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The Adaptor Molecule CIN85 Regulates Syk Tyrosine Kinase Level by Activating the Ubiquitin-Proteasome Degradation Pathway

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Triggering of mast cells and basophils by IgE and Ag initiates a cascade of biochemical events that lead to cell degranulation and the release of allergic mediators. Receptor aggregation also induces a series of biochemical events capable of limiting FcεRI-triggered signals and functional responses. Relevant to this, we have recently demonstrated that Cbl-interacting 85-kDa protein (CIN85), a multidomain protein mainly involved in the process of endocytosis and vesicle trafficking, regulates the Ag-dependent endocytosis of the IgE receptor, with subsequent impairment of FcεRI-mediated cell degranulation. The purpose of this study was to further investigate whether CIN85 could alter the FcεRI-mediated signaling by affecting the activity and/or expression of molecules directly implicated in signal propagation. We found that CIN85 overexpression inhibits the FcεRI-induced tyrosine phosphorylation of phospholipase Cγ, thus altering calcium mobilization. This functional defect is associated with a substantial decrease of Syk protein levels, which are restored by the use of selective proteasome inhibitors, and it is mainly due to the action of the ubiquitin ligase c-Cbl. Furthermore, coimmunoprecipitation experiments demonstrate that CIN85 overexpression limits the ability of Cbl to bind suppressor of TCR signaling 1 (Sts1), a negative regulator of Cbl functions, while CIN85 knockdown favors the formation of Cbl/Sts1 complexes. Altogether, our findings support a new role for CIN85 in regulating Syk protein levels in RBL-2H3 cells through the activation of the ubiquitin-proteasome pathway and provide a mechanism for this regulation involving c-Cbl ligase activity. The Journal of Immunology, 2007, 179: 2089–2096.

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Supporting information

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2 G.P. and R.M. contributed equally to this work.
3 Current address: Department of Histology and Medical Embryology, University “La Sapienza,” Rome, Italy.
4 Address correspondence and reprint requests to Dr. Rossella Paolini, Department of Experimental Medicine, University “La Sapienza,” Viale Regina Elena 324, Rome, Italy. E-mail address: rossella.paolini@uniroma1.it
5 Abbreviations used in this paper: PTK, protein tyrosine kinase; anti-pTyr, anti-phosphotyrosine; Ub, ubiquitin; CIN85, Cbl-interacting 85-kDa protein; SH3, Src homology 3; WT, wild type; PLC, phospholipase C; HSA, human serum albumin; [Ca2+]i, intracellular calcium ion concentration; RT-Q-PCR, real-time quantitative PCR; siRNA, small interfering RNA; PCc, C-terminal proline-rich and coiled coil; Sts, suppressor of TCR signaling.

Department of Experimental Medicine, Institute Pasteur-Fondazione Cenci Bolognetti, University “La Sapienza,” Rome, Italy

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Syk to the proteasome for degradation, thus providing another molecular mechanism for attenuating FcεRI-mediated positive signals (16).

More recently, we have shown that Cbl could promote FcεRI internalization via a pathway that is functionally separable from its Ub ligase activity and is dependent on Cbl interaction with a multidomain protein, Cbl-interacting 85-kDa protein (CIN85; Ref. 17). CIN85 is a member of a newly discovered subfamily of broadly expressed adaptor proteins that share the presence of several domains able to promote multiple protein-protein interactions (18–20). CIN85 binding to Cbl is mediated by its Src homology 3 (SH3) domains and is largely dependent on the tyrosine phosphorylation of Cbl, whereas the proline-rich region of CIN85 acts as an interaction module for additional SH3 domain-containing proteins (21, 22). We have generated transfectants stably overexpressing CIN85 using the RBL-2H3 rat mast cell line, and demonstrated that CIN85 overexpression accelerates the redistribution of engaged receptor complexes, their sorting in early endosomes, and their delivery to a lysosomal compartment for degradation (17). RBL transfectants were also impaired in their ability to degranulate after Ag stimulation, suggesting that the accelerated down-regulation of activated receptors contributes to dampen the functional response.

The purpose of the present study was to further evaluate the function of CIN85 as a negative regulator of FcεRI-mediated degranulation. In particular, we analyzed whether exogenous CIN85 overexpression could affect the activity and/or expression of molecules directly implicated in Ag-mediated signaling.

We found that wild-type (WT) CIN85 overexpression reduces Syk protein levels, thus affecting the FcεRI-mediated functional responses. Our results support previous evidence for proteasome-dependent pathways in the regulation of Syk tyrosine kinase expression (16, 23–25) and provide a mechanism for this regulation involving the action of CIN85 and Cbl proteins.

Materials and Methods

Chemical reagents and Abs

All chemical and drugs were obtained from Sigma-Aldrich, unless otherwise mentioned.

The rabbit polyclonal anti-CIN85 (raised against the C terminus), anti-suppressor of TCR signaling (Scts) 1 and anti-Sts2 Abs were a gift from Dr. I. Dikic (Goethe University Medical School, Frankfurt, Germany); the mouse monoclonal anti-FcεRI α-chain (BC4) was purchased from BD Biosciences; the mouse monoclonal anti-CIN85 (clone 84) and anti-phosphotyrosine (anti-pTyr) 4G10 Abs were purchased from UBI; rabbit anti-Chi C-15, anti-Syk N-19, anti-Lyn 44, anti-phospholipase C (PLC)γ1 530, and anti-PLCγ2 Q-20 polyclonal Abs, and the anti-Fyn 15 mAb were purchased from Santa Cruz Biotechnology; anti-FLAG M2 and anti-β-actin AC15 mAbs, and monoclonal anti-ΔNP-specific mouse IgG (clone SPE-7) were purchased from Sigma-Aldrich. The proteasome inhibitors epoxomicin and PI-116 and the mouse monoclonal anti-Ub P2K (PWW810) were purchased from Affinity Research Products. G418 was from Invitrogen Life Technologies. Fluo 3-AM and Pluronic F-127 were obtained from Molecular Probes. Rabbit reticulocyte lysates (L415/1-3) were purchased from Promega.

Cell culture and stimulation

The RBL-2H3 mast cell line was cultured in monolayers as described previously (14). The Syk-negative variant of RBL-2H3 cells was kindly provided by Drs. J. Zhang and R. P. Siragian (National Institutes of Health, Bethesda, MD; Ref. 16).

Stable transfectants overexpressing FLAG-tagged human WT CIN85 or CIN85-C-terminal proline-rich and coiled coil (PCC) mutant were generated as described previously (17), established as polyclonal cell lines by culture in the presence of 700 μg/ml G418 (Invitrogen Life Technologies), and used in all the experiments presented. Transfected cell clones were also generated by limiting dilution.

Adherent cells were incubated with 0.5 μg/ml monomeric anti-DNP mouse IgE for 12 h at 37°C. The cells were then harvested, resuspended at 10^6/ml in prewarmed EMEM, and stimulated by adding DNP coupled to human serum albumin (HSA; 1 μg/ml) for the indicated lengths of time. Stimulation was stopped on ice by addition of cold PBS, and cells (25 × 10^6/ml) were lysed in a buffer (pH 8) containing 0.5% Triton-X-100, 200 mM boric acid, 160 mM NaCl, 5 mM EDTA, 1 mM PMFS, 1 mM Na_2VO_3, 50 mM NaF, 5 mM N-ethylmaleimide, and 5 μg/ml each of aprotinin, leupeptin, and pepstatin as previously described (16). Lysates were cleared of debris by centrifugation at 15,000 × g for 20 min; the protein concentration was determined using the Bradford protein assay (Bio-Rad) with BSA (Amresco) as standard, and the normalized samples were used as whole cell lysates or for immunoprecipitation.

For experiments requiring inhibition of proteasome degradation, cells were pretreated with 10 μM epoxomicin or 25 μM PI-116 for 8 or 12 h as specified, washed in cold PBS, and directly lysed in hot Laemmli buffer (75 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 1% 2-ME).

Immunoprecipitation, electrophoresis, and immunoblotting

For immunoprecipitation, postnuclear supernatants were first preclotted by mixing with protein G- (Sigma-Aldrich), or protein A-Sepharose beads (Amersham Pharmacia Biotech Italia) for 1 h at 4°C and then immunoprecipitated with the indicated Abs prebound to protein G- or protein A-Sepharose beads. After gentle rotation at 4°C for 2–12 h, the beads were washed five times with low-salt buffer, and bound proteins were eluted with Laemmli buffer, resolved by SDS-PAGE on precasted minigels (7.5 or 10% Tris-HCl; Bio-Rad), and transferred electrophoretically to nitrocellulose filters. After blocking nonspecific reactivity, filters were probed with specific Abs diluted in 20 mM Tris-HCl pH 8, 150 mM NaCl and 0.05% Tween 20 (TBS-T). After extensive washing in TBS-T, the membranes were incubated with HRP-labeled goat anti-mouse Ig or goat anti-rabbit Ig Abs (Amersham Biosciences), and immunoreactivity was visualized by using the ECL system (Amersham Biosciences).

Densitometric analysis of the films was performed using the NIH Image 1.62f software.

[Ca^{2+}]_i analysis

RBL-2H3 cells were washed once in RPMI 1640 containing 1% FCS. This medium was used during the entire procedure. The cells (20 × 10^6/ml) were loaded with 7 μM Fluo 3-AM and 1 μg/ml Pluronic F-127 in the dark for 45 min at 37°C and 5% CO_2. After two washes, cells were resuspended at the concentration of 20 × 10^6/ml. Aliquots of 1 × 10^6 cells were warmed to 37°C for 5 min, stimulated by adding 0.5 μg/ml of BC4, and immediately analyzed by flow cytometry with a FACScan (FACSCalibur; BD Biosciences). The green fluorescence emission was measured on a logarithmic scale every 3 s for kinetic study as indicated. Unstimulated cells were considered as calibrator.

Relative Syk mRNA amount of each transfectant, normalized with 2-microglobulin was amplified using a common primer set (Applied Biosystems).

mRNA expression analysis

Total RNA was isolated with the RNeasy Mini Kit (Qiagen). Two micrograms of total RNA were reverse transcribed with murine leukemia virus reverse transcriptase and random hexamers (Applied Biosystems). Rat Syk mRNA expression was analyzed by real-time quantitative PCR (RT-Q-PCR) using a commercial TaqMan assay reagent (Applied Biosystems).

PCR were performed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems) according to the manufacturer’s instructions.

For each amplification run, a standard curve was generated using five serial dilutions of total cDNA. All amplification reactions were performed in triplicate, and the averages of the threshold cycles were used to interpolate standard curves and to calculate the transcript amount in samples using SDS version 1.7a software (Applied Biosystems).

Relative Syk mRNA amount of each transfectant, normalized with β2-microglobulin, was expressed as arbitrary units and referred to empty vector-transfected cells considered as calibrator.
In vitro ubiquitination assay

Cells (5 × 10^7/ml) were lysed in a buffer (pH 8) containing 1% Triton-X-100, 0.1% SDS, 200 mM boric acid, 160 mM NaCl, 5 mM EDTA, 1 mM PMSEP, and 5 μg/ml each of aprotinin, leupeptin, and pepstatin as previously described (27). c-Cbl was immunoprecipitated from cells transfected with empty vector or with WT CIN85 and used as E3 ligase; Syk was immunoprecipitated from untransfected RBL-2H3 cells and used as substrate. The immunoprecipitates were washed separately five times with lysis buffer and then mixed before performing the assay. After an additional wash with 1× ubiquitination buffer (50 mM Tris (pH 7.5), 0.5 mM MgCl₂, 0.1 mM ATP, 0.1 mM DTT, 1 mM creatine phosphate), the beads were incubated in 40 μl of the same buffer supplemented with 70% (v/v) rabbit reticulocyte lysates, 10 U of creatine phosphokinase, and 10 μg of Ub for 2 h at 30°C. The samples were washed three times with lysis buffer, eluted with SDS-sample buffer, resolved by SDS-PAGE, and transferred electrophoretically to nitrocellulose filters.

Small interfering RNA (siRNA)

CIN85 siRNAs (siGenome SMART pool rat CIN85 (L-080145-01), a mixture of four different siRNAs, those that proved to be theoretically and/or empirically effective in gene knockdown) and a control siRNA (siCON Universal Buffer) were purchased from Dharmacon. siRNA duplexes were resuspended in 1× siRNA Universal Buffer.

CIN85 knockdown was achieved by transfecting RBL-2H3 cells with CIN85 siRNA duplexes. The transfection was performed by electroporation (310 V, 960 μF) incubating 10 × 10⁶ cells with 2.5 μM siRNA in 500 μl of serum-free MEM. Controls included mock transfection in the absence of siRNA as well as using the nontargeting siRNA. After 24 and 48 h, total RNA was isolated with RNeasy Mini Kit (Qiagen), and CIN85 mRNA expression was analyzed by RT-Q-PCR using a primer endogenous controls for normalization. siRNA-transfected RBL-2H3 clones were analyzed by immunoblotting with anti-PLCγ2 or anti-PLCγ1 antibodies.

Results

CIN85 overexpression inhibits the FcεRI-induced increase in intracellular calcium and PLCγ tyrosine phosphorylation

FcεRI-mediated activation of mast cells results in the release of preformed mediators from cytoplasmic granules (3, 5). We have previously observed in RBL-2H3 cells a substantial inhibition of multivalent Ag-induced degranulation by overexpression of WT but not mutant forms of CIN85 interfering with membrane receptor endocytic processes (17). Similar results were also obtained upon stimulation of RBL cells with an anti-FcεRI α-chain mAb (BC4) (data not shown).

Mast cell degranulation requires a calcium response that involves both the release of calcium from intracellular stores and calcium influx from the medium through channels in the plasma membrane (3–6). Therefore, we analyzed the FcεRI-induced transient rise in free intracellular [Ca²⁺]i concentrations in cells overexpressing CIN85. Cells were labeled with Fluo-3 and subjected to FcεRI clustering by addition of the anti-FcεRI α-chain BC4 mAb. The induced [Ca²⁺]i changes were monitored by flow cytometry. The rapid response to BC4 was suppressed by 40% after overexpression of the WT, but not a mutant form of CIN85 only containing the PCc domain (Fig. 1, A and B). A similar result was obtained when RBL-2H3 clones generated by limiting dilution from the polyclonal population of CIN85 transfectants were analyzed (data not shown).

Stimulation of all transfectants with ionomycin resulted in comparable levels of Ca²⁺ mobilization, indicating that they were able to mobilize Ca²⁺ to similar extents (data not shown). These results suggest that overexpression of CIN85 affects an early stage of FcεRI-induced cell activation.

The aggregation of FcεRI results in tyrosine phosphorylation and activation of both PLCγ1 and PLCγ2, which generate inositol 1,4,5-trisphosphate that in turn mediates the increase in intracellular calcium (6). To compare the phosphorylation status of PLCγ in the different CIN85 transfectants, adherent cells were incubated overnight with anti-DNP IgE mAb and stimulated (or not) with the multivalent Ag DNP-HSA for 1 min. Cell lysates (3 × 10⁶/sample) were immunoprecipitated with anti-PLCγ1 or anti-PLCγ2 polyclonal Ab, resolved by 7.5% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-pTyr mAb. As a consequence of WT CIN85 overexpression, the extent of tyrosine phosphorylation of both PLCγ1 and PLCγ2 upon receptor engagement was lower than in cells transfected with the empty vector, or the mutant form of CIN85 (Fig. 1C). The membranes were reprobed for PLCγ1 and PLCγ2, respectively, to verify an equal loading of proteins. These results suggest that CIN85-mediated inhibition of PLCγ phosphorylation lowers inositol 1,4,5-trisphosphate production and hence reduces the amplitude of the transient [Ca²⁺]i rise.
Overexpression of CIN85 affects the expression level of Syk

The decrease of Ag-induced PLCγ tyrosine phosphorylation observed after WT CIN85 overexpression suggests a possible alteration in activity and/or expression of PTKs, and in particular of Syk which is required for both PLCγ1 and PLCγ2 activation (6, 28).

To determine the phosphorylation status of this kinase in RBL-2H3 transfected cells, cell lysates prepared from control and CIN85 overexpressing cells were resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted as indicated (Fig. 2A). The substantial decrease in Syk tyrosine phosphorylation observed upon WT CIN85 overexpression could affect the steady state protein level of Syk by promoting proteasome degradation. This alteration is involved in Syk degradation.

Proteasome inhibitors restore Syk protein levels in CIN85-overexpressing cells

Work by many research groups including our own, implicates proteasome-dependent mechanisms in the regulation of Syk tyrosine kinase expression levels in both resting and activated human basophils and RBL-2H3 cells (16, 24, 25). To investigate whether CIN85 overexpression could affect the steady state protein level of Syk by promoting proteasome degradation, cells were treated with cell-permeable protease inhibitors or with a corresponding volume of the vehicle DMSO as control and analyzed after 8 h for the expression of Syk by Western blotting on whole cell lysates. Incubation with epoxomicin, a selective and irreversible inhibitor of the proteasome proteolytic activities, restores Syk protein expression in the transfected cells overexpressing WT CIN85 (Fig. 3A), in absence of receptor stimulation, suggesting that overexpression of WT CIN85 was implicated in the down-regulation of Syk protein levels.

Therefore, we extended our analysis comparing the basal level of Syk in the cell lysates obtained by the different transfected clones. The immunoblotting with the anti-CIN85 mAb that detects both the FLAG-tagged human CIN85 overexpressed forms and the endogenous rat CIN85 isoform demonstrated a 5-fold increase of CIN85 expression, whereas the immunoblotting with the anti-FLAG mAb showed a comparable level of overexpressed CIN85 proteins (Fig. 2B). A decrease of Syk expression (>70%) was observed only in the transfected cells overexpressing WT CIN85 when compared with cells transfected with empty vector or the mutant form of CIN85 (Fig. 2C). The same membranes were sequentially probed with Abs specific to other molecules known to be involved in the FceRI-induced secretory response. The expression levels of none of them were affected (Fig. 2C and data not shown). Similar results were also obtained when RBL-2H3-transfected clones were analyzed (data not shown).

FIGURE 2. CIN85 regulates the expression level of the tyrosine kinase Syk. A, RBL-2H3 transfectants were loaded with anti-DNP IgE and stimulated or not with 1 μg/ml DNP-HSA (Ag) for 1 min at 37°C. Cell lysates (3 × 10^3/sample) were immunoprecipitated with anti-Syk polyclonal Ab, resolved by 7.5% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-pTyr and anti-Syk Abs, as indicated. The position of m.w. markers is indicated. B and C, Total cell lysates (TCL) from each transfectant were resolved by 10% SDS-PAGE, transferred to nitrocellulose and immunoblotted with the indicated Abs. The position of m.w. markers is indicated on the left. B and C. Two micrograms of total RNA obtained from the different transfectants were reverse transcribed, and rat Syk mRNA expression was analyzed by RT-Q-PCR. The endogenous gene rat β2-microglobulin was also amplified to normalize each sample. A Syk-deficient cell line (Syk<sup>-</sup>) was used as negative control. Relative Syk mRNA amount of each transfectant was normalized with 2-microglobulin and referred to empty vector-transfected cells considered as calibrator. Data are expressed as the mean ± SD obtained from three independent experiments.

FIGURE 3. Syk proteasome degradation in CIN85-overexpressing cells. RBL-2H3 cells transfected with empty vector or WT CIN85 were pretreated for 8 h with DMSO (control), 10 μM epoxomicin or 25 μM PI-116 (A), and for 12 h with DMSO (control) or 25 μM PI-116 (B). Cells were then directly lysed with hot Laemmli buffer, and total cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-Syk Ab (top). The membranes were stripped and reprobed with anti-actin (bottom) to verify equal protein loading. Results are representative of one of three independent experiments.

Down-regulation of Syk expression by CIN85

Sucrose density centrifugation analysis of MHC class I expression levels in absence of receptor stimulation, suggesting that overexpression of WT CIN85 was implicated in the down-regulation of Syk protein levels.
Materials and Methods

SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-Cbl (lane 1) and anti-Cbl and anti-Syk (lane 2) Abs were used in the absence of cell extracts as negative control. After the reaction proteins were eluted and resolved by 7.5% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the indicated Abs. B, RBL-2H3 cells transfected with empty vector, WT CIN85, or CIN85 PCc mutant were loaded with anti-DNP IgE and left stimulated or not with 1 μg/ml DNP-HSA (Ag) for 1 min at 37°C. Cell lysates (3 × 10³/sample) were immunoprecipitated with anti-CIN85 or anti-FLAG mAbs, resolved by 7.5% SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-Cbl (top) and anti-CIN85 CT (bottom) polyclonal Abs. The position of m.w. markers is indicated on the left. Results represent one of three independent experiments.

Another specific proteasome inhibitor, PI-116, caused only a modest increase of Syk protein level. However, when the pretreatment in the presence of the last inhibitor was prolonged (12 h), a total restoration of Syk expression was induced (Fig. 3B). Caspase and calpain inhibitors as well as ammonium chloride known to inhibit lysosome function had no detectable effects on Syk levels (data not shown). The membranes were reprobed for actin to verify an equal loading of proteins (Fig. 3, bottom). These results suggest that overexpression of CIN85 affects the expression level of Syk mainly by promoting its proteasome degradation.

**CIN85 overexpression increases c-Cbl ligase activity and promotes a constitutive c-Cbl/CIN85 association**

The finding that CIN85 exerts a regulatory effect on Syk expression by promoting its proteasome-dependent degradation prompted us to analyze whether CIN85 overexpression can activate ligase(s) able to ubiquitinate Syk.

We decided to focus our attention on c-Cbl, because we have previously demonstrated that it acts as E3 ligase mediating Ag-induced ubiquitination of Syk on RBL-2H3 cells (16).

We have compared c-Cbl ligase activity in empty vector and WT CIN85-transfected cells by immunoprecipitating the enzyme from total cell extracts and performing an in vitro ubiquitination assay (Fig. 4A). We have used rabbit reticulocyte lysates as source of E1 and E2 enzymes and Syk immunoprecipitated from unstimulated RBL-2H3 cells as substrate, because we could never detect in vivo Syk ubiquitination in resting cells (16).

Immunoblotting with anti-Syk and anti-Ub Abs revealed the presence of Syk molecular species modified by a few Ub molecules together with a smear in the high-m.w. region, characteristic of polyubiquitination. An increase of Syk ubiquitination was observed when c-Cbl ligase was immunoprecipitated from WT CIN85-overexpressed cell lysates (Fig. 4; compare lanes 4 and 6). Anti-Cbl (lane 1) or a mix of anti-Cbl and anti-Syk (lane 2) protein A-Sepharose conjugated beads were used in the absence of cell extracts as negative control. This result demonstrates that CIN85 overexpression increases c-Cbl E3 ligase activity promoting a more robust in vitro Syk ubiquitination.

To investigate the mechanism that regulates c-Cbl ligase activity, we first decided to examine whether overexpression of CIN85 can affect the formation of c-Cbl/CIN85 complexes. We have previously reported the presence of constitutive c-Cbl/CIN85 complexes in RBL-2H3 cells, and we have also demonstrated that the level of c-Cbl/CIN85 association correlates with that of c-Cbl tyrosine phosphorylation induced upon FceRI engagement (17).

Cell lysates obtained before and after FceRI stimulation were subjected to immunoprecipitation with anti-CIN85 mAb to precipitate the endogenous rat CIN85 or with anti-FLAG mAb to precipitate only the overexpressed FLAG-tagged forms of human CIN85, separated by SDS-PAGE, and analyzed by immunoblotting with anti-Cbl polyclonal Ab (Fig. 4B). An Ag-inducible association of endogenous CIN85 with c-Cbl was observed in RBL-2H3 cells transfected with empty vector, confirming our previous finding (17). WT CIN85 overexpression caused the formation of additional c-Cbl/CIN85 complexes in resting cells, and this association was increased upon receptor aggregation. The mutant form of CIN85 (CIN85-PCc) failed to interact with endogenous c-Cbl, confirming the requirement of CIN85 SH3 domains to bind Cbl. Similar results were obtained when anti-Cbl immunoprecipitation and anti-CIN85 immunoblotting was performed (data not shown). These results demonstrate that CIN85 overexpression favors the formation of additional c-Cbl/CIN85 complexes in resting cells.

**CIN85 affects the formation of Cbl/Sts1 complexes**

It has been recently suggested that the Cbl-interacting proteins belonging to the Sts family, Sts1 and Sts2, act as modulators of
biological responses elicited by TCR and receptor tyrosine kinases, by regulating Cbl functions (29, 30). Interaction between Cbl and Sts is independent on Cbl tyrosine phosphorylation and is mediated by the SH3 domains of Sts binding to the proline-rich region of Cbl (30). CIN85 is also composed of SH3 domains that are involved in interaction with Cbl (19). Therefore, we investigated whether exogenous overexpressed CIN85 could compete with Sts1 in c-Cbl binding.

To analyze the presence of Sts proteins in mast cells, lysates from RBL-2H3 cells were immunoprecipitated with anti-Sts1 or anti-Sts2 Abs or normal rabbit serum as control. Immunoblotting revealed the presence of a 70-kDa specific form detected after anti-Sts1 but not anti-Sts2 immunoprecipitation and on total cell lysates (Fig. 5A). To investigate whether Sts1 could interact with c-Cbl, lysates obtained from cells transfected with empty vector or CIN85 proteins were subjected to immunoprecipitation with a rabbit anti-Cbl polyclonal Ab, separated by SDS-PAGE, and analyzed by immunoblotting with anti-Sts1 Ab (Fig. 5B, right). We found that Sts1 constitutively interacts with c-Cbl on RBL-2H3 cells (data not shown) and on cells transfected with empty vector. Following overexpression of WT CIN85, we observed a decrease of c-Cbl/Sts1 complexes, whereas the mutant form of CIN85 unable to bind Cbl did not alter the c-Cbl/Sts1 complex formation (Fig. 5, B and C).

The expression level of Sts1 was not affected by CIN85 overexpression (Fig. 5B, left). These results suggest that overexpressed CIN85 competes with endogenous Sts1 for binding to c-Cbl.

We next assessed the role of endogenous CIN85 in limiting the formation of c-Cbl/Sts1 complexes by performing siRNA-mediated knockdown of CIN85 expression. We found that CIN85 protein expression cannot be completely suppressed in RBL-2H3 cells (we reproducibly observed ~60% inhibition; Fig. 6A). However, siRNA-mediated reduction of CIN85 increased the amount of c-Cbl/Sts1 complexes (Fig. 6B), suggesting that endogenous CIN85 can compete with Sts1 to bind c-Cbl.

**Discussion**

Because of its identification as a Cbl-interacting protein (18), CIN85 has been found to interact with several adaptor molecules mainly implicated in the process of endocytosis and vesicle trafficking, which is the central mechanism for receptor down-regulation (21, 22, 31–33).

In this respect, we have recently proposed a role for CIN85 in controlling the clearance of FceRI engaged receptor complexes from the cell surface of mast cells, thus providing a mechanism to attenuate the intracellular signaling initiated by IgE receptors (17).

More recent evidence suggest that in addition to promote clathrin-mediated receptor internalization, CIN85 can also regulate the activity of several enzymes responsible for signal propagation (34–37).

The impairment in Ca2+ mobilization and PLCγ tyrosine phosphorylation observed upon stable overexpression of WT CIN85 in RBL-2H3 cells (Fig. 1) strongly indicates that CIN85 interferes with early signaling components in FceRI signal transduction. Indeed, we found a reduction of Syk expression level in WT CIN85 overexpressing cells when compared with control cells (Fig. 2).

In a rodent model, the use of Syk-specific inhibitors and Syk-negative mast cell lines has demonstrated an obligatory role for this kinase in FceRI-mediated signaling (28, 38–41). In humans,
a minority of normal blood donors contain basophils that fail to degranulate, and these nonreleaser basophils express normal level of FcεRI but contain very low levels of Syk protein (42, 43). Thus, it is very likely that the reduction of Syk protein level observed upon CIN85 overexpression may account for the impairment of FcεRI-induced functional responses.

Despite the basal low level of Syk present in CIN85-overexpressing cells, the kinase is tyrosine phosphorylated upon receptor engagement (Fig. 2A). This result suggests that the enzymes acting upstream to Syk are not affected by CIN85 overexpression. In support of this conclusion, we found no alteration in the expression level of Lyn and Fyn (Fig. 2C). Furthermore, the ligand-induced tyrosine phosphorylation of FcεRI subunits was not affected by CIN85 overexpression (data not shown), indicating a normal activity of Lyn.

After CIN85 overexpression, we have observed an alteration of Syk mRNA levels that does not correlate with the strong reduction of Syk protein levels (Fig. 2; compare D and C), evoking the action of a posttranslational mechanism mainly responsible for Syk degradation.

Evidence from several laboratories has demonstrated that Syk is highly susceptible to the Ub proteasome-mediated proteolysis in both resting and activated hemopoietic cell types (16, 24, 25, 27). In the present investigation, we have found that proteasome inhibitors restored Syk expression in CIN85-overexpressing cells (Fig. 3), strongly implicating the Ub-proteasome pathway in the regulation of Syk stability. However, we fail to observe a concurrent restoration of FcεRI-induced degranulation (data not shown). The explanation very likely lies in additional effect(s) of proteasome inhibitors occurring upstream and/or downstream to Syk. Relevant to this, Youssif et al. (24) have reported a dramatic impairment of receptor phosphorylation on human basophils treated with proteasome inhibitor I.

The Syk binding Ub ligase c-Cbl has been implicated in Syk degradation both in RBL-2H3 cells and B cells (10, 16, 44). In particular, we have demonstrated that upon FcεRI engagement, c-Cbl mediates Syk ubiquitination and marks the kinase for proteasome degradation (16).

c-Cbl expression levels were not affected upon CIN85 overexpression (Fig. 2C); however, we found a more robust in vitro Syk ubiquitination when c-Cbl was immunoprecipitated from cells transfected with WT CIN85 than from control cells (Fig. 4A), suggesting that c-Cbl ligase activity contributes to the instability of Syk protein levels.

It has been recently demonstrated that the ligase activity of Cbl proteins can be negatively regulated by different families of scaffold proteins, including Sts adaptors (45, 29, 30). We have found that Sts1 constitutively associates with c-Cbl on RBL-2H3 cells and that this association decreases upon CIN85 overexpression and increases after CIN85 knockdown (Figs. 5B and 6B, respectively). Both Sts1 and CIN85 contain SH3 domains directly involved in the interaction with c-Cbl proline-rich region (20, 30); thus, it is likely that the two adaptors compete to bind Cbl. In agreement with this hypothesis, we found an enhanced formation of CIN85/Cbl complexes upon CIN85 overexpression (Fig. 4B), likely affecting the action of Sts1 as negative regulator of c-Cbl ligase activity.

Although CIN85 knockdown favors the formation of c-Cbl/Sts1 complexes, we were unable to appreciate any increase of Syk protein levels (data not shown). Thus, it remains possible that other mechanisms operate to control Syk expression in resting RBL-2H3 cells. In this respect, Siegel et al. (46) have recently described a new mechanism regulating Syk protein stability on B cells that implicates a direct interaction between unphosphorylated forms of Syk and the transcriptional factor OCA-B.

A second member of Cbl mammalian protein able to act as an E3 ligase, namely Cbl-b, is also expressed on RBL-2H3 cells and has been reported to act, together with c-Cbl, as a negative regulator of mast cell functions (47, 48). We found that upon CIN85 overexpression Cbl-b can bind to CIN85 (data not shown), thus likely implying also its contribution to the instability of Syk protein.

The interaction between c-Cbl and CIN85 increases upon FcεRI engagement (Fig. 4B). Furthermore, as a consequence of WT CIN85 overexpression, the Ag-induced decrease of Syk protein level is greater than in cells expressing the empty vector or the mutant form of CIN85 unable to bind Cbl (data not shown). This result suggests that overexpressed CIN85 in addition to control the basal level of Syk can also contribute to limit the FcεRI-mediated signal by accelerating the Ub-proteasome degradation of Syk induced upon receptor engagement.

In summary, our finding supports a new role for CIN85 in regulating Syk protein levels in RBL-2H3 cells through the activation of the Ub-proteasome pathway involving the action of c-Cbl. A regulated expression of Syk protein has been previously reported in several hemopoietic cells, including T and B cells (49, 50). We have already mentioned the case of the nonreleaser basophils that express normal level of FcεRI but contain low levels of Syk proteins compared with releaser basophils (42, 43). It is important to verify in the future whether there is a correlation between the expression level of CIN85 and the integrity of the molecular machinery that regulates Syk stability.

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Disclosures

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References


