Zap-70 to cause tyrosine phosphorylation of p120^{c-cbl} was dependent on Lck- or FynT-mediated signals. As c-Cbl can associate with several other signaling molecules, it may couple Zap-70 to downstream effectors during T cell activation.

Stimulation of T lymphocytes by antigen or anti-TCR antibodies causes a rapid tyrosine protein phosphorylation signal (for review, see references 1–4). Accumulating evidence shows that this signal is initiated by p56^{lck} and p59^{fynT}, two members of the Src family abundantly expressed in T lymphocytes (for review, see references 1–5). This function presumably reflects the ability of Lck and FynT to phosphorylate specific tyrosine residues in the cytoplasmic domains of the CD3 and ζ chains of the TCR complex. Subsequent to this event, Zap-70, a member of the Syk/Zap-70 family of cytoplasmic tyrosine protein kinases, associates with tyrosine-phosphorylated CD3 and ζ through its tandem amino-terminal Src homology 2 (SH2) domains (6–9). This association is paralleled by an increase in the tyrosine phosphorylation and catalytic activity of Zap-70. The importance of Zap-70 in TCR signaling is highlighted by the finding that a subset of humans with severe combined immunodeficiency has mutations in the *zap-70* gene (10–12).

The identity of the targets of Zap-70 in T lymphocytes is not known. However, we previously found that Zap-70 associates with a 120-kD tyrosine-phosphorylated polypeptide (p120) in activated T cells (13, 14). This association was shown to involve a pool of Zap-70 molecules not associated with TCR. Moreover, it seemingly required the

presence of phosphorylated tyrosine residues, as it could be efficiently competed by addition of phosphotyrosine to cell lysates. Whereas the identity of p120 could not be established in this earlier study, evidence was provided that it was distinct from phosphatidylinositol 3' kinase, GTPase-activating protein of p21^{ras} and focal adhesion kinase (Fak). Furthermore, while the 120-kD product of the *c-cbl* protooncogene (p120^{c-cbl}) is prominently tyrosine phosphorylated in activated T cells, we failed to detect Zap-70 in anti-Cbl immunoprecipitates, suggesting that the Zap-70-associated p120 was also distinct from c-Cbl.

The *cbl* gene was initially identified in a rearranged form, as the oncogene of the Cas-Br-M murine leukemia virus, which causes pre-B cell lymphomas and myeloid leukemias in mice (15). Cloning of the *c-cbl* protooncogene showed that it encodes a 120-kD protein bearing a proline-rich domain and a possible carboxy-terminal leucine zipper (16). While *c-cbl* is expressed in several cell types, it preferentially accumulates in thymus and testis, as well as in hemopoietic cells (17). A role for c-Cbl in hemopoietic cell signaling was suggested by the recent finding that it undergoes prompt tyrosine phosphorylation after stimulation of the T cell receptor for antigen (18), the Fc receptor for IgG (19), and the receptors for GM-CSF and erythropoietin (20). Furthermore, it has been documented that c-Cbl can bind a multitude of SH2 and/or SH3 domain-containing proteins, through its tyrosine-phosphorylated region and proline-rich domain, respectively (18). These Cbl-binding

Dr. Weil's present address is Département de Biologie Moléculaire, Institut Pasteur, 75724 Paris, Cédex 15, France.

partners include FynT, the adaptor proteins Grb2 and Nck, phosphatidylinositol 3' kinase, and phospholipase C- γ , which are likely involved in regulating and/or mediating Cbl-related functions.

Materials and Methods

Cells. BI-141 is a class II MHC-restricted, beef insulin-specific mouse T cell hybridoma, which lacks expression of CD4 and CD8 (21, 22). It was propagated in RPMI 1640 medium supplemented with 10% fetal bovine serum and, if indicated, the aminoglycoside G418 (0.6 mg/ml). BI-141 derivatives expressing an activated version of p56^{lck} (tyrosine 505 to phenylalanine 505 [F505] Lck) have been described elsewhere (21). Cos-1 cells were propagated in α MEM with 10% fetal bovine serum.

Antibodies. Antibodies against p120^{c-cbl} were produced by immunizing New Zealand White rabbits with a TrpE fusion protein encompassing amino acids 541–886 of the mouse c-Cbl sequence. Rabbit antisera directed against the unique domain of p56^{lck} (23) or p59^{fn} (22), or the "linker" region of Zap-70 (13), were described elsewhere. Affinity-purified rabbit antiphosphotyrosine antibodies were previously reported (21, 22).

Antibody-mediated T Cell Activation. BI-141 cells (1×10^7) were stimulated for 2 min with saturating amounts of anti-TCR V β 8 mouse mAb F23.1 (24) and sheep anti-mouse (SAM) IgG, as outlined elsewhere (21, 22). After stimulation, cells were lysed in 2 \times TNE buffer (100 mM Tris, pH 8.0, 2% NP-40, and 40 mM EDTA), supplemented with 20 μ g/ml each of the protease inhibitors leupeptin, aprotinin, *N*-tosyl-L-phenylalanine chloromethyl ketone, *N*-*p*-tosyl-L-lysine chloromethyl ketone, and PMSF, as well as the phosphatase inhibitors sodium fluoride (100 mM) and sodium orthovanadate (2 mM).

Immunoprecipitations and Immunoblots. Unless indicated, post-nuclear supernatants were precleared with *Staphylococcus aureus* protein A. Then, lysates were immunoprecipitated with the appropriate antisera, according to a previously described protocol (25). For depletion experiments, lysates were immunoprecipitated three times with either normal rabbit serum (NRS) or anti-Cbl antibodies, before a final immunoprecipitation with anti-Zap-70 or anti-Cbl antibodies. Immunoblots were performed as detailed elsewhere (23, 25).

Coexpression in Cos-1 Cells. cDNAs encoding mouse Zap-70 (8), p56^{lck} (26), or p59^{fnT} (27) were reported elsewhere. A c-cbl cDNA was generated by PCR from mouse thymus RNA, based on the published sequence (our unpublished data). cDNAs were

individually inserted in the multiple cloning site of pXM139, which bears the SV40 origin of replication. Cos-1 cells were transfected by the DEAE-dextran method, using a fixed total amount of DNA (12 μ g) (28). After 12 h, cells were incubated with chloroquine (60 μ g/ml) for 5 h. After an additional growth period of 48 h, cells were harvested by scraping, and lysed in 1 \times TNE buffer (50 mM Tris, pH 8.0, 1% NP-40, and 2 mM EDTA), supplemented with the protease and phosphatase inhibitors described above. Then, lysates were subjected to immunoblotting or immunoprecipitation, as outlined earlier.

Results

To evaluate better the possibility that Zap-70 associates with the c-Cbl protein, we generated a highly sensitive rabbit antiserum against the Cbl protein (see Materials and Methods). This reagent was used to study further the potential association of Zap-70 with c-Cbl in BI-141 cells expressing an activated version of p56^{lck} (F505 Lck; 21), which exhibit a greatly enhanced TCR-induced tyrosine protein phosphorylation signal. After activation with anti-TCR antibodies, cells were lysed in nonionic detergent-containing buffer. Postnuclear lysates were then immunoprecipitated with various antibodies, and subjected to immunoblotting with antiphosphotyrosine (Fig. 1 A) or anti-Cbl (Fig. 1 B) antibodies. As previously reported (13, 14), activated BI-141 cells contained easily detectable amounts of tyrosine-phosphorylated Zap-70 (Fig. 1 A, lane 2). Additionally, Zap-70 immunoprecipitates exhibited a 120-kD tyrosine-phosphorylated protein (p120). Also consistent with our earlier report (13), anti-Cbl immunoprecipitates (lane 3) from activated BI-141 cells contained a 120-kD tyrosine-phosphorylated product, representing the c-Cbl protein. However, no tyrosine-phosphorylated p70 was detected in these immunoprecipitates. When identical immunoprecipitates were immunoblotted with anti-Cbl antibodies (Fig. 1 B), significant quantities of c-Cbl were noted in anti-Zap-70 immunoprecipitates (lane 2). It was estimated that 5–10% of c-Cbl was present in anti-Zap-70 immunoprecipitates. Coimmunoprecipitation of Zap-70 and c-Cbl was also observed in normal mouse thymocytes (our unpublished data).

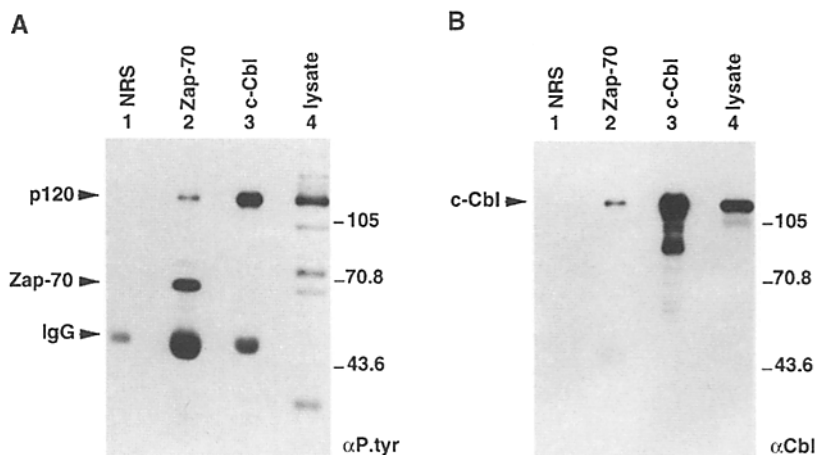


Figure 1. Association of Zap-70 with c-Cbl in activated BI-141 T-cells. BI-141 cells expressing activated p56^{lck} molecules were stimulated for 2 min with anti-TCR mAb F23.1 and SAM IgG. After extraction in nonionic detergent-containing buffer, lysates were subjected to immunoprecipitation with the indicated antibodies, and immunoblotting with antiphosphotyrosine (A) or anti-Cbl (B) antibodies. The positions of p120, Zap-70, heavy chain of IgG, and c-Cbl are indicated on the left, while those of prestained molecular mass markers are shown on the right. Exposures: 15 h.

To demonstrate clearly that the Zap-70-associated p120 was c-Cbl, depletion experiments were conducted (Fig. 2). Lysates from activated BI-141 cells were subjected to repeated immunoprecipitations with either NRS (lanes 1–3) or anti-Cbl antibodies (lanes 4–6). After three cycles, the remaining cellular proteins were immunoprecipitated with either anti-Cbl (lanes 7 and 8) or anti-Zap-70 (lanes 9 and 10) serum, and probed by immunoblotting with antiphosphotyrosine antibodies. Using this approach, >90% of tyrosine-phosphorylated c-Cbl molecules was removed with one Cbl immunoprecipitation (lane 4), and nearly 100% was eliminated after two precipitations (lane 8). After three c-Cbl immunoprecipitations, we found that <10% of Zap-70-associated p120 remained in cell lysates (lane 10; data not shown). In combination, these results indicated that the Zap-70-associated p120 mostly represented p120^{c-cbl}. However, as longer autoradiographic exposures clearly showed that ~10% of p120 remained after immunodepletion of c-Cbl (data not shown), it is possible that one or more additional p120s were also associated with Zap-70. While the identity of these products is not known, they may correspond to the recently described focal adhesion kinase-related FakB (29).

We also investigated whether the association of Zap-70 with c-Cbl was constitutive, or was induced by T cell activation. Cells were either left unstimulated, or stimulated with anti-TCR antibodies. Anti-Cbl immunoblotting of

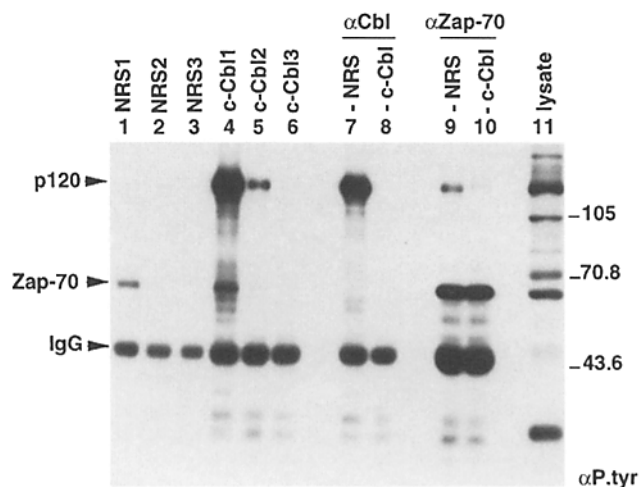


Figure 2. Effects of immunodepletion of c-Cbl on the recovery of Zap-70-associated p120. Lysates from activated BI-141 cells were subjected to repeated immunoprecipitations with NRS or anti-Cbl antibodies, before a final immunoprecipitation with anti-Cbl (lanes 7 and 8) or anti-Zap-70 (lanes 9 and 10) antibodies. Tyrosine-phosphorylated proteins were detected by immunoblotting with antiphosphotyrosine antibodies. The presence of a tyrosine-phosphorylated 70-kD protein in immunoprecipitates obtained with normal rabbit serum (lane 1) or anti-Cbl antibodies (lane 4) was due to recovery of TCR-associated Zap-70 molecules with the combination of mAb F23.1 and SAM IgG used for TCR aggregation. Because a preclearing step was not used in this experiment, the anti-TCR immune complexes were nonspecifically precipitated by the *Staphylococcus aureus* protein A. The positions of p120, Zap-70, and heavy chain of IgG are shown on the left, while those of prestained molecular weight markers are indicated on the right. Exposures: 24 h.

Zap-70 immunoprecipitates revealed that the association of Zap-70 with c-Cbl was undetectable in unstimulated cells (Fig. 3, lane 1), and that it was induced by TCR stimulation (lane 2). Anti-Cbl immunoblotting of parallel Cbl immunoprecipitates demonstrated that the abundance of p120^{c-cbl} was not augmented during T cell activation (lanes 3 and 4).

To examine the specificity of the interaction between Zap-70 and c-Cbl, we assessed the ability of Cbl to bind p56^{lck} and p59^{fynT}, the two other tyrosine protein kinases implicated in TCR signaling (for review, see references 1–4). After detergent extraction, lysates from activated BI-141 cells were immunoprecipitated with antibodies directed against the unique domain of Lck or FynT, and were immunoblotted with anti-Cbl serum (Fig. 4 A). We found that anti-Fyn immunoprecipitates (lane 5) contained appreciable quantities of p120^{c-cbl}. However, these amounts were consistently lower than those observed in anti-Zap-70 immunoprecipitates (lane 2; data not shown). In contrast, no c-Cbl was detected in anti-p56^{lck} immunoprecipitates (lane 4) or in immunoprecipitates generated with preimmune serum (lane 1). Similar results were obtained with BI-141 cells expressing a constitutively activated form of p59^{fynT} (data not shown). Given these results, we wanted to establish whether the binding of c-Cbl to FynT was also regulated by TCR stimulation. Evaluation of both unstimulated and TCR-stimulated BI-141 cells revealed that the interac-

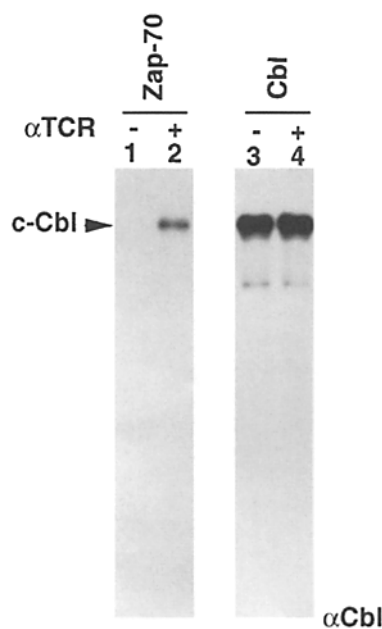


Figure 3. Association of Zap-70 with c-Cbl is induced by T cell activation. BI-141 cells were either left unstimulated (lanes 1 and 3), or stimulated with mAb F23.1 and SAM IgG (lanes 2 and 4). The abundance of Zap-70-associated p120^{c-cbl} was determined by immunoblotting of anti-Zap-70 immunoprecipitates with anti-Cbl antibodies (lanes 1 and 2), while the cellular levels of c-Cbl were determined by anti-Cbl immunoblotting of anti-Cbl immunoprecipitates (lanes 3 and 4). The migration of c-Cbl is indicated on the left. Exposures: lanes 1 and 2; 18 h; lanes 3 and 4; 5 h.

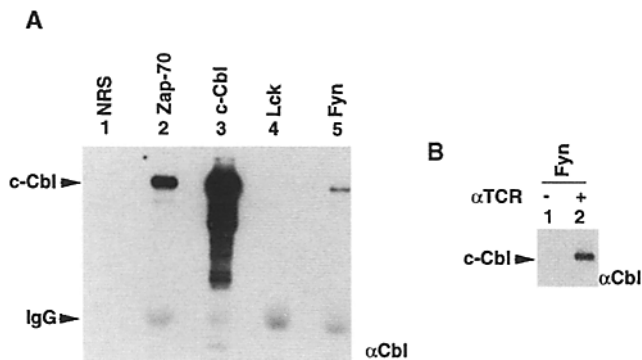


Figure 4. Association of c-Cbl with FynT, but not Lck, in activated BI-141 cells. (A) BI-141 cells containing F505 Lck were stimulated with anti-TCR mAb F23.1 and SAM IgG for 2 min. Lysates were then immunoprecipitated with various antibodies, followed by immunoblotting with anti-Cbl antibodies. The migrations of c-Cbl and heavy chain of IgG are shown on the left. Exposure: 84 h. (B) Same as in A, except that cells were either left unstimulated or stimulated with anti-TCR mAb F23.1 and SAM IgG. Lysates were immunoprecipitated with anti-Fyn antibodies, and immunoblotted with anti-Cbl serum. The position of c-Cbl is indicated on the left. Exposure: 24 h.

tion of c-Cbl with p59^{FynT} was absent before TCR stimulation (Fig. 4 B, lane 1). Therefore, as is the case for the Zap-Cbl association, the detection of FynT-Cbl complexes required activation of BI-141 T cells.

To understand better the respective roles of Zap-70, p56^{Lck}, and p59^{FynT} in the tyrosine phosphorylation of c-Cbl, we attempted to reconstitute their functional interactions in nonlymphoid Cos-1 cells. cDNAs encoding these kinases were transfected either individually or in combination, in the presence of a mouse *c-cbl* cDNA. After 3 d, cells were lysed in detergent-containing buffer, and postnuclear lysates were directly probed by immunoblotting (Fig. 5). Using a panel of antisera directed against c-Cbl, Zap-70, Fyn, and Lck, it was confirmed that all cells expressed the appropriate cDNAs (Fig. 5 A). Through antiphosphotyrosine immunoblotting of total cell lysates (Fig.

5 B), we also determined that c-Cbl was not tyrosine phosphorylated in Cos-1 cells in the absence of coexpression of a tyrosine protein kinase (lane 2). Introduction of FynT (lane 3) or Lck (lane 4) provoked an appreciable augmentation of the phosphotyrosine content of c-Cbl. In contrast, expression of Zap-70 alone (lane 5) had no impact. Interestingly though, Zap-70 increased by approximately threefold the effects of FynT (lane 6) and Lck (lane 7) on c-Cbl tyrosine phosphorylation. This consequence was observed in three independent experiments, and was confirmed by immunoprecipitation of p120^{c-cbl} (data not shown). While phosphorylation of c-Cbl by these tyrosine protein kinases could be reconstituted in Cos-1 cells, it is worth noting that we were unable to show association of c-Cbl and Zap-70 in this system (data not shown).

Discussion

The results of our experiments showed that a significant amount (5–10%) of the protooncogene product p120^{c-cbl} could be detected in anti-Zap-70 immunoprecipitates from either activated BI-141 T cells (this report) or activated mouse thymocytes (our unpublished data). Moreover, depletion experiments confirmed that the majority (>90%) of the Zap-70-associated p120 was removed by anti-Cbl antibodies, implying that it largely represented p120^{c-cbl}. Despite this firm evidence, we consistently failed to detect Zap-70 in anti-Cbl immunoprecipitates (13 and this report). Although the precise basis for this phenomenon is not determined, the anti-Cbl antibodies used in our experiments may not efficiently recognize c-Cbl molecules complexed to Zap-70. This possibility appears unlikely however, since equivalent results were obtained with an antiserum directed against another domain of c-Cbl (our unpublished data). Alternatively, the lack of detection of Zap-70 in anti-Cbl immunoprecipitates could indicate that only a small fraction of Zap-70 was bound to c-Cbl in activated T cells. We favor this possibility.

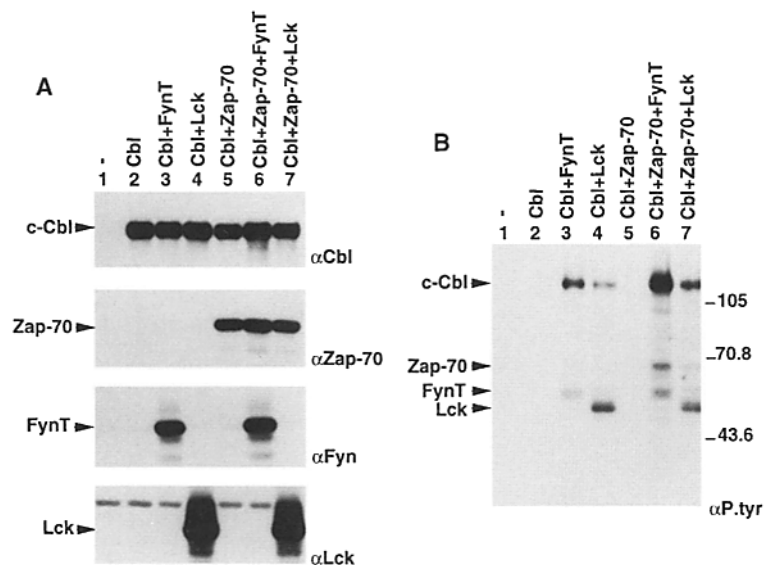


Figure 5. Reconstitution of interactions between c-Cbl, Zap-70, and Src family kinases in Cos-1 cells. Cos-1 cells were transfected with the indicated cDNAs. After 3 d, protein expression was determined by immunoblotting of total cell lysates (A). Levels of phosphotyrosine were ascertained by immunoblotting with antiphosphotyrosine antibodies (B). The migrations of c-Cbl, Zap-70, FynT, and Lck are indicated on the left, whereas those of pre-stained molecular weight markers are shown on the right. Exposures: (A) top, 15 h; top middle, 15 h; bottom middle, 10 h; bottom, 10 h; (B) 48 h.

The formation of Zap-Cbl complexes was shown to be induced by T cell activation. Therefore, it is probable that this association required modification of either or both of these products in the course of T cell activation. Further support for this view was lent by our finding that phosphotyrosine (but not phosphoserine) dissociated Zap-Cbl complexes (13 and our unpublished data), which implied that phosphorylated tyrosine residues were crucial for the stability of the interaction. Because both Zap-70 and p120^{c-cbl} are tyrosine phosphorylated in activated T cells (6, 18), either could act as a docking site for the other. One obvious possibility is that the SH2 motifs of Zap-70 interact with sites of tyrosine phosphorylation on c-Cbl. Unfortunately, however, we were unable to show binding of tyrosine-phosphorylated c-Cbl to recombinant Zap-70 SH2 domains in vitro (our unpublished data). Conversely, it is plausible that phosphorylated tyrosines on Zap-70 were central to the interaction. As c-Cbl does not bear any SH2 domain, this model would require the participation of an intermediary molecule linking tyrosine phosphorylated Zap-70 to c-Cbl. This idea would also be consistent with the inability of Zap-70 to associate with c-Cbl in Cos-1 cells, even in the presence of Src-related enzymes. Since c-Cbl possesses binding sites for SH2 and SH3 domains (18), it may interact with Zap-70 through an "adaptor" molecule such as Grb-2, Crk, or Nck (30). These polypeptides contain both SH2 and SH3 domains, and are known to mediate associations between signal transduction molecules.

By coexpression in Cos-1 cells, evidence was adduced that Zap-70 could not phosphorylate c-Cbl in the absence

of Lck or FynT. This result was reminiscent of that of Chan et al. (6), which showed that phosphorylation of cellular proteins by Zap-70 in Cos-7 cells was also dependent on coexpression of a Src-like kinase. Importantly, we found that Zap-70 augmented the extent of c-Cbl tyrosine phosphorylation induced by Lck or FynT. On this basis, we hypothesized that Zap-70 and Lck/FynT cooperate to induce optimal tyrosine phosphorylation of p120^{c-cbl} during T cell activation. The relative contribution of each enzyme, as well as the possibility that they phosphorylate distinct sites on p120^{c-cbl}, remain to be examined.

What is the potential role of c-Cbl in Zap-70-mediated signal transduction? Previous studies have shown that c-Cbl is a potent growth regulator that has oncogenic potential towards cells of hemopoietic and fibroblastic lineages (15, 16, 31, 32). While its exact mode of action is not defined, p120^{c-cbl} has the ability to associate with various polypeptides containing SH2 or SH3 domains (18). Therefore, c-Cbl may function as a large docking protein linking Zap-70 to biochemical events such as Ras activation and lipid metabolism. In this context, the formation of Zap-Cbl complexes may represent a crucial step in T cell activation. Alternatively, it is possible that the binding of c-Cbl affects the regulation of Zap-70. For example, by associating with both Zap-70 and p59^{fynT}, c-Cbl could enhance the ability of FynT to phosphorylate (and activate) Zap-70. This may be especially important for non-TCR-associated Zap-70 molecules, which are actually known to preferentially associate with c-Cbl (13). These two possibilities are currently being tested.

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Address correspondence to André Veillette, McGill University, McIntyre Medical Sciences Building, Room 715, 3655 Drummond Street, Montréal, H3G 1Y6, Canada.

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