Temporal Activation of Nontransmembrane Protein-tyrosine Kinases following Mast Cell FcεRI Engagement*

(Received for publication, May 3, 1995, and in revised form, June 16, 1995)

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One of the primary responses observed following antigen-induced cross-linking in mast cells is an increase in the phosphorylation of certain cellular proteins on tyrosine residues. Stimulation of protein-tyrosine kinase activity appears to be necessary for induction of downstream responses such as degranulation. The role of nonreceptor protein-tyrosine kinases in the signal transduction pathway initiated by FcεRI engagement in an interleukin-3-dependent mast cell line has been examined. The results presented here show that the enzymatic activity of Lyn is increased within seconds of receptor engagement. Syk activity also undergoes a rapid and transient increase, reaching a peak at approximately 30 s. Similarly, the activity of Fes, representing a third class of nontransmembrane protein-tyrosine kinase increases as well, with its activity peak reached at 1 min poststimulation. The enzymatic activities of Syk and Fes were found to correspond to anti-phosphotyrosine antibody reactivity. Phosphorylation of tyrosine residues of the β and γ chains of FcεRI increased concomitant with increased protein-tyrosine kinase activity. These results indicate that at least three classes of nontransmembrane protein-tyrosine kinases are involved in mast cell FcεRI signaling and that the activation of these classes of enzymes is temporally regulated.

Mast cells are the primary effectors in immediate type hypersensitivity reactions. Upon exposure to allergen, mast cells are induced to rapidly secrete preformed mediators from cytoplasmic granules. Mast cells bind IgE avidly via specific, high affinity Fc receptors, termed FcεRI. FcεRI are heterotetrameric complexes comprising an IgE-binding α subunit, a β subunit, and two γ subunits (1, 2). The cytoplasmic domains of the β and γ subunits each contain two tyrosine residues, which are located within a conserved consensus sequence termed the immunoreceptor tyrosine activation motif (ITAM) or antigen recognition activation motif (3–6). Phosphorylation of this pair of conserved tyrosines is required for successful signal transduction in mast cells (7) and other hemopoietic cell types (8, 9). Cell activation follows cross-linking of surface-bound IgE molecules by multivalent allergen (10).

Phosphorylation of a select set of cellular proteins on tyrosine residues is a hallmark of the activation of cells through engagement of multichain immune recognition receptors (MIRR) and is one of the earliest detectable events following stimulation of mast cells through FcεRI (11–15). Protein tyrosine phosphorylation following engagement of the FcεRI is thought to be mediated by the nontransmembrane protein-tyrosine kinases Lyn and Syk. Lyn, a member of the Src family of protein-tyrosine kinases, is constitutively associated with FcεRI (15), binding to the unactivated β subunit (7). Syk is a 72-kDa protein-tyrosine kinase (16) that, along with p70SGK (Zap) (9), defines a family of cytoplasmic enzymes distinguished by the presence of tandem SH2 domains located amino-terminal to the catalytic domain. Syk becomes activated upon ligation of both B-cell (17) and mast cell MIRRs (7, 18) and associates with phosphorylated ITAMs present within the cytoplasmic sequences of the MIRRs. In mast cells, Syk binds to the phosphorylated ITAMs of FcεRI γ (19–22).

Fes is a ubiquitously expressed 94-kDa cytoplasmic protein-tyrosine kinase related to Fes (23). At present, little is known about the involvement of Fes in cellular signaling pathways.

Temporal differences in the activation of nonreceptor protein-tyrosine kinases following MIRR engagement have been reported in B-cell and T-cell lines (17, 24). The purpose of this study was to gain a further understanding of mast cell stimulation by examining the timing and order of activation of nontransmembrane protein-tyrosine kinases following FcεRI engagement. We find that Lyn and Syk are activated sequentially and that Fes also undergoes transient activation.

MATERIALS AND METHODS

Antibodies—Rabbit antisera directed against unique sequences of Lyn and Syk have been described previously (24, 25). Antisera directed against FcεRI β amino acids 201–243, Fes α amino acids 27–68, and Fer amino acids 201–400 were produced as glutathione S-transferase fusion proteins (Pharmacia Biotech Inc.) and used to immunize rabbits. Mouse monoclonal anti-phosphotyrosine (APT) SH1 and rabbit polyclonal APT have been described previously (24, 26).

Cells—PT18 cells were sensitized by a 1-h preincubation with monoclonal anti-DNP IgE. The cells were washed in serum-free medium and resuspended at a density of 1 × 10⁷ cells/ml. Activation was triggered with the addition of dinitrophenol-conjugated human serum albumin (DNP-HSA).

Ca²⁺ Mobilization—The concentration of intracellular free Ca²⁺ was measured using the Ca²⁺-sensitive dye indo-1 (Molecular Probes, Eugene, OR) and a Coulter EPICS Elite flow cytometer. The procedure used has been described in detail elsewhere (27). The data were analyzed using Multitimesoftware (Phoenix Flow Systems, San Diego, CA).

Immunoprecipitation, Kinase Assays, and Immunoblotting—Cell lysis, immunoprecipitation, immune complex kinase assays, and immunoblotting were performed as described previously (28, 29).

RESULTS

Mast Cell Signal Transduction Pathways Are Stimulated by FcεRI Engagement—PT18 mouse mast cells were sensitized by...
incubating the cells for 1 h with an allergen-specific IgE (anti-DNP). The sensitized cells were then stimulated by the addition of DNP-HSA and lysed as a function of time over the course of 30 min. The lysates were analyzed by anti-phosphotyrosine immunoblotting to establish the extent and timing of protein tyrosine phosphorylation (Fig. 1A). Immunoblot analysis reveals a complex pattern of protein tyrosine phosphorylation peaking and ebbing with different kinetics. FcεRI engagement results in the immediate tyrosine phosphorylation of a number of proteins, with the most prominent signals appearing at molecular masses of 30, 38–40, 50, 55–60, 72, 90, and 110 kDa. In general, the wave of tyrosine phosphorylation begins very soon after stimulation, reaches a peak at 1–3 min, and has returned to unstimulated levels at 10–30 min.

The reactivity of IgE-sensitized PT-18 cells to DNP-HSA was also examined by monitoring changes in the concentration of intracellular free calcium \([\text{Ca}^{2+}]\). The concentration-response curve for DNP-HSA is Gaussian in that addition of low levels of ligand produce small increases in \([\text{Ca}^{2+}]\), moderate levels produce a maximal increase in \([\text{Ca}^{2+}]\), that is sustained, and higher levels produce a maximal \([\text{Ca}^{2+}]\), rise that lacks the sustained phase. The response of PT 18 cells to moderate (2.5 ng/ml) to high (100 ng/ml) concentrations of DNP-HSA are shown in Fig. 1B.

Tyrosine Phosphorylation of FcεRI Component Proteins—Among the proteins phosphorylated on tyrosine following receptor cross-linking are the \(\beta\) and \(\gamma\) subunits of FcεRI. To determine the kinetics of receptor activation, the tyrosine phosphorylation of \(\beta\) and \(\gamma\) was evaluated as a function of time after receptor engagement (Fig. 2). Tyrosine phosphorylation of FcεRI-\(\beta\) and FcεRI-\(\gamma\) can be detected as quickly as 10 s following FcεRI engagement. Phosphorylation of both \(\beta\) and \(\gamma\) follows the same kinetics. The peak of tyrosine phosphorylation occurs at 1 min and diminishes to near basal levels by 30 min post-stimulation. The abundance of FcεRI-\(\beta\) and FcεRI-\(\gamma\) was unchanged over the time course, thus indicating that changes in FcεRI-\(\beta\) and FcεRI-\(\gamma\) APT reactivity reflect increased tyrosine phosphorylation. Interestingly, upon activation, a lower mobility form of FcεRI-\(\gamma\) appears. This tyrosine-phosphorylated form of FcεRI-\(\gamma\) has an apparent molecular mass of approximately 10 kDa compared with an apparent molecular mass of 6 kDa for the nonphosphorylated form. By comparing the abundance of FcεRI-\(\gamma\) detected by immunoprecipitation/FcεRI-\(\gamma\) blot (Fig. 2B, lower panel) with the APT reactivity (Fig. 2B, upper panel), it is evident that the phosphorylated form that appears after activation accounts for only a small amount of the FcεRI-\(\gamma\) in the cell.

Activation of Lyn, Syk, and Fer—PT18 cells were stimulated by treatment with IgE/DNP-HSA, and lysates were prepared over a time course as described above. The enzymatic activity of protein-tyrosine kinases in these samples was established through the use of in vitro kinase autophosphorylation assays (Fig. 3). The kinase activity of Lyn increased immediately following receptor engagement, reaching its maximum level at the initial time point of 10 s. Only a brief spike of Lyn activation is observed, with activity falling back to an unstimulated level by 30 s (Fig. 3A, upper panel). The Src family member Fyn is also produced by PT18 cells but at a barely detectable level (data not shown).

The catalytic activity of Syk was also found to increase following FcεRI engagement. Syk activity was elevated immediately, but it did not reach its peak level of activity until about 30 s poststimulation (Fig. 3B, upper panel). The activity then slowly decayed, returning to basal levels by 30 min.

The kinase activity of Fer was also examined to determine if Fer could be responsible for the protein band(s) at approximately 94 kDa detected by APT immunoblotting. The protein kinase activity of Fer was found to increase following addition of DNP-HSA. Fer activity was increased by 10 s, with a maximum level attained by 1 min. Fer activity was reduced to unstimulated levels by 10–30 min.

The activity profiles of Lyn, Syk, and Fer determined by autophosphorylation were confirmed by evaluating the ability of these enzymes to phosphorylate an exogenous substrate. In each case, the ability of each enzyme to catalyze the phosphorylation of enolase roughly paralleled changes in autophosphorylation demonstrated in the previous assays. Lyn was again activated immediately, but the magnitude of the difference between unstimulated and stimulated activities toward enolase is much smaller than observed in the autophosphorylation assays. The capacity of Syk to phosphorylate enolase correlated with its ability to catalyze autophosphorylation. Similarly, the kinase activity profile of Fer toward enolase matched that observed in the autophosphorylation assay.

The correlation between the enzymatic activation and tyrosine phosphorylation states of these enzymes was established by determining reactivity of each protein with APT antibodies following FcεRI engagement. The results of these experiments are presented in Fig. 4. Syk was unreactive to APT antibody when isolated from unstimulated cells, but its immunoreactivity increased significantly following receptor engagement (Fig.
APT immunoreactivity was high from 30 s to 3 min, and then it decreased to a low but detectable level by 30 min. The abundance of Syk was not altered in the course of these experiments. Thus, the reactivity of Syk with APT antibody paralleled its enzymatic activation as determined by both autophosphorylation and phosphorylation of exogenous substrate. The APT reactivity of Fer also increased upon stimulation of the PT18 cells, reaching a maximum at 30 s to 1 min and decreasing to unstimulated level by 30 min. The abundance of Fer was constant over this time course. As observed with Syk, changes in the tyrosine phosphorylation state of Fer correlate with changes in its enzymatic activity.

**DISCUSSION**

Protein tyrosine phosphorylation is a well established initiator of signal transduction pathways triggered in response to receptor engagement in hemopoietic cells. The concept of an ordered activation of enzymes being necessary to propagate such signals has been recently established in both B- and T-cells (17, 24). We have found that this observation extends to mast cells, albeit with a compressed time scale. Furthermore, we report evidence that in addition to Lyn and Syk, the non-receptor protein-tyrosine kinase Fer participates in mast cell signaling and is part of the ordered activation scheme.

The results of our immune complex kinase assays show that the enzymatic activation of the Src family member Lyn after FcεRI cross-linking is immediate and transient. The activation of Src family enzymes in MIRR signal transduction is a well established phenomenon. Lck and Fyn are activated in response to T-cell receptor ligation (24), the enzymatic activities of Lyn and Blk are increased following stimulation of B-cell lines through the B-cell receptor (17), and Lyn has been shown to be activated in stimulated mast cell and basophilic cell lines (7, 15). Lyn is the most abundant Src family member in PT 18 cells and other mast cell and basophilic cell lines and appears to send the initiating signal following FcεRI engagement. The location of Lyn at the cytoplasmic subsurface of the plasma membrane and the observation that this protein-tyrosine kinase is constitutively bound to the FcεRI-β chain places this enzyme in the appropriate subcellular location to respond rapidly to receptor engagement.

The peak levels of Syk and Fer activation were found to follow that of Lyn. The peak of Syk activity coincides with the time at which FcεRI-γ tyrosine phosphorylation is approaching maximal levels. This finding is consistent with a model where
Lyn is somehow activated by receptor cross-linking and phosphorylates the β and γ subunits of the receptor. Tyrosine-phosphorylated γ ITAMs would provide a docking site for Syk via its tandem SH2 domains (21, 22). Recent evidence indicates that binding of Syk to phosphorylated ITAMs induces a conformational change in the protein, leading to its enzymatic activation (30). The overall timing of the tyrosine-phosphorylation response and the characteristics of Syk activation in mast cells are quite different from those observed in B-cells (17). B-cells present a less complex pattern of tyrosine-phosphorylated proteins following engagement of the B-cell antigen receptor, and the tyrosine phosphorylation develops more slowly. Furthermore, the activation state of Btk following receptor cross-linking or activation or an increase in tyrosine phosphorylation of Btk may mask changes in the activation state of Btk following receptor cross-linking or limit our ability to detect such changes.

In summary, we have shown that tyrosine phosphorylation in PT 18 mast cells is characterized by a more compressed time frame, the total cell tyrosine phosphorylation response as well as the activation of protein-tyrosine kinases returns to unstimulated levels of activity by 30 min poststimulation. This time frame is consistent with the physiological role of the mast cell to provide an immediate hypersensitivity reaction and then turn off.

The protein-tyrosine kinase Fer is also activated following engagement of the FcRI. The catalytic activation and tyrosine phosphorylation of Fer follows that of Syk. This is the first known example of Fer activation in response to external stimuli. Additional studies are being performed to clarify the role of Fer in hemopoietic cell signal transduction. Another protein-tyrosine kinase reported to play a role in mast cell signal transduction is Btk (31). Although we found Btk to be an abundant enzyme in PT18 cells, we have been unable to detect activation or an increase in tyrosine phosphorylation of Btk following stimulation of PT18 cells (data not shown). The Btk present in PT 18 cells appears to have a high level of constitutive tyrosine phosphorylation. This high level of tyrosine phosphorylation in the absence of stimulation may mask changes in the activation state of Btk following receptor cross-linking or limit our ability to detect such changes.