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The Product of the Proto-Oncogene c-*cbl*: A Negative Regulator of the Syk Tyrosine Kinase

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Engagement of antigen and immunoglobulin receptors on hematopoietic cells is directly coupled to activation of nonreceptor protein tyrosine kinases (PTKs) that then phosphorylate critical intracellular substrates. In mast cells stimulated through the FcεRI receptor, activation of several PTKs including Syk leads to degranulation and release of such mediators of the allergic response as histamine and serotonin. Regulation of Syk function occurred through interaction with the Cbl protein, itself a PTK substrate in this system. Overexpression of Cbl led to inhibition of Syk and suppression of serotonin release from mast cells, demonstrating its ability to inhibit a nonreceptor tyrosine kinase. Complex adaptor proteins such as Cbl can directly regulate the functions of the proteins they bind.

Cbl, the product of the proto-oncogene c-*cbl*, is a prominent substrate of the cellular PTKs activated by multiple immune and growth factor receptors (*1*, *2*). Both the retroviral gag-v-cbl fusion protein and a Cbl protein containing a 17–amino acid internal deletion, which was isolated from the 70Z pre–B cell tumor line, are transforming in fibroblast and pre–B cells (*3*). However, the function of the proto-oncogene product remains undefined. The fact that it undergoes tyrosine phosphorylation and that it binds critical signaling molecules such as PTKs and adaptor molecules such as Grb2 and the phosphoinositide-3 kinase subunit p85, and the observation that SLI-1, a *Caenorhabditis elegans* homolog, has an inhibitory effect on the Ras pathway suggest that Cbl has a critical function in signal transduction (*4*). Cbl is rapidly tyrosine phosphorylated in the rat basophilic leukemia cell line RBL-2H3 after antigen-induced aggregation of the FcεRI receptor (*5*). In

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this system, long studied as a model of allergen-induced mast cell degranulation (6), we demonstrated that Syk is involved in the phosphorylation of tyrosine residues in the COOH-terminal 250 amino acids of Cbl. Cbl and Syk form a complex in which the NH_2-terminal half of Syk containing its tandem SH2 domains bind to Cbl residues 1–655 (7). After receptor engagement, the fraction of Cbl bound to Syk had decreased tyrosine phosphorylation relative to that observed on total Cbl. These and additional observations suggest a regulatory effect of Cbl in this system, and led us to determine whether Cbl overexpression could affect Syk kinase activity and mast cell function.

Overexpression of Cbl, Syk, and relevant mutant forms of each was accomplished with recombinant vaccinia constructs (5, 8). RBL-2H3 cells were infected with constructs encoding Syk and either Cbl or empty vector alone (9). Detection of Cbl and Syk by immunoblotting demonstrated that infection with recombinant Cbl constructs resulted in a threefold increase in the amount of Cbl protein (Fig. 1A) (10). Addition of the antigen dinitrophenyl–human serum albumin (DNP-HSA) to aggregate FcεRI receptors occupied by monoclonal immunoglobulin E (IgE)–binding DNP led to tyrosine phosphorylation of Syk detectable after 1 min (Fig. 1A). Overexpression of Cbl led to decreased tyrosine phosphorylation of Syk. Infection with vector alone had no effect on the amount of tyrosine phosphorylation (5). Infection with increasing amounts of recombinant Cbl virus led to greater amounts of Cbl expression in RBL-2H3 cells (Fig. 1B). This in turn led to a dose-dependent decrease in the amount of Syk tyrosine phosphorylation. The highest dose of virus resulted in nearly complete inhibition of Syk tyrosine phosphorylation, and was thus used in subsequent experiments.

Because Syk tyrosine phosphorylation reflects enzyme activation (11), overexpression of Cbl should lead to inhibition of Syk PTK activity. RBL-2H3 cells were infected with recombinant Syk constructs and with recombinant virus encoding Cbl, a form of Cbl containing a 17–amino acid internal deletion, or empty vector (Fig. 2A). Amounts of Syk were comparable under all conditions, and infection with either wild-type Cbl or the 70Z form led to more than tenfold overexpression of protein. Overexpression of Cbl led to inhibition of antigen-induced Syk tyrosine phosphorylation and Syk activity, measured by autophosphorylation and the phosphorylation of the cytoplasmic fragment of erythrocyte band 3 (10). Expression of the mutant form or vec-

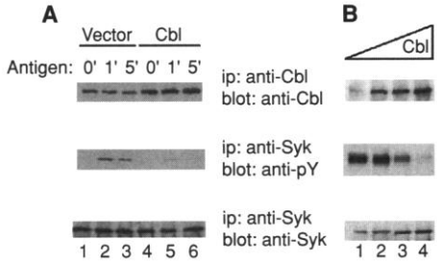

Fig. 1. Overexpression of Cbl leads to inhibition of Syk tyrosine phosphorylation. (**A**) RBL-2H3 cells were infected with recombinant Syk vaccinia and either recombinant Cbl vaccinia or vaccinia vector, washed, and incubated with monoclonal IgE. Minutes of exposure to antigen are indicated above each lane. (9). Protein from cell lysates were immunoprecipitated with the indicated antibody and immunoblotted with antibodies specific for Cbl, phosphotyrosine (pY), or Syk. (**B**) Cells were infected with Syk vaccinia recombinants and the following ratios of Cbl and empty vector (in PFU/cell): 0/25, 3/22, 8.5/16.5, and 25/0 (lanes 1 through 4). The cells were loaded with IgE for 6 hours and stimulation with DNP-HSA was for 2 min. Immunoprecipitation and blotting were done with the indicated antibodies.

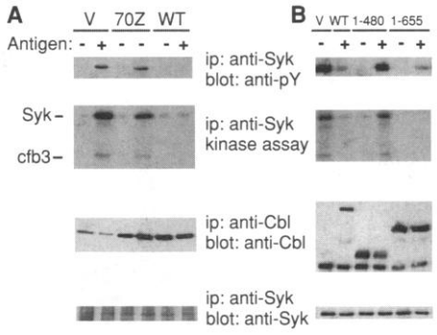

Fig. 2. Overexpression of Cbl inhibits Syk activity. RBL-2H3 cells were infected with Syk vaccinia recombinant virus (5 PFU/cell) and empty vector (25 PFU/cell), 70Z Cbl vaccinia (**A**), Cbl truncation mutants (**B**), or wild-type Cbl vaccinia viruses. After infection for 2 hours, cells were incubated with IgE and stimulated with DNP-HSA. Proteins were immunoprecipitated with antibodies to Syk or Cbl as indicated and were detected by immunoblotting with antibodies to phosphotyrosine (pY), Cbl, or Syk. For the in vitro kinase assay, anti-Syk immunoprecipitates were incubated in kinase buffer with cfb3. The phosphorylated proteins were resolved with SDS-PAGE.

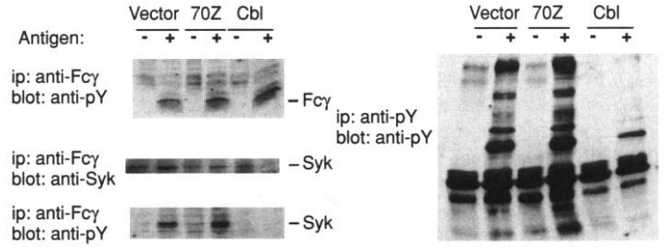

Fig. 3. Overexpression of Cbl blocks Syk assembly with the Fc receptor and intracellular signaling. RBL-2H3 cells were infected with Syk vaccinia recombinant virus and empty vector, 70Z Cbl vaccinia, or wild-type Cbl vaccinia viruses; incubated with IgE; and stimulated with DNP-HSA as in Fig. 2. Proteins from cellular lysates were immunoprecipitated with antibodies to the Fcγ component of the FcεRI receptor or to phosphotyrosine and were detected by immunoblotting with antibodies to Syk or phosphotyrosine.

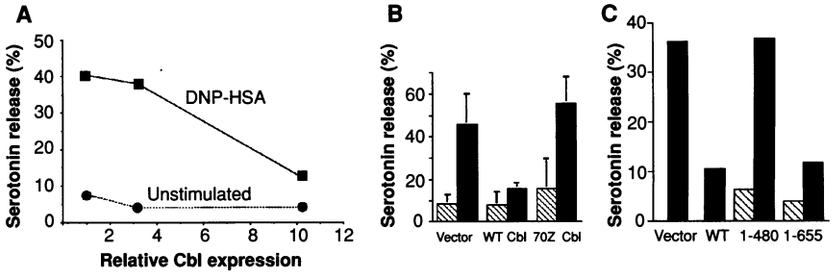

Fig. 4. Inhibition of RBL-2H3 cellular function after overexpression of Cbl. (**A**) Cells were coinfected with Syk vaccinia recombinants (5 PFU/cell) and with the following proportions of wild-type Cbl and vaccinia vector (in PFU/cell ratios): 0/25, 5/20, and 25/0. Cbl protein was detected by blotting with antibodies to Cbl and quantified by densitometry. Without wild-type overexpression, the amount of Cbl is arbitrarily one unit. With a ratio of 5/20, the amount of Cbl was 3.2 times that found without exogenous Cbl. Infection with 25 PFU/cell led to 10.3-fold overexpression. The cells were incubated with IgE and tritiated serotonin for 6 hours, stimulated with DNP-HSA for 45 min, or left unstimulated. Serotonin release was measured by scintillation counting. (**B**) RBL-2H3 cells were infected with 5 PFU/cell of Syk vaccinia recombinant virus and 25 PFU/cell of vector, 70Z Cbl vaccinia, or wild-type Cbl vaccinia viruses. After infection for 2 hours, cells were then incubated with IgE and tritiated serotonin for 6 hours, and stimulated (solid bars) with DNP-HSA for 45 min or left unstimulated (hatched bars). (**C**) RBL-2H3 cells were infected with Syk vaccinia recombinant virus (5 PFU/cell) and vector (25 PFU/cell), wild-type Cbl, or Cbl truncation mutant vaccinia viruses and treated as in (B).

tor alone had no effect. The failure of the 70Z form to block Syk activity demonstrates the specificity of the interaction, because only a small internal deletion resulted in failure of kinase inhibition, although both wild-type and 70Z Cbl bound Syk (*5*, *12*).

The region of Cbl between residues 481 and 655 is required for interaction with Syk, and after overexpression, for inhibition of tyrosine phosphorylation of endogenous Cbl (*5*). Expression of either the Cbl 1–655 truncation mutant or wild-type Cbl had the same inhibitory effect on FcεRI-mediated Syk tyrosine phosphorylation and Syk activity (Fig. 2B). The 1–480 mutant failed to inhibit Syk activity. Thus, the capacity to bind Syk and inhibit its activity maps to the same region of Cbl.

Antigen-induced aggregation of the FcεRI leads to activation of the Lyn PTK, which tyrosine phosphorylates the receptor Fcγ chain. Syk becomes activated by binding the phosphorylated receptor subunit through its tandem SH2 domains (*6*, *11*). To further evaluate the mechanism of Cbl inhibition of Syk, we investigated Fcγ phosphorylation and Syk interaction with the Fcγ subunit after Cbl overexpression. There was no effect on antigen-induced Fcγ chain tyrosine phosphorylation when either wild-type or mutant Cbl was overexpressed (Fig. 3). Thus, Cbl had no effect on this Lyn function. Syk was found to be pre-associated with the receptor, though the level of association increased slightly after antigen activation. Overexpression of the 70Z form of Cbl had no effect on Syk association, but overexpression of wild-type Cbl blocked association of Syk detected with antibodies to either Syk or phosphotyrosine. The interaction of Cbl with Syk, by blocking association with the FcεRI and blocking kinase activity, would be expected to block cellular activation. Examination of the pattern of intracellular tyrosine phosphorylation confirmed this prediction. A marked decrease in the number of phosphorylated substrates was detected after overexpression of wild-type but not 70Z Cbl.

FcεRI-induced degranulation in mast cells is dependent on Syk expression and activity. RBL-2H3 cells selected for failure to express this enzyme do not respond to FcεRI by release of histamine (*13*). Inhibition of Syk interaction with the FcεRI receptor also blocks degranulation (*14*). We thus tested the effect of Cbl overexpression on the functional response of RBL-2H3 cells to receptor cross-linking (*15*). RBL-2H3 cells were infected with various amounts of recombinant Cbl to test for a dose-dependent effect on cellular function (Fig. 4). Recombinant vaccinia containing vector alone was added as needed to keep total viral load constant. Immunoblotting revealed increasing amounts of Cbl protein (*12*). Receptor-induced serotonin release in the absence of exogenous Cbl was comparable to that seen without infection (*12*, *16*). Expression of recombinant Cbl led to a dose-dependent inhibition of receptor-mediated release of serotonin. Higher levels of Syk overexpression overcame the Cbl-mediated inhibition (*12*). Serotonin release in response to receptor cross-linking was also inhibited by the Cbl 1–655 truncation mutant. The 1–480 truncation mutant, which failed to bind Syk, failed to block serotonin release. Overexpression of the 70Z form of Cbl fails to block and reproducibly led to a slight stimulation of release. Thus, the Cbl mutants that block Syk enzymatic activity are the same as those that inhibit RBL-2H3 function.

The observation that Cbl is rapidly tyrosine phosphorylated in response to ligation of various receptors and that it is associated with multiple signaling molecules has led to much interest in uncovering the function of this protein (*1*, *2*). The capacity of distinct mutations of Cbl to lead to transformation and the observation that a homolog in *C. elegans* has a negative regulatory role in controlling Ras function support the idea that the Cbl protein has important functions in signal transduction (*3*, *4*). Overexpression of Cbl reveals an undiscovered role for this protein. It blocks Syk assembly with the activated receptor and blocks Syk enzymatic activity. Cbl can thus serve as an endogenous regulator of signal transduction pathways and cellular activation in the mast cell. Perhaps in this system, endogenous levels of Cbl set a ceiling on Syk activity. The multiple interactions that Cbl forms in various systems suggests that it can be viewed as a complex adaptor protein, much like the insulin receptor substrates IRS-1 and IRS-2 (*17*). All of these molecules are capable of linking multiple signaling elements in a highly defined manner. Our data demonstrate that the function of such adaptor molecules can extend beyond regulation of signaling through assembly. Complex adaptors can also directly regulate the activity of the molecules they bind, thereby directly affecting cellular function.

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10. The cells were lysed in 1% Brij with protease and phosphatase inhibitors (*5*), and lysates were centrifuged at 12,000*g* for 15 min. Protein supernatants were subjected to immunoprecipitation with antibody to Syk bound to protein A–agarose and washed three times with lysis buffer. Syk kinase assays were done at 37°C for 6 min in kinase reaction buffer containing 25 mM tris (pH 6.8), 150 mM NaCl, 10 mM $MnCl_2$, and 50 μM adenosine triphosphate by adding 5 μCi [γ-^{32}P]ATP and 1 μg of cfb3 as an exogenous substrate. The reaction was terminated by addition of 4× reducing sample buffer. The samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE), and the gel was subjected to autoradiography.
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