

CIN85 Regulates the Ligand-Dependent Endocytosis of the IgE Receptor: A New Molecular Mechanism to Dampen Mast Cell Function¹

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Ligation of the high-affinity receptor for IgE (FcεRI), constitutively expressed on mast cells and basophils, promotes cell activation and immediate release of allergic mediators. Furthermore, FcεRI up-regulation on APC from atopic donors is involved in the pathophysiology of allergic diseases. In consideration of the clinical relevance of the IgE receptor, the down-modulation of FcεRI expression in mast cells may represent a potential target for handling atopic diseases. In an effort to identify new molecular mechanisms involved in attenuating FcεRI expression and signaling, we focused our attention on CIN85, a scaffold molecule that regulates, in concert with the ubiquitin ligase Cbl, the clathrin-mediated endocytosis of several receptor tyrosine kinases. In the present study, we show that endogenous CIN85 is recruited in Cbl-containing complexes after engagement of the FcεRI on a mast cell line and drives ligand-induced receptor internalization. By confocal microscopic analysis, we provide evidence that CIN85 directs a more rapid receptor sorting in early endosomes and delivery to a lysosomal compartment. Furthermore, biochemical studies indicate that CIN85 plays a role in reducing the expression of receptor complex. Finally, we demonstrate that CIN85-overexpressing mast cells are dramatically impaired in their ability to degranulate following Ag stimulation, suggesting that the accelerated internalization of activated receptors by perturbing the propagation of FcεRI signaling may contribute to dampen the functional response. This role of CIN85 could be extended to include other multimeric immune receptors, such as the T and B cell receptors, providing a more general molecular mechanism for attenuating immune responses. *The Journal of Immunology*, 2005, 175: 4208–4216.

The high-affinity receptor for IgE (FcεRI) plays a central role in the induction and maintenance of allergic reactions (1). The FcεRI expressed on the cell surface of mast cells and basophils is a tetrameric complex composed of the IgE-binding α-chain and the β- and γ-chains, both able to transduce signals via the paired tyrosine residues located in their cytoplasmic motifs termed ITAMs (2, 3). Notably, the β-chain plays an essential role in setting the level of cellular response to IgE and Ag through its capacity to amplify both the γ-chain signaling and FcεRI cell surface expression (4, 5). FcεRI engagement leads to β and γ subunits tyrosine phosphorylation through the Src family protein tyrosine kinase (PTK)⁴, Lyn, allowing the recruitment and activation of the cytoplasmic Syk kinase (6, 7). The activation of Syk is required for all known FcεRI-

mediated responses, including the secretion of allergic mediators and the induction of cytokine and chemokine gene transcription (3, 7–9). Evidence collected during the past years have also reported the expression of a trimeric form of FcεRI, lacking the β subunit, on human-dedicated APCs such as Langerhans cells and monocytes (10, 11). APCs bearing trimeric FcεRI can efficiently present IgE-bound Ags to T cells putatively playing an important role in the amplification of inflammatory response (12).

Current therapeutic strategies for the treatment of allergic diseases rely on the use of anti-inflammatory drugs (13). Although these treatments can be highly effective at controlling disease, they are often associated with systemic side effects at higher doses and variable patient response. Therefore, efforts have been undertaken to develop novel therapies that specifically target the allergic process. Because it is now clear that exposure to high levels of monomeric IgE, beyond simply “sensitizing” cells to recognize specific Ag, can also markedly increase the surface expression of FcεRI (14), one promising approach involves the use of humanized anti-IgE mAbs able to block IgE binding to cell surface FcεRI (15, 16).

As a different strategy, some laboratories, including our own, have attempted to identify molecules able to directly regulate FcεRI cell surface expression and/or signaling.

One such molecule, the cytoplasmic PTK Syk, has been targeted by various approaches, including a small selective inhibitor that specifically abrogates mast cell degranulation (17).

More recently, a variety of multidomain adaptor proteins that function to promote multiple protein-protein or protein-lipid interactions, thus regulating mast cell activation, have been described (8).

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⁴ Abbreviations used in this paper: PTK, protein tyrosine kinase; GAM, goat anti-mouse; HSA, human serum albumin; IR, immune receptor; MVB, multivesicular body; RTK, receptor tyrosine kinase; SH3, Src homology 3; Tf, transferrin; WT, wild type.

Among them, the Cbl family of ubiquitin ligases has attracted considerable interest due to the recent finding that it controls the intensity and duration of receptor-generated signals by specific ubiquitin modification of the activated receptors (18–20).

Polyubiquitination, a modification in which a chain of ubiquitin is appended to the substrate, drives targeting for proteasomal degradation (21). Receptor mono- and multiubiquitination, instead, can act as a signal for endocytic trafficking, both at the stage of receptor internalization at the plasma membrane and in the endosomal compartment, where ubiquitinated receptors are finally sorted to the lysosome for degradation (22–27).

In regard to the FcεRI, we have demonstrated that Cbl is responsible for the ligand-induced ubiquitination of the FcεRI β and γ subunits and have suggested a role for this modification in receptor down-modulation (28, 29).

More recent evidence indicates that Cbl could promote internalization of receptor tyrosine kinases (RTKs) via a pathway that is functionally separable from its ubiquitin ligase activity and is dependent on Cbl interaction with a multidomain protein CIN85 (Cbl-interacting protein of 85 kDa) (30). CIN85 belongs to a newly discovered subfamily of broadly expressed adaptor proteins that share the presence of several domains able to promote multiple protein-protein interactions (31–35).

CIN85 is composed of three Src-homology 3 (SH3) domains at the N terminus that are involved in interactions with various signaling molecules, a central proline-rich region acting as an interaction module for additional SH3 domain-containing proteins, and a coiled-coil domain in the C terminus implicated in oligomerization.

CIN85 binding to Cbl is mediated by its SH3 domains and is enhanced by RTK-induced tyrosine phosphorylation of Cbl, whereas the proline-rich region of CIN85 constitutively interacts with endophilins, a class of regulatory components of clathrin-coated pits (36). Phosphorylated Cbl mediates the association of CIN85/endophilin complexes to activated RTKs, whereas endophilin in concert with clathrin adaptors promotes clathrin-mediated receptor internalization (30, 36, 37).

In regard to FcεRI expressed on mast cells, it has been demonstrated previously that upon Ag stimulation, engaged receptors are removed from the cell surface by endocytosis through clathrin-coated pits (38, 39). It is presently unknown whether CIN85 and Cbl could cooperate in the clathrin-mediated internalization of engaged receptors.

To establish whether CIN85 can function as a negative regulator of mast cell functions by controlling receptor internalization, we have generated transfectants stably overexpressing CIN85 using the rat basophilic leukemia cell line, RBL-2H3, that expresses high levels of FcεRI.

Overexpression of wild-type (WT) CIN85 in RBL cells accelerates FcεRI internalization and sorting into early endosomes and subsequent receptor delivery to lysosomes for degradation. These events are associated with an impaired ability of RBL transfectants to degranulate following Ag stimulation, suggesting that CIN85 is involved in limiting the FcεRI-triggered mast cell functional response.

Materials and Methods

Chemical reagents and antibodies

All chemicals and drugs were obtained from Sigma-Aldrich unless otherwise noted.

Anti-FcεRI α subunit (BC4) and anti-FcεRI β subunit mAbs (JRK) were kindly provided by Dr. R. Siraganian (National Institutes of Health, Bethesda, MD) and by Dr. J.-P. Kinet (Beth Israel Deaconess Medical Center, Boston, MA), respectively; the rabbit polyclonal anti-CIN85 Ab raised

against the C terminus was described previously (36). Anti-CIN85 mAbs 179.1.E1 and 84, anti-phosphotyrosine (anti-pTyr) 4G10 mAb, and anti-FcεRI γ-chain polyclonal Ab were purchased from Upstate Biotechnology; rabbit polyclonal anti-Cbl C15 Ab was purchased from Santa Cruz Biotechnology; anti-FLAG M2 mAb, anti-β actin AC15 mAb, and monomeric anti-DNP-specific monoclonal mouse IgE were purchased from Sigma-Aldrich.

Transferrin (Tf)-Texas Red and Lyso-Tracker Red were purchased from Molecular Probes; 4% paraformaldehyde/PBS was purchased from Electron Microscopy Sciences; FITC-conjugated goat anti-mouse (GAM) IgG was obtained from Cappel Research Products; and G418 was from Invitrogen Life Technologies.

Cell lines and stable transfectants

The rat basophilic leukemia RBL-2H3 cells were cultured in monolayers in MEM supplemented with 16% FCS, penicillin (100 IU/ml), as described previously (28).

RBL-2H3 cells were transfected with empty vector (pcDNA3) or constructs encoding FLAG-tagged human WT CIN85, CIN85-3SH3, and CIN85-PCc mutants described previously (36). The transfection was performed by electroporation (310 V, 960 μF) incubating 5×10^6 cells with 20 μg of DNA in 500 μl of serum-free MEM. Stable transfectants were established as polyclonal cell lines by culture in the presence of G418 (700 μg/ml) (Invitrogen Life Technologies) and used in all the experiments presented. Where indicated, transfectant cell clones generated by limiting dilution were also used.

Cell stimulation, immunoprecipitation, and immunoblotting

Adherent cells were incubated with 0.5 μg/ml monomeric anti-DNP mouse IgE for 12 h at 37°C. Cells were then harvested, resuspended at 10^7 /ml in serum-free prewarmed EMEM, and stimulated by adding DNP coupled to human serum albumin (DNP-HSA) (1 μg/ml) for the indicated lengths of time. Alternatively, where indicated, the stimulation was performed by first incubating the harvested cells with anti-FcεRI α-chain (BC4) mAb (0.5 μg/ 10^6 cells) for 20 min on ice. After washing out the unbound Ab, cells were resuspended at 10^7 /ml in serum-free medium and stimulated for the indicated lengths of time at 37°C. Stimulation was stopped on ice by addition of cold PBS, and cells (25×10^6 /ml) were then lysed as described previously (29). Lysates were cleared of debris by centrifugation at $15,000 \times g$ for 20 min, the protein concentration was determined using the Bradford protein assay (Bio-Rad), and the normalized samples were used as whole cell lysates or for immunoprecipitation.

In experiments of protein degradation, BC4-stimulated cells were washed with cold PBS and directly lysed (15×10^6 /ml) in hot Laemmli buffer (75 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 1% 2-ME).

For immunoprecipitation, postnuclear supernatants were first precleared by mixing with protein G-Sepharose beads (Sigma-Aldrich) or protein A beads (Amersham Biosciences) for 1 h at 4°C and then immunoprecipitated with the indicated Abs prebound to protein G beads (mouse Abs) or protein A beads (rabbit Abs). The beads were washed five times with lysis buffer, and bound proteins were eluted with Laemmli buffer, resolved by SDS-PAGE on precast minigels (7.5% Tris-HCl gels; Bio-Rad), and transferred electrophoretically to nitrocellulose filters. After blocking nonspecific reactivity with 5% BSA diluted in TBS-T (20 mM Tris-HCl (pH 8), 150 mM NaCl, and 0.05% Tween), filters were probed with the indicated primary Abs diluted in TBS-T according to the manufacturer's instructions. After washing in TBS-T, the membranes were incubated with HRP-labeled GAM Ig or goat anti-rabbit Ig Abs (Amersham Biosciences), and immunoreactive signals were visualized by the ECL system (Amersham Biosciences).

For experiments requiring membrane stripping, the membrane was treated in a buffer containing 62.5 mM Tris-HCl, 2% SDS, and 100 mM 2-ME at 60°C for 30 min and then extensively washed.

Densitometric analysis of the films was performed with NIH Image 1.62f software. The relative protein amount was referred to the unstimulated sample.

FcεRI down-modulation assay

Cells/sample (5×10^5) were incubated with BC4 mAb on ice for 30 min and, after extensive washing, resuspended in 50 μl of serum-free prewarmed medium and incubated for different lengths of time at 37°C to induce receptor internalization. Endocytosis was stopped by addition of 0.1% Na₂S₂O₃ in cold PBS for 5 min. Control samples were kept on ice for the same time points in the presence of 0.1% Na₂S₂O₃ in cold PBS after BC4 incubation. To evaluate FcεRI surface expression, samples were labeled with FITC-conjugated GAM IgG, and the cytofluorometric analysis was performed with a FACSCalibur flow cytometer (BD Biosciences Immunocytometry Systems).

Immunofluorescence and confocal microscopic analysis

Cells were grown for 24 h in MEM 16% FBS on round glass coverslips coated with 2% gelatin (150×10^3 /well) before incubation with BC4 mAb ($1.5 \mu\text{g}/\text{sample}$) at 4°C for 1 h in serum-free MEM. After extensive washing in cold medium, cells were kept on ice or stimulated at 37°C for the indicated lengths of time to induce receptor internalization.

For Tf internalization assay, cells were incubated with $50 \mu\text{g}/\text{ml}$ Tf-Texas Red at 37°C for 10 min to identify sorting endosomes. For Lyso-Tracker internalization assay, cells were incubated with 100 nM Lyso-Tracker Red for 1 h at 37°C to identify late endosomes and lysosomes.

Stimulation was blocked washing with cold PBS and fixing for 30 min at room temperature in 4% paraformaldehyde/PBS. The fixed cells were then permeabilized for 5 min with 0.1% saponin in PBS. Coverslips were rinsed with PBS, incubated for 30 min with FITC-conjugated GAM IgG, and then mounted with 90% glycerol in PBS. FITC-labeled Ag-Ab complexes were analyzed with a Zeiss Axiophot epifluorescence microscope ($\times 40$ magnification) (Zeiss). Fluorescence images were recorded with a charge-coupled device color camera SPOT-2 (Diagnostic Instruments) and analyzed by a IAS 2000/H1 software (Delta Sistemi).

Colocalization of the fluorescence signals was analyzed by a Zeiss LSM5 Pascal Laser scan microscope ($\times 60$ magnification) using the Zeiss KS 300 3.0 Image Processing system. The mean $\pm$ SD percent of colocalization was calculated analyzing a minimum of 30 cells randomly taken for each experiments, and *p* values were calculated using Student's *t* test.

All images were assembled using Photoshop software and processed using Adobe Photoshop version 6.0, and figures were compiled with Adobe Indesign, version 2.0.

Degranulation assay

Degranulation of the different transfectants was determined by the measurement of β -hexosaminidase release, as described previously (29). Briefly, cells cultured overnight with $0.5 \mu\text{g}/\text{ml}$ monomeric anti-DNP IgE were harvested and seeded in 24-well plates (0.5×10^6 /well), washed twice with Tyrode's buffer (10 mM HEPES (pH 7.4), 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl_2 , 1 mM MgCl_2 , 5.6 mM glucose, and 0.1% BSA), and then stimulated in the same buffer with different concentrations of DNP-HSA for the indicated lengths of time or with 1 mM thapsigargin for 1 h. The enzymatic activity of β -hexosaminidase in supernatants and cell pellets was measured by adding *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide in 0.1 M sodium citrate (pH 4.5) for 60 min at 37°C . The reaction was stopped by addition of 0.2 M glycine (pH 10.7). The release of the product *p*-nitrophenol was detected by measuring absorbance at 405 nm. The Ag-induced β -hexosaminidase release was expressed as percentage of the maximal release induced by $1 \mu\text{g}/\text{ml}$ ionomycin plus $50 \text{ ng}/\text{ml}$ PMA.

Results

CIN85 is expressed in RBL-2H3 cell line and recruited to Cbl complexes upon FcεRI stimulation

To investigate the presence of CIN85 in mast cells, cell lysates from RBL-2H3 were immunoprecipitated with anti-CIN85 (C terminus) or normal rabbit serum as control or with two different mAbs both recognizing CIN85 SH3 domains. The immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with polyclonal (C terminus) or monoclonal (clone 179) anti-CIN85 Ab (Fig. 1A). Immunoblotting demonstrated the presence of different CIN85 isoforms in the RBL-2H3 mast cell line, likely representing alternative mRNA splice variants with the main 85-kDa form equally recognized by the two anti-CIN85 Abs. The same CIN85 isoforms were also detected in total cell lysates (data not shown). The existence of different isoforms of CIN85 has been proposed previously based on cDNA analysis and recently demonstrated by the use of different mAbs in murine cell lines (40, 41).

CIN85 constitutively interacts with endophilin and, following RTK activation, binds to Cbl via its SH3 domains (36, 37). This binding is controlled by ligand-induced tyrosine phosphorylation of Cbl (31, 36). To investigate whether the FcεRI engagement that induces c-Cbl tyrosine phosphorylation on RBL cells (42) could control CIN85/Cbl interaction, adherent RBL-2H3 cells were incubated overnight with anti-DNP IgE mAb and stimulated (or not) with the multivalent Ag DNP-HSA for the indicated lengths of time. Cell lysates were subjected to immunoprecipitation with a

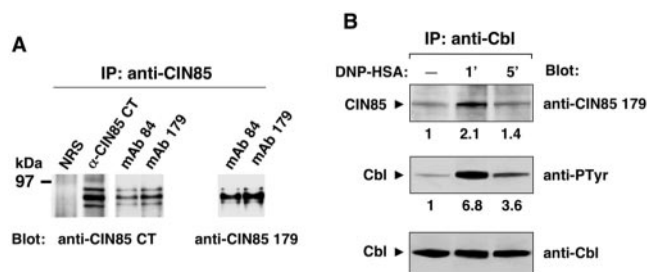


FIGURE 1. Expression of CIN85 and its association with c-Cbl in RBL-2H3. **A**, Cell lysates (3×10^7 /sample) were immunoprecipitated with polyclonal or monoclonal anti-CIN85 Ab, resolved by 7.5% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the indicated Abs. Normal rabbit serum (NRS) was used as negative control. **B**, RBL-2H3 cells (5×10^7 /sample) were loaded with anti-DNP IgE and stimulated for the indicated lengths of time with $1 \mu\text{g}/\text{ml}$ DNP-HSA at 37°C . Cell lysates were immunoprecipitated with anti-Cbl polyclonal Ab, resolved by 7.5% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-CIN85 mAb after stripping with anti-pTyr mAb or anti-Cbl polyclonal Ab. The relative protein amount was referred to the unstimulated sample and indicated at the bottom of the anti-CIN85 and anti-pTyr blots. The position of m.w. markers is indicated. Results shown in **B** are representative of three independent experiments.

rabbit anti-Cbl polyclonal Ab, separated by SDS-PAGE, and analyzed by immunoblotting with anti-CIN85 mAb (Fig. 1B). The relative amount of CIN85 associated with Cbl changed in a time-dependent manner: it was maximal at 1 min after stimulation and decreased after 5 min. The level of c-Cbl/CIN85 association correlates with that of c-Cbl tyrosine phosphorylation, as shown by the anti-pTyr blot. Immunoblotting with anti-Cbl Ab demonstrates that equivalent amounts of c-Cbl were immunoprecipitated at all time points examined.

Similar results were also obtained upon stimulation of RBL cells with an anti-FcεRI α -chain mAb (BC4) (data not shown). Taken together, these findings demonstrate a ligand-inducible association of endogenous CIN85 with c-Cbl in RBL-2H3 cells.

FcεRI down-modulation is enhanced in CIN85-overexpressing cells

It has been demonstrated previously in RBL cells that Ag stimulation induces internalization of engaged FcεRI through clathrin-coated pits (38, 39). However, the mechanisms responsible for receptor endocytosis are poorly understood.

To investigate whether CIN85 can control internalization of Ag-activated FcεRI, we stably transfected RBL-2H3 cells with empty vector or with constructs encoding FLAG-tagged human WT CIN85 or CIN85 3SH3 and PCc mutants containing the three N-terminal SH3 domains or the C-terminal proline-rich and coiled-coil domains, respectively. Both mutants have been shown to interfere with RTK endocytosis and are unable to form the Cbl/CIN85/endophilin trimolecular complex (36).

Cell lysates obtained from the different transfectants were first analyzed by immunoblotting with anti-FLAG mAb to select those with the highest and more comparable levels of CIN85 proteins (data not shown and Fig. 2A, top panel). The immunoblotting with the anti-CIN85 mAb that detects both the overexpressed forms of human CIN85 and the endogenous rat CIN85 isoforms demonstrates a 5-fold increase of CIN85 expression (Fig. 2A, bottom panel).

All the selected transfectants show comparable levels of FcεRI cell surface expression as evaluated by cytofluorimetric analysis (Fig. 2B).

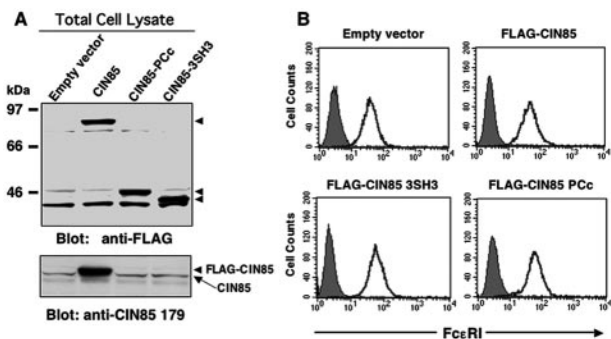


FIGURE 2. CIN85 overexpression and FcεRI surface expression in transfected RBL cells. *A*, Total cell lysates from cells transfected with empty vector or the expression constructs for FLAG-tagged WT CIN85, CIN85-3SH3, and CIN85-PCc mutants were resolved by 10% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-FLAG (upper panel) or anti-CIN85 (lower panel) mAb. The FLAG-tagged, the endogenous CIN85 molecular species, and the m.w. marker position are indicated. *B*, Cells were stained with anti-FcεRI α-chain mAb (BC4) for 30 min at 4°C and with FITC-conjugated GAM (open histograms) or an isotype control Ab (closed histograms) and analyzed by flow cytometry.

To determine whether CIN85 overexpression could affect the ligand-induced FcεRI down-modulation, we stimulated each transfectant with the anti-FcεRI α-chain BC4 mAb for different lengths of time at 37°C and analyzed changes in cell surface FcεRI expression by FACS analysis. Enhanced receptor down-modulation was observed following overexpression of the WT but not the mutant forms of CIN85 at 30 min (Fig. 3) and 60 min (data not shown) after stimulation.

A similar result was obtained when RBL-2H3 clones obtained from the heterogeneous population of CIN85 transfectants were analyzed (data not shown). These findings suggest the involvement of the complex Cbl/CIN85/endophilin in regulating FcεRI surface expression upon stimulation.

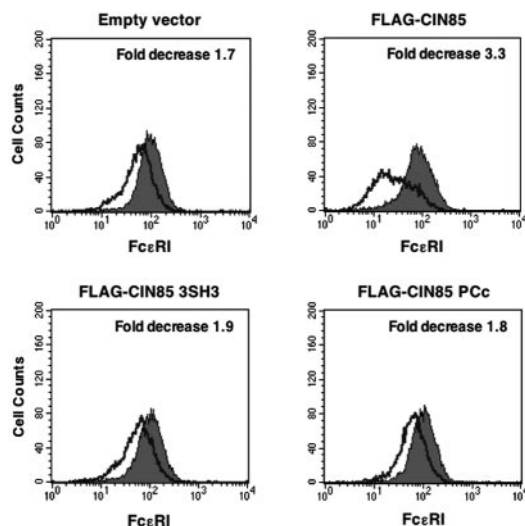


FIGURE 3. CIN85 overexpression enhances FcεRI down-modulation. Different RBL transfectants were labeled with BC4 mAb on ice and, after washing the unbound Ab, stimulated for 30 min at 37°C. FcεRI expression of unstimulated (closed histograms) and stimulated (open histograms) cells was evaluated by flow cytometric analysis performed after the addition of FITC-conjugate GAM Ab. Fold decrease was calculated as ratio of the mean fluorescence intensity of unstimulated vs stimulated samples. Results shown are representative of three independent experiments.

To further investigate whether CIN85 could directly control receptor endocytosis, we decided to study our transfectants for their ability to modulate ligand-induced FcεRI internalization by using immunofluorescence and microscopic analysis. Cells were incubated with BC4 mAb for 1 h at 4°C, left on ice or stimulated for the indicated lengths of time at 37°C to allow receptor/mAb complex endocytosis, and then fixed and permeabilized. The engaged receptors were then visualized using a GAM IgG-FITC (Fig. 4). In all transfectants treated at 4°C, we observed a diffuse distribution of the receptors over the whole membrane. Incubation at 37°C for 10 min already resulted in a rapid receptor redistribution with some clustered zones visible on the cell surface. After 20 and particularly 30 min of stimulation, a different redistribution of the receptors was evident with a dash-like pattern on the plasma membrane of the empty vector and CIN85 mutant transfectants and dots on the plasma membrane of the WT CIN85 transfectants (Fig. 4, *A* and *B*). After 40 min of stimulation, the receptors were concentrated in intracellular peripheral spots in the cells overexpressing WT CIN85, whereas they were still localized in dots on the surface of cells transfected with the empty vector (Fig. 4*B*).

At 1 h of stimulation, the receptors localized in punctate intracellular endocytic dots at the cell periphery and, at a longer time (2 h), were found concentrated in larger spots in the central perinuclear area of the cell in all the transfectants analyzed (Fig. 4).

All together, our results strongly suggest a role for CIN85 in controlling the ligand-induced FcεRI internalization process.

CIN85 overexpression accelerates FcεRI entry into the early endosomes and the lysosomal compartment

To determine whether the accelerated ligand-induced FcεRI internalization promoted by CIN85 overexpression could result in a more rapid sorting of the internalized receptors in the endosomal compartments, we performed a confocal microscopic study.

To this aim, cells overexpressing empty vector or WT CIN85 were treated with BC4 mAb as above and with Texas Red-conjugated Tf as a marker of early endosomes or with Lyso-Tracker Red as a marker of more acidic compartments (late endosomes and lysosomes) and stained with GAM IgG-FITC (green) to visualize FcεRI receptors along the endocytic pathway.

After 1 h in the presence of BC4 at 4°C (data not shown) and after an additional 10 min of stimulation at 37°C, the receptors were distributed on the plasma membrane and did not colocalize with Tf both in control empty vector and WT CIN85-transfected cells (Fig. 5*A*). In agreement with the results shown in Fig. 4, after 30 min of stimulation, the receptor was distributed mainly in dots present on the cell surface and intracellularly in the transfectants overexpressing WT CIN85, while it was still visualized in dashed areas in empty vector transfectant cells. Colocalization with Tf-Texas Red (yellow spots) was observed after 40 min of stimulation, and became more evident at longer times (1 h). Quantitative analysis indicates that after 40 min of BC4 stimulation, ~31% of the FcεRI receptor punctate signal colocalizes with Tf signal in WT CIN85 overexpressing cells as compared with 9.4% in control cells (Fig. 5*B*).

Thus, overexpression of CIN85 induces a more rapid FcεRI sorting into early endosomes following receptor stimulation.

To follow the fate of the internalized receptors, RBL cells transfected with empty vector or WT CIN85 were stimulated for longer time periods with BC4 mAb. Following 60 min of stimulation, we

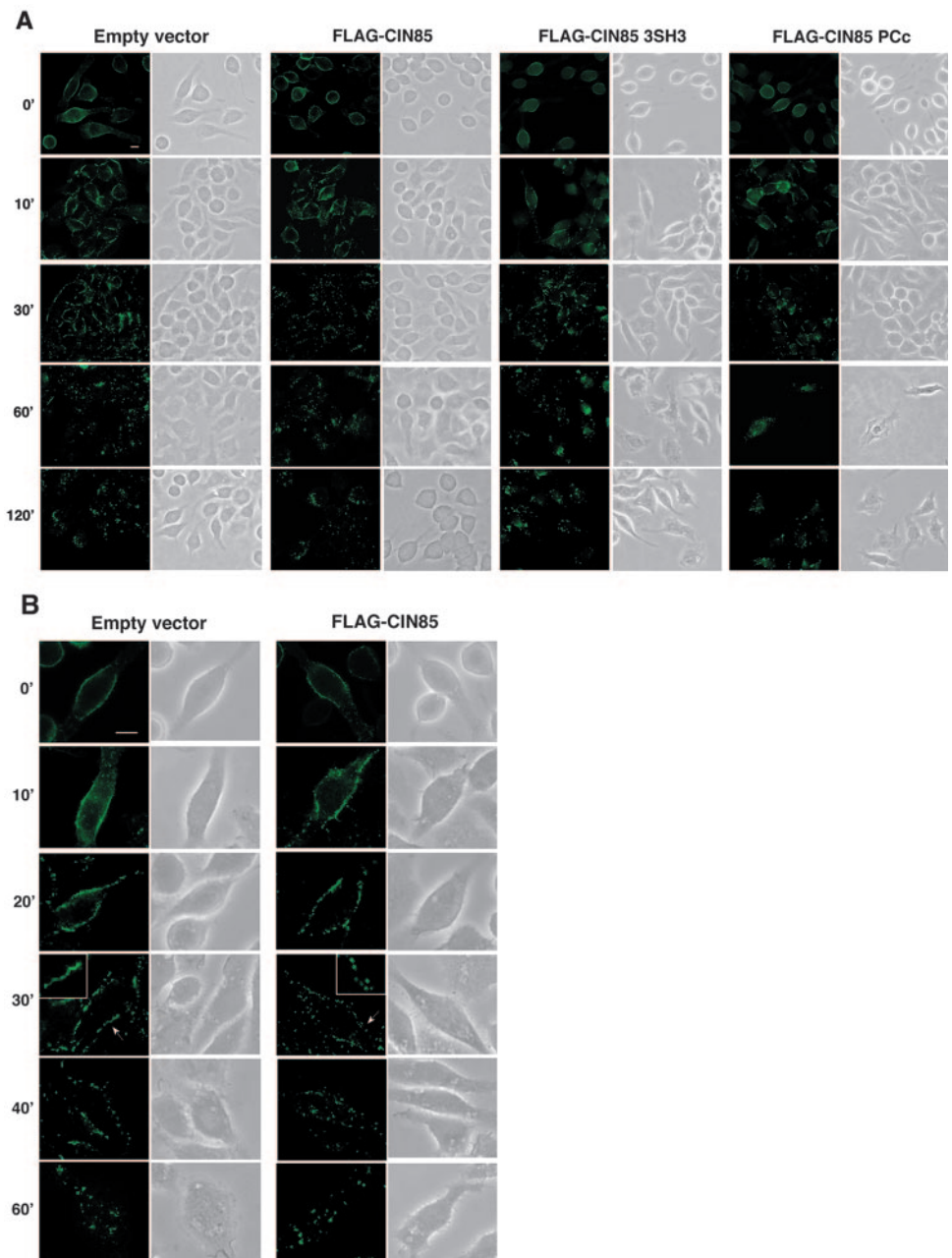


FIGURE 4. CIN85 overexpression accelerates FcεRI endocytosis. The transfected RBL cells were incubated with BC4 mAb for 1 h at 4°C, left on ice or allowed to internalize after shifting the temperature to 37°C, fixed, and permeabilized. The receptor/mAb complex was stained with a GAM IgG-FITC (green) and then visualized by microscopic analysis. The differential interference contrast images of labeled cells are also shown. *B*, Representative images from >30 cells examined are shown, and the *insets* show enlargements of the areas marked by arrowheads. Bars: 10 μm. Results shown are representative of three independent experiments.

observed little or no colocalization of the receptors with Lyso-Tracker Red-positive acidic compartments. After 90 min, a partial colocalization of the internalized receptors with Lyso-Tracker was detected (yellow spots) and became more evident after 2 h of stimulation. Notably, after 90 min of stimulation, the colocalization of the internalized receptors with Lyso-Tracker in WT CIN85-overexpressing cells was greater than in control cells (55 vs 28% on control cells) (Fig. 5*B*).

These results strongly suggest that overexpression of WT CIN85 induces a more rapid sorting of FcεRI from early endosomes to late endosomes and lysosomes following receptor stimulation.

Overexpression of the mutant forms of CIN85 did not alter the kinetics of receptor sorting along the endocytic pathway, as compared with that observed in empty vector transfectants (Fig. 5*C*).

Taken together, our results indicate that overexpression of CIN85 accelerates FcεRI internalization and sorting to early endosomes. Moreover, CIN85 overexpression affects the late steps of

FcεRI endocytosis, inducing a more rapid targeting of activated receptor complexes to lysosomes for degradation.

CIN85 overexpression augments the ligand-induced decrease of FcεRI β and γ subunit expression levels

To compare the FcεRI subunit expression level in the different transfectants, cells were left untreated or stimulated with BC4 mAb for the indicated lengths of time, and the expression of β- and γ-chains was evaluated by Western blotting of whole cell lysates (Fig. 6). The time-dependent decrease of both β and γ protein level observed upon receptor engagement suggests that during the endocytosis process FcεRI is internalized and transported as an intact complex to the lysosomal compartment for degradation. As a consequence of WT CIN85 overexpression, the ligand-induced decrease of β and γ protein level was greater than in cells expressing the empty vector (Fig. 6, *top* and *middle panels*) or the mutant forms of CIN85 (data not shown).

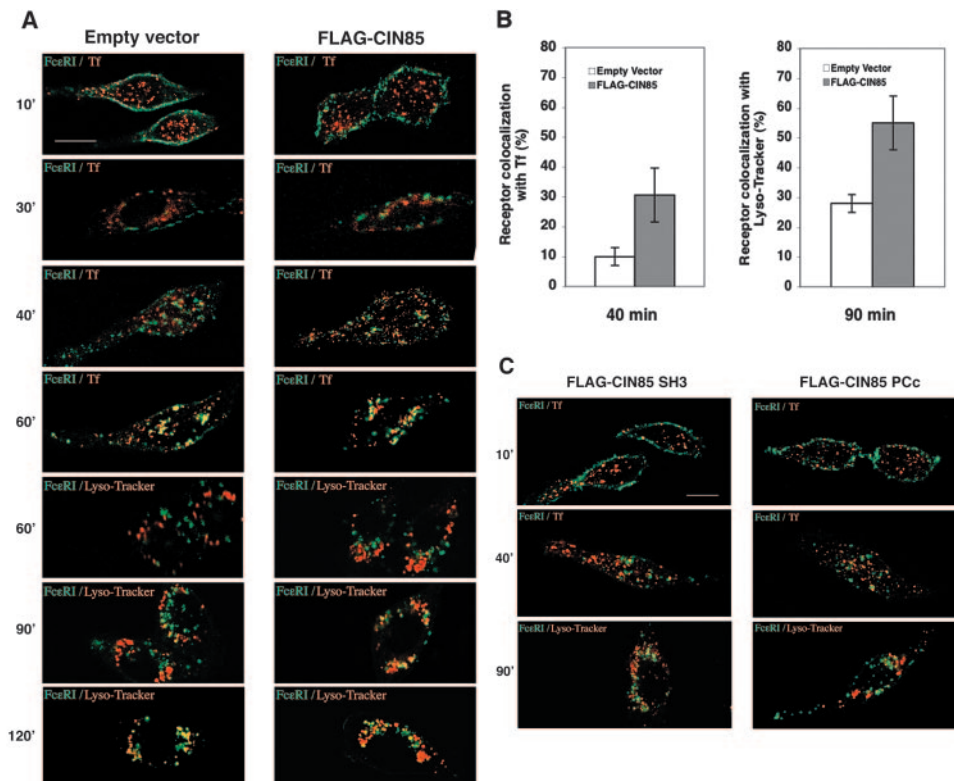


FIGURE 5. Confocal analysis of FcεRI distribution during early and late steps of endocytosis. Cells overexpressing empty vector, FLAG-CIN85 (A and B), FLAG-CIN85 3SH3, or FLAG-CIN85 PCc (C) were incubated with BC4 mAb as previously described, stimulated for the indicated lengths of time at 37°C, stained with Texas Red-conjugated Tf to identify early endosomes or with Lyso-Tracker Red to identify late endosomes and lysosomes, doubly stained with GAM IgG-FITC to visualize FcεRI complex along the endocytic pathway, and analyzed by confocal microscopy. The results of the analysis are shown as a color merge of a single confocal plane (A and C). Yellow indicates coincident green and red labels. Representative images from >30 cells examined for each time point are shown. Bar: 10 μm. Results shown are representative of three independent experiments. B, Quantitative analysis of the results shown in A indicates that after 40 min of stimulation $31 \pm 9\%$ of internalized receptor signals colocalized with Tf in FLAG-CIN85-overexpressing cells as compared with $9.4 \pm 3\%$ on control cells (empty vector). After 90 min of stimulation $55 \pm 8.7\%$ of internalized receptor signals colocalized with Lyso-Tracker in FLAG-CIN85-overexpressing cells as compared with $28 \pm 4\%$ on control cells. The error bars represent the SD. The statistical analysis demonstrates a significant difference ($p < 0.002$ both at 40 and 90 min).

The membranes were reprobed for actin to verify an equal loading of proteins (Fig. 6, bottom panels). The above results suggest that CIN85 accelerates Ag-induced FcεRI sorting to the lysosome for degradation.

CIN85 negatively regulates mast cell degranulation

FcεRI-mediated activation of mast cells results in the release of preformed mediators from cytoplasmic granules. Therefore, to examine the effect of CIN85 overexpression on RBL cell function, we evaluated the extent of Ag-induced degranulation by assaying the release of the granule-associated enzyme β-hexosaminidase (Fig. 7). We first analyzed the response of the different transfectants following 1 h of stimulation to increasing doses of Ag. Cells overexpressing WT CIN85 were impaired in their ability to degranulate showing a very low response even at the highest dose of Ag, when compared with cells transfected with empty vector or CIN85 mutants (Fig. 7A). The time course analysis (Fig. 7B) confirmed a dramatic decrease (> 80%) of mast cell degranulation at all time points tested in WT CIN85 transfectant cells. A similar decrease of Ag-induced release was seen when RBL-2H3 clones obtained from the heterogeneous population of WT CIN85 transfectants were analyzed (data not shown). These effects were dependent on receptor engagement because all the transfectants exhibited a similar response to 1 μM thapsigargin, a potent cell-permeable intracellular calcium releaser (Fig. 7C). This latter result also demonstrates that cells overexpressing WT CIN85 are

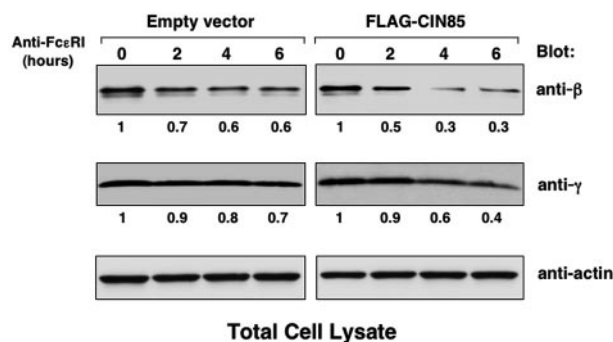


FIGURE 6. CIN85 regulates the expression level of FcεRI β and γ subunits. RBL cells transfected with empty vector or WT CIN85 were incubated with BC4 mAb for 30 min on ice. After extensive washing, cells were stimulated at 37°C for the indicated lengths of time. Stimulation was blocked by washing twice with cold PBS, and cells were directly lysed with hot Laemmli buffer. Lysates were resolved by 10–20% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-β (top panels), anti-γ (middle panels), or anti-actin (bottom panels) Ab. The relative protein amount, normalized with the band intensity of actin, was referred to the unstimulated sample and indicated at the bottom of the figures. Results shown are representative of three independent experiments.

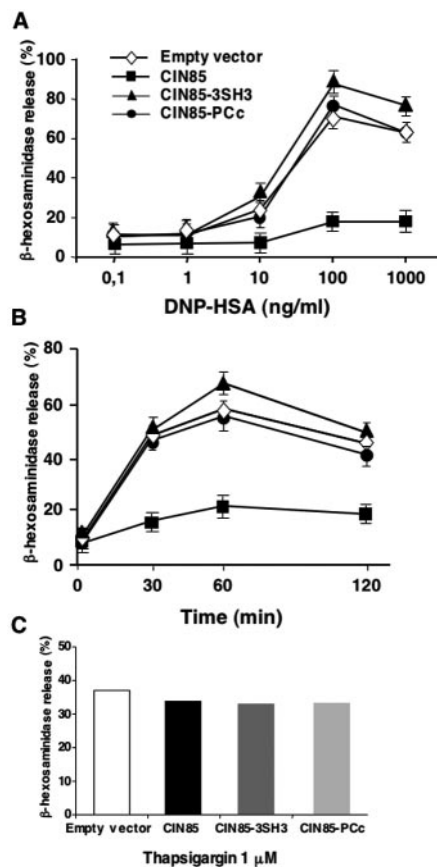


FIGURE 7. CIN85 overexpression inhibits ligand-induced degranulation. RBL cells transfected with empty vector or with WT CIN85 were loaded with anti-DNP IgE and stimulated with the indicated concentrations of DNP-HSA for 1 h (A), or with 100 ng/ml DNP-HSA for the indicated time periods (B), or with 1 μ M thapsigargin for 1 h (C). The extent of degranulation was determined measuring the release of the granule-associated enzyme β -hexosaminidase. Ag-induced β -hexosaminidase release was expressed as a percentage of the maximal release induced by ionomycin plus PMA. Ionomycin plus PMA-induced release among the different transfected cells was comparable. Data are expressed as the mean $\pm$ SD obtained from five independent experiments.

still capable of degranulating indicating that their intracellular calcium stores are not affected.

All together, our results indicate that CIN85 overexpression negatively regulates FcεRI-mediated mast cell functions.

Discussion

The identification of the mechanisms involved in the down-modulation of FcεRI cell surface expression may provide new insights into how mast cells may be manipulated to achieve therapeutic ends in the treatment of allergic diseases.

Work by many research groups has revealed that the ubiquitin ligase Cbl and the family of CIN85 proteins play a central role in the ligand-dependent down-regulation of several RTKs (20, 30, 34). Moreover, recent findings have suggested a critical role for Cbl in the ubiquitin-dependent down-regulation of ITAM-containing immune receptors, including the FcεRI (29, 43), but it is still largely unknown whether CIN85 could operate in concert with Cbl to coordinate the clathrin-mediated internalization of engaged FcεRI.

In this study, we have demonstrated that overexpression of WT CIN85 results in enhanced down-modulation of the FcεRI upon Ag stimulation of a rat mast cell line. Furthermore, we provide

evidence that FcεRI triggering results in an increased association of CIN85 with tyrosine phosphorylated c-Cbl pool (Fig. 1B). Based on previous observations suggesting that c-Cbl is recruited to the FcεRI upon engagement (42), it is likely that the CIN85/Cbl association is a critical event for CIN85 recruitment to the engaged receptor complex. However, the mechanism responsible for this recruitment remains to be identified.

Our previous findings demonstrate a role for c-Cbl in ligand-induced receptor ubiquitination and down-regulation (29). In addition, we have found that overexpression of Cbl in RBL cells accelerates ligand-induced receptor internalization (data not shown).

All together, our results support the involvement of CIN85 and a dual role for c-Cbl as ubiquitin ligase and as adaptor protein in FcεRI endocytosis.

We have further investigated the role of CIN85 in controlling the intracellular distribution and the fate of internalized receptor complexes. Previous studies have suggested that engaged FcεRI is endocytosed through clathrin-coated pits and transported to the endosomal system for degradation (38, 39). In agreement with these studies, we found that RBL treatment with hypertonic medium that is known to perturb clathrin-coated pit formation (44) dramatically impairs receptor endocytosis (data not shown), and our confocal microscopic analysis further supports the notion that the receptor is transported to endocytic compartments upon stimulation (Fig. 5A). Furthermore, we demonstrate that overexpression of WT CIN85 accelerates both FcεRI entry into early endosome and receptor trafficking to a lysosomal compartment.

The molecular mechanism by which CIN85 controls the trafficking of internalized FcεRI remains to be further investigated.

The scaffolding properties of CIN85 allow a rapid exchange of CIN85 binding partners, depending on their local concentration, cellular location, and/or posttranslational modifications (35, 45). All these different spatial and temporal interactions orchestrated by CIN85 could in turn control endosomal sorting and targeting of activated receptor complexes to lysosomes, a process essential for receptor degradation and attenuation of intracellular signaling.

We could not observe attenuation of receptor internalization when mutated forms of CIN85 unable to interact with Cbl (FLAG-CIN85 PCc) or endophilin (FLAG-CIN85 3SH3) were overexpressed (Fig. 5C). The lack of a dominant negative effect does not depend on their expression because both mutants were expressed at levels comparable to that of WT CIN85 (Fig. 2A, top panel). We tend to rule out the possibility that in the presence of interfering forms of CIN85, the FcεRI down-modulation occurs through a clathrin-independent mechanism because treatment with hypertonic medium similarly affects ligand-induced receptor endocytosis in all the transfectants examined (data not shown). A more likely explanation for the lack of inhibition is that adaptor molecules other than CIN85 may control the entry of the receptor and its sorting. In agreement with this latter possibility, we have evidence that RBL cells also express CD2AP (R. Molfetta, unpublished observation), another CIN85 family member that controls TCR down-modulation in activated T cells (46).

Thus, redundant mechanisms of receptor entry may exist in RBL cells, and their contribution to clathrin-mediated endocytosis needs further investigation. Moreover, it is also conceivable that mono-ubiquitination itself can serve as an internalization signal (24–27). In this regard, we have preliminary results indicating that both β and γ FcεRI chains are preferentially mono- and multiubiquitinated by Cbl upon receptor engagement (R. Molfetta, unpublished data).

FcεRI is composed of multiple subunits that undergo coordinate turnover upon IgE binding (47). To investigate whether following

receptor stimulation the engaged FcεRI is transported as an intact tetrameric complex to the lysosome for degradation, we have analyzed whether CIN85 overexpression may affect the expression levels of the β and γ receptor subunits because the confocal microscopic analysis we performed allowed us to follow only the fate of the α -chain. The result shown in Fig. 6 revealed the ability of FcεRI engagement to decrease the expression level of both β - and γ -chains, suggesting that the FcεRI subunits remain associated and are processed as an intact complex during endocytosis and intracellular trafficking. In addition, we have also provided evidence supporting a role for CIN85 in accelerating FcεRI tetrameric complex sorting and degradation in that overexpression of CIN85 resulted in a greater reduction of the level of both β and γ FcεRI subunits.

Interestingly, recent findings have shown that proteasome activity is required for sorting of RTKs to inner membranes of multivesicular bodies and subsequent receptor degradation (48), and our previous observations indicate that treatment of RBL cells with proteasome inhibitors blocks ligand-induced degradation of FcεRI (29). All together, these results suggest that the proteasome function may be critical for proper targeting of activated FcεRI complexes to lysosomes.

Internalization of stimulated receptors and lysosomal degradation of the ITAM-containing receptor subunits appears to be an important regulatory mechanism to ensure a timely limited activity of engaged FcεRI. This conclusion is supported by the results presented in Fig. 7: in cells overexpressing WT CIN85, a dramatic decrease of β -hexosaminidase release was detected after FcεRI stimulation, suggesting that the more rapid internalization of activated receptors thereby perturbing the intracellular signaling contributes to limit mast cell functional response.

On the other hand, we cannot rule out that receptor endocytosis is the only mechanism by which CIN85 may control cell function. Indeed, more recent evidence suggest that the activity of other substrates involved in the signal propagation could be possibly regulated by CIN85 (49–51) and additional work will be devoted to their identification in RBL cells. Our preliminary results indicate that the tyrosine phosphorylation of both FcεRI β and γ subunits is not affected by CIN85 overexpression (data not shown), suggesting a normal activity of the Src family PTK, Lyn, mainly involved in receptor phosphorylation.

In conclusion, our data strongly favor a model in which after receptor engagement the Cbl/CIN85 complex is rapidly recruited to the cell membrane where it can drive internalization of engaged FcεRI and subsequent sorting into endocytic compartments, a process required for receptor degradation.

All together our data support a role for CIN85 in controlling a rapid clearance of engaged receptor complexes from the cell surface, thus contributing to the inhibition of the intracellular signaling initiated by IgE receptors. It remains to be investigated whether a similar mechanism could also operate to regulate the FcεRI cell surface expression on human mast cells and APCs and whether CIN85 holds therapeutic promise for disease intervention.

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Disclosures

The authors have no financial conflict of interest.

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