Activation of Syk Tyrosine Kinase Is Required for c-Cbl-mediated Ubiquitination of FceRI and Syk in RBL Cells*

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The activation of protein-tyrosine kinases (PTKs)1 is an essential event in the transduction of intracellular signals from immune receptors (IR), including the T and B cell antigen receptors (TCR and BCR, respectively), the high affinity receptor for IgE (FcεRI) and γ subunits ubiquitination by an as yet undefined mechanism. Here we show that, upon FcεRI engagement on RBL-2H3 cells Syk undergoes ubiquitination and Syk kinase activity is required for its own ubiquitination and that of FcεRI β and γ chains. This requirement was demonstrated by overexpression of Syk wild-type or its kinase-dead mutant in RBL cells or using an Syk-deficient RBL-derived cell line transfected with wild-type or a kinase inactive form of Syk. We also identify c-Cbl as the E3 ligase responsible for both Syk and receptor ubiquitination. Furthermore, we demonstrate that Syk controls tyrosine phosphorylation of Syk-associated Cbl induced after receptor engagement. These data suggest a mutual regulation between Syk and Cbl activities. Finally, we show that a selective inhibitor of proteasome degradation induces persistence of tyrosine-phosphorylated receptor complexes, of activated Syk, and of FcεRI-triggered degranulation. Our results provide a molecular mechanism for down-regulation of engaged receptor complexes by targeting ubiquitinated FcεRI and activated Syk to the proteasome for degradation.

Engagement of the high affinity receptor for IgE (FcεRI) on mast cells and basophils results in FcεRI β and γ subunits ubiquitination by an as yet undefined mechanism. Here we show that, upon FcεRI engagement on RBL-2H3 cells Syk undergoes ubiquitination and Syk kinase activity is required for its own ubiquitination and that of FcεRI β and γ chains. This requirement was demonstrated by overexpression of Syk wild-type or its kinase-dead mutant in RBL cells or using an Syk-deficient RBL-derived cell line transfected with wild-type or a kinase inactive form of Syk. We also identify c-Cbl as the E3 ligase responsible for both Syk and receptor ubiquitination. Furthermore, we demonstrate that Syk controls tyrosine phosphorylation of Syk-associated Cbl induced after receptor engagement. These data suggest a mutual regulation between Syk and Cbl activities. Finally, we show that a selective inhibitor of proteasome degradation induces persistence of tyrosine-phosphorylated receptor complexes, of activated Syk, and of FcεRI-triggered degranulation. Our results provide a molecular mechanism for down-regulation of engaged receptor complexes by targeting ubiquitinated FcεRI and activated Syk to the proteasome for degradation.

The activation of protein-tyrosine kinases (PTKs)1 is an essential event in the transduction of intracellular signals from immune receptors (IR), including the T and B cell antigen receptors (TCR and BCR, respectively), the high affinity receptor for IgE (FcεRI), and the widely distributed receptors for IgG.

These IRs contain multiple subunits: some, distinct for each receptor, are used for ligand binding, whereas others share conserved cytoplasmic motifs that are critical for the process of cell activation (immune receptor tyrosine-based activation motif, ITAM) (1–6). The IRs lack intrinsic kinase activity; however, within seconds of their engagement, PTKs are activated leading to phosphorylation of various substrates, including IR subunits (7–12). ITAM phosphorylation by the Src family PTKs provides docking sites for the tandem pair of Src homology 2 (SH2) domains of a second class of PTKs belonging to the Syk family (3–6). This family includes only two members: Syk, which is present in most hematopoietic cells and ZAP-70, which is exclusively expressed in T and NK cells. As documented by several studies, the expression of Syk and ZAP-70 is essential for lymphocyte development and signal transduction via IRs (13–16). The association of phosphorylated ITAMs with SH2 domains of Syk family PTKs leads to the activation of Syk and ZAP-70 mainly by autophosphorylation (17), thus allowing the propagation of IR signaling.

We and others have demonstrated that Syk and ZAP-70 as well as IR subunits are subjected to an additional covalent modification following IR engagement in that they become modified by ubiquitin (Ub) (18–22). Moreover, we have also provided evidence suggesting a direct correlation between IR-induced Syk and ZAP-70 ubiquitination and degradation (21).

Ubiquitination, which consists in the covalent modification of a protein by Ub or Ub chains, involves several types of enzymes (23–25). The ubiquitin-protein isopeptide ligases (E3s) provide specificity to the Ub system: they are responsible for substrate recognition and for promoting Ub ligation to the target protein. A recently identified class of E3 ligases is characterized by the presence of a RING finger domain that serves as the binding site for specific E2 Ub carrier proteins (26). The protooncogene c-Cbl belongs to this class of E3 ubiquitin ligases: it can recognize specific substrates due to the presence of multiple protein recognition domains, and recruits a specific E2 enzyme via its RING finger domain (27, 28). Thus, Cbl can associate with and negatively regulate a number of receptor and non-receptor PTKs targeting them for ubiquitination and degradation (22, 29–34).

Recent evidence indicates that c-Cbl promotes ubiquitination of cytoplasmic kinases (22, 34) and of IR subunits, as demonstrated by the Cbl-mediated control of TCR ζ chain ubiquitination in a 293T cell system (35). However, the mechanism responsible for activating Cbl ligase activity after IR engagement is largely unknown. Thus, the aim of our study was to investigate the molecular mechanism regulating the Cbl-dependent ubiquitination of Syk family PTKs and receptor subunits in...
duced after IR engagement.

As an in vivo system we choose a rat basophilic leukemia cell line (RBL-2H3) that constitutively expresses the high affinity receptor for IgE (FcεRI). This is a tetrameric complex composed of a ligand-binding α chain, a β chain, and a homodimer of γ chains (36). The β chain plays an essential role in setting the level of cellular response to IgE and antigen, through its capacity to amplify both cell surface FceRI expression (37) and signaling (38). The FcεRI γ chain is also part of other IRs (39–41) and is homologous to the TCR γ subunit, which is critical for signal transduction (4). FcεRI engagement leads to the immediate tyrosine phosphorylation of β and γ subunits through the tyrosine kinase of the Src family, Lyn, allowing Syk binding to the phosphorylated ITAM-containing subunits. The consequent activation of Syk is essential for all known FcεRI-mediated responses, including secretion of allergic mediators and induction of cytokine gene transcription (42–45).

In the present study we demonstrate that Syk kinase activity is an indispensable requisite for Cbl-dependent ubiquitination of both FcεRI and Syk induced after IR engagement.

**EXPERIMENTAL PROCEDURES**

**Chemical Reagents and Antibodies**—All chemicals and drugs, unless otherwise mentioned, were obtained from Sigma (Sigma-Aldrich, Italy). Anti-FcεRI α subunit Ab (BC4) has been previously described (46); anti-FcεRI β subunit Ab JRK (47) was kindly provided by Dr. J.-P. Kinet (Beth Israel Deaconess Medical Center, Boston, MA). Goat anti-mouse IgG F(ab')

2 fragment (GAM) was purchased from Cappel Laboratories (ICN Biomedicals, Opera, Milan, Italy). Anti-phosphotyrosine (anti-PY) 4G10 mAb and anti-FcεRI γ chain polyclonal Ab were purchased from UBI (Lake Placid, NY); rabbit polyclonal anti-Syk (N-19), anti-Chi C15, and anti-α-chain Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); dinitrophenyl-specific monoclonal mouse IgE was purchased from Sigma; rat anti-mouse IgE mAb was purchased from Pharmingen (San Diego, CA). The anti-Ub mAb FK2 (PW8810) and the proteasome inhibitors were purchased from Affiniti Research Products Limited (Mamhead, Exeter, United Kingdom).

**Cell Cultures and Stimulation**—The rat basophilic leukemia RBL-2H3 cells were cultured in monolayers as previously described (19). The B2 sub-clone derived by a Syk-negative variant of RBL-2H3 cells was used in this study. Adherent RBL-2H3 cells were cultured in monolayers as previously described (48).

In the experiments where FcεRI immunoprecipitation was performed, RBL cells were stimulated with IgE plus Ag (Figs. 1, 3, and 7). Briefly, adherent cells were incubated with 0.5 μg/ml monomeric monoclonal anti-DNP mouse IgE for 12 h at 37 °C. The cells were then harvested at 10^7/ml in prewarmed EMEM supplemented with 10% FCS for 2 h in EMEM containing 0.1% bovine serum albumin and 10 mM Hepes pH 7.4, before stimulation. The cell monolayers were then washed twice with Tyrode’s buffer (10 mM Hepes buffer 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% bovine serum albumin) and stimulated with DNP-HSA at concentrations from 0.1 to 1000 ng/ml or with 1 μM thapsigargin for the indicated lengths of time in the same buffer.

The enzymatic activity of β-hexosaminidase in supernatants and cell pellets was solubilized with 0.5% Triton X-100 in Tyrode’s buffer and processed for β-hexosaminidase activity. RBL cells were infected with three plaque-forming units/cell of the indicated viruses.

**RESULTS**

**FcεRI β and γ Subunits and the Tyrosine Kinase Syk Are Rapidly Ubiquitinated after Receptor Engagement on RBL Cells**—We first wanted to examine whether, in addition to the ligand-induced FcεRI β and γ ubiquitination, the tyrosine kinase Syk is subjected to modification by Ub upon receptor engagement. Adherent RBL-2H3 cells were incubated overnight with anti-DNP IgE mAb and stimulated (or not) with the multivalent DNP-lysine acetate (DNP) to detect Ub-lysine acetate (DNP).

In panel A confirm the presence of β and γ species modified by addition of Ub molecules (indicated by parentheses). A better characterization of all the modified receptor species was reported in our previous study (19). Additional bands were also detected by the anti-Ub blot; they likely represent ubiquitinated proteins coprecipitating with the receptor subunits.

The time course experiment presented in panel B shows that receptor ubiquitination occurs as an early event of the signaling cascade, paralleling the induction of β and γ tyrosine phosphorylation. In agreement with our previous study (50), we show that the level of receptor subunits decreases after receptor engagement and with the time of stimulation (panel B, lower).

This may be due to cellular redistribution of engaged receptors to a relatively Triton-insoluble compartment and/or to a degradation process promoted by receptor engagement.
Immunoblotting of electrophoresed and transferred Syk precipitates demonstrates that, besides the main form migrating around 72,000 daltons, additional bands of higher molecular mass reacting with anti-Syk are rapidly induced upon receptor aggregation (Fig. 1C). Most of these induced forms (indicated by asterisks) are tyrosine-phosphorylated and/or -ubiquitinated as shown by the anti-PY and the anti-Ub blots, respectively. The same immunoprecipitates blotted with an isotype-matched control mAb do not show any reactivity in the 72,000-dalton or higher molecular mass range (data not shown). Similar results were obtained when RBL cells were stimulated with lower levels of multivalent antigen or with an anti-FcεRI chain mAb (BC4) (data not shown). Taken together these findings indicate that FcεRI engagement on RBL cells promotes both receptor and Syk ubiquitination.

**Ligand-induced Ubiquitination of Syk Requires Syk Kinase Activity**—The results presented in Fig. 1C suggest that the activation of Syk may be required to promote Syk ubiquitination.

To directly test this hypothesis, RBL cells were infected with recombinant vaccinia viruses encoding either porcine wild-type Syk or a kinase-inactive mutant form of Syk (Syk-KI). After infection, cells were left untreated or incubated with the anti-FcεRI α chain mAb and then stimulated with a goat anti-mouse IgG F(ab')2 fragment (GAM). Anti-Syk immunoprecipitates were resolved on SDS-PAGE and immunoblotted with the same anti-Syk Ab used for immunoprecipitation or anti-Ub mAb (Fig. 2A). Ligand-induced ubiquitinated forms of Syk accumulated only when the wild-type but not the kinase inactive form of Syk was overexpressed indicating that Syk ubiquitination depends on its own kinase activity. Furthermore, we found that a mutation in the C-terminal SH2 domain of Syk affecting phosphotyrosine recognition (48) significantly decreases the ligand-induced ubiquitination of Syk (data not shown). Comparable levels of Syk and Syk-KI were overexpressed as detected by immunoblotting the total cell lysates with an anti-Syk polyclonal Ab reacting with both rat and porcine Syk (B). Taken together, our results indicate that the ligand-induced ubiquitination of Syk depends on its membrane recruitment and activation.

**The Expression of Active Syk Kinase Is Required for FcεRIβ Subunit Ubiquitination**—We then asked whether the Syk kinase activity was also necessary for FcεRI Ag-induced ubiquitination. To address this question we employed a Syk-deficient RBL-2H3-derived cell line (B2) and clones obtained by stable transfection of the B2 cell line with wild-type or a kinase-dead mutant form of rat Syk (KI). The clones were stimulated and lysed as in Fig. 1A, and the effect of Ag stimulation on the FcεRI β chain tyrosine phosphorylation and ubiquitination was evaluated (Fig. 3). As previously reported (48), the expression of Syk is not required for β chain phosphorylation that was similar in all the clones. The β chain was also extensively ubiquitinated upon receptor engagement when WT Syk was present. In contrast, there was no detectable β ubiquitination in the absence of Syk or in the presence of its kinase-dead mutant. Although we observed a decrease in the level of β chain after receptor engagement (see also Fig. 1), the amounts of immunoprecipitated β chain were comparable in each clone (lower panel). Thus, the kinase activity of Syk is required not only for its own ligand-induced ubiquitination but also for receptor ubiquitination.

**c-Cbl Is the Ubiquitin Ligase for Syk**—It has become increasingly evident that c-Cbl, acting as an E3 ligase, controls the ligand-induced ubiquitination of several receptor and non-receptor PTKs (22, 29–34). In RBL cells c-Cbl constitutively associates with Syk and becomes tyrosine-phosphorylated upon receptor engagement (49). We therefore tested whether Cbl could act as the ubiquitin ligase for Syk. For this purpose RBL cells were infected with recombinant vaccinia viruses encoding porcine Syk in combination with human HA-tagged wild-type Cbl, a form of Cbl containing a 17-amino acid deletion in the RING finger domain (70Z) or the empty vector (WR) (Fig. 4).

Cell lysates from unstimulated or anti-FcεRI α chain-stimulated cells were immunoprecipitated with anti-Syk polyclonal Ab, and the immunoprecipitates were resolved on SDS-PAGE and immunoblotted with anti-Syk Ab or anti-Ub mAb (A). Overexpression of wild-type Cbl leads to an accumulation of ligand-induced ubiquitinated forms of Syk, whereas the RING finger mutant of Cbl has a dominant negative effect on Syk ubiquitination. We show that the 70Z mutant of Cbl is still able to interact with Syk (lower panel), as previously reported (51). Comparable levels of Syk and Cbl were overexpressed, as revealed by the anti-Syk and anti-HA blots (B). Thus, both wild-type Cbl and 70Z-Cbl bind to the tyrosine kinase Syk but only Cbl with the intact RING finger can transfer ubiquitin to the substrate.

**c-Cbl Is the Ubiquitin Ligase for FcεRI β and γ Subunits**—It has been demonstrated that SH2-dependent Syk is recruited by the cell membrane and is rapidly activated after FcεRI engage-
Syk and Cbl Control Ubiquitination in RBL Cells

Fig. 2. Syk kinase activity is required for ligand-induced ubiquitination of Syk. RBL-2H3 cells were infected with recombinant vaccinia viruses encoding wild-type Syk (Syk), a kinase-inactive form of Syk (SYK KI), or empty vector (WR). Cells were left untreated (−) or incubated with DNP-HSA for 30 min at 4 °C and then stimulated with GAM for 1 min at 37 °C. A, cell lysates were immunoprecipitated with anti-Syk Ab, resolved on SDS-PAGE, transferred to nitrocellulose, and sequentially immunoblotted, after stripping, with anti-Ub mAb (right panel) and anti-Syk polyclonal Ab (left panel). The positions of molecular weight markers are indicated. These results represent one out of three independent experiments.

We therefore investigated whether, in addition to being the ligase for Syk, Cbl could also control FcεRI ubiquitination. For this goal we analyzed the level of receptor ubiquitination induced in RBL cells after overexpression of recombinant viruses encoding wild-type Cbl or its 70Z mutant (Fig. 5). Upon infection, cell lysates from unstimulated or Ag-stimulated samples were immunoprecipitated with anti-IgE polyclonal antibody, resolved by SDS-PAGE, and immunoblotted with anti-β mAbs, anti-γ polyclonal Ab, or anti-Ub mAb, as indicated (Fig. 5A). Upon overexpression of Cbl, we observed an increase of ubiquitinated β and γ forms, suggesting the involvement of Cbl in the control of receptor ubiquitination. This last modification requires the intact RING finger domain of Cbl, because a dramatic decrease of ubiquitinated receptor species in response to FcεRI engagement was observed when the 70Z-Cbl was overexpressed. Comparable levels of Cbl proteins were overexpressed, as revealed by the anti-HA blot (Fig. 5B). Taken together (Figs. 4 and 5), our results demonstrate the role of c-Cbl in promoting both FcεRI and Syk ubiquitination.

The Tyrosine Phosphorylation of Cbl Associated with Syk Is Controlled by Syk—In addition to a physical association with the IR-activated PTKs, Cbl also serves as their substrate (49, 52). It has been previously reported that both Lyn and Syk control Cbl tyrosine phosphorylation induced after FcεRI engagement on RBL cells (49, 53). Furthermore, it has been recently demonstrated that in vitro induced tyrosine phosphorylation of Cbl is required for its own ligase activity (33). Because we have demonstrated the importance of Syk activity for promoting Syk and receptor ubiquitination, we investigated whether Syk controls, in vivo, the tyrosine phosphorylation of the Syk-associated Cbl pool.

To directly test this hypothesis, RBL cells were infected with recombinant vaccinia viruses encoding either porcine wild-type Syk or a kinase inactive mutant form of Syk (SYK KI). After infection, the cells were stimulated and lysed as in Fig. 2. Anti-Syk immunoprecipitates were resolved on SDS-PAGE and immunoblotted with anti-PY mAbs, anti-Cbl, and anti-Syk polyclonal Ab, as indicated. The positions of molecular weight markers are indicated. These results represent one out of three independent experiments.
Proteasome Inhibition Prolongs Antigen-induced Tyrosine Phosphorylation of FcγRI and Syk Kinase Activity and Positively Regulates FcγRI-mediated Degranulation—Ubiquitination of cellular proteins leads to their degradation by the proteasome system (23–25). We have recently shown that both Syk and ZAP-70 PTKs are subjected to Ub/proteasome-dependent degradation following CD16 engagement on human NK cells (21). Moreover, it has been suggested that the ligand-induced ubiquitination of RTKs such as epidermal growth factor receptor is involved in their down-regulation (29–32). We therefore examined whether inhibition of Ub/proteasome-dependent degradation would result in the persistence of FcγRI signaling.

For this purpose RBL cells were incubated with or without the specific proteasome inhibitor, epoxomicin, and stimulated...
Ubiquitination and the consequent degradation of the ubiquitinated proteins play an important role in the control of many cellular processes, including signal transduction (23–25). In the present study, we demonstrate a regulatory role for the tyrosine kinase Syk in controlling its own ubiquitination and that of FceRI \( \beta \) and \( \gamma \) chains, events induced upon receptor engagement on RBL cells. Most of the FceRI- and Syk-ubiquitinated forms are also tyrosine-phosphorylated (Fig. 1), suggesting that ubiquitination preferentially affects engaged receptor and the activated forms of Syk. This last assumption is supported by the result shown in Fig. 2 where the kinase-inactive mutant of Syk is not modified by Ub following receptor engagement. This result is consistent with observations indicating that only the active forms of Src are specifically targeted for Ub-dependent degradation (54). Collectively, these findings suggest that the activation of Syk induces quantitative and/or qualitative changes of Syk phosphorylation required for its own ubiquitination.

We also show that the presence of an active form of Syk is required to promote receptor ubiquitination (Fig. 3), suggesting that Syk may directly activate the ubiquitin system by phosphorylating the enzymes responsible for FceRI and its own ubiquitination. In this regard it has been previously reported that both Lyn and Syk PTKs are essential for the tyrosine phosphorylation of the E3 ligase c-Cbl after FceRI engagement in RBL cells (49, 53).

Herein, we identify c-Cbl as the E3 ligase responsible for the ligand-induced ubiquitination of both Syk and FceRI (Fig. 4 and 5): Cbl-mediated ubiquitination is dependent on Syk activity and requires an intact RING finger domain of Cbl. Syk and Cbl have been previously reported to be constitutively associated in RBL cells, and this association apparently does not change upon receptor engagement (49). However, it cannot be ruled out that, following Syk recruitment and autoprophosphorylation, new transient Syk-Cbl complexes are formed but are not easily detectable. Regardless, all our results suggest that the Syk-Cbl interaction is important because it allows the enzymes to become reciprocal substrates.

On one hand, Syk can control the tyrosine phosphorylation of the associated Cbl (Fig. 6), and this modification is likely to be required for Cbl ligase activity. In accordance with our data, recent evidence from \textit{in vitro} ubiquitination studies show a marked increase of Cbl ligase activity when the adaptor is tyrosine-phosphorylated (33). However, it cannot be ruled out that other adaptors and/or enzymes, being substrates of Syk, may also indirectly control the ligase activity of Cbl.

On the other hand, Cbl can control Syk ubiquitination thus providing a mechanism for selective down-regulation of the active pool of Syk. In fact, despite the lack of a detectable reduction in Syk levels, after overexpression of Cbl we observed a decrease of Ag-induced tyrosine phosphorylated forms of Syk and in particular of the active pool of Syk (data not shown). Our results are in accordance with previous findings demonstrating that overexpression of Cbl inhibits Syk kinase activity and serotonin release in RBL cells without decreasing the total level of Syk (51).
We also provide evidence supporting the involvement of Syk/Cbl-dependent ubiquitination in the regulation of the level of engaged receptors. We observed a time-dependent decrease of FcεRI β chain upon stimulation, which was almost completely reverted in the presence of epoxomicin, reflecting a persistence of phosphorylated receptors (Fig. 7). Thus, it is possible that the persistence of active forms of Syk observed in the presence of epoxomicin may be also due to continuous and
durable kinase recruitment via the longer-lasting tyrosine-phosphorylated receptors. This finding suggests that Ag-induced receptor ubiquitination by regulating the half-life of activated receptor complexes may contribute to the down-regulation of signaling leading to effector functions. This conclusion is supported by the results of Fig. 8: in epoxomicin-treated cells an increase of β-hexosaminidase release was detected after FcεRI stimulation, indicating that proteasome-dependent degradation of FcεRI and Syk could affect receptor-mediated effector function.

In conclusion, our data strongly favor a model in which after receptor engagement the Syk-Cbl complex is recruited by the cell membrane where the activation of both enzymes allows receptor subunits and Syk ubiquitination. The consequent proteasome-dependent degradation of ubiquitinated receptor complexes and activated Syk could contribute to the down-regulation of the intracellular signaling initiated by multisubunit IRs.

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