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Roles of Src-family kinase isoforms, Lyn, Fyn, Fgr, and c-Src on degranulation in RBL-2H3 mast cells

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The rat RBL-2H3 mast cells contain various Src-family kinases. Previous reports with this cell line indicated that Lyn activation is an important initial signaling for the activation of the cells. However, the role and location of other Src-family kinase isoforms which are expressed in the cells are not clear. In this study, we now show that isoforms of Src-family kinases, Lyn, Fyn, Fgr, c-Src, and Yes are differentially expressed and located differently in the cells as indicated by RT-PCR, immunoblotting analysis, and confocal microscopy. Lyn and Fgr were located on plasma membrane but on the other hand c-Src and Yes were located on intracellular organelle. All of Src-family kinases were cloned and overexpressed for investigating the roles of the isoforms. Overexpression of Fyn and Fgr, not Lyn and c-Src, stimulated Ag-induced degranulation in the cells. Our findings strongly suggest for the first time that each of Src-family kinase isoform can regulate differentially FcεRI-mediated signaling in RBL-2H3 mast cells.

Key words – Src-family kinase, Lyn, Fyn, Fgr, c-Src, Mast cells

Introduction

Mast cells are responsible for a variety of allergic disorders [1,21]. These cells are stimulated by IgE-directed antigens through the high affinity receptor for IgE, namely Fc εRI, and release granules that contain preformed inflammatory mediators and inflammatory lipids and cytokines. Lyn, a Src-family kinase (SFK), is regarded as an essential SFK in mast cell degranulation [2,3,20]. The aggregation of antigens with the IgE/FcεRI complex results in the recruitment and activation of Src kinases and subsequently other tyrosine kinases. The function of Lyn kinase in mast cells has been studied in the RBL-2H3 cell line, which is now known to be an analog of rat mucosal mast cells [16]. In a widely accepted model [18], it is well established that receptor aggregation leads to the activation of β subunit-associated Lyn, and Lyn phosphoryalates tyrosine residues in the Immunoreceptor tysosine-based activation motifs (ITAMs) in the cytoplasmic regions of β and γ subunits. Phosphorylated β and γ ITAMs recruit Lyn and Syk, respectively [18]. Another Src family kinase, Fyn, was also shown to associate with FcεRI and to play a complementary role via activating Gab2 and PI3K [13]. In contrast with the positive role of Lyn for activation of mast cell [18], a negative role of Lyn was shown by numerous studies, particularly those of B cell signaling and mast cells [12,13,24]. Despite the rapid progress in our understanding of FcεRI signaling, the role of Lyn in mast cells has been controversial [7,8,11] and roles of other SFK such as Fyn, Fgr, c-Src, Hck, and Yes still remained unclear.

In this paper, various endogenous SFKs were detected in RBL-2H3 mast cells, and they were differently located in the cells: Lyn, Fyn, and Fgr on plasma membrane; c-Src, Hck, and Yes on intracellular organelles. Furthermore, Fyn and Fgr positively regulate degranulation in antigen-stimulated RBL-2H3 cells. In contrast, Lyn exhibited a negative role on degranulation in the cells.

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Materials and Methods

Materials

Reagents were purchased from the following sources: PP2 was from Calbiochem (La Jolla, CA); antibody against c-Src was from Upstate Biotechnology (Lake Placid, NY); antibodies against Lyn, Fyn, and Fgr were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); cell culture reagents were from GIBCO/Invitrogen (Carlsbad, CA); Tris-glycine polyacrylamide gels were from Novex (San Diego, CA); trinitrophenyl (DNP)-specific monoclonal IgE and

DNP-BSA were from Sigma (St. Louis, MO).

Extraction of RNA and RT-PCR

Total RNA was isolated from RBL-2H3 cells using Trizol Reagent (Invitrogen, Carlsbad, CA) and was reverse transcribed with the Superscript first strand synthesis system (Invitrogen) according to the manufacturer's protocol. PCR was performed at 94°C for 45 sec, 55°C for 45 sec, and 72°C for 60 sec for 30 cycles. The primers for the various SFKs were designed to make the same size PCR products. The following primers were used: rat Lyn forward 5'-CCC TCA AGC CTG GCA CCA TGT-3', reverse 5'-CCG AAG GAC CAC ACG TCA GA-3'; rat Fyn forward 5'-CTC TTA AGC CAG GCA CAA TGT-3', reverse 5'- CCA AAG GAC CAC ACG TCA GA-3'; rat Fgr forward 5'- CGC TGA AGC CAG GCA CCA TGT-3', reverse 5'- CCA AAG GAC CAC ACG TCT GA-3'; rat Src forward 5'- CCC TGA AGC CAG GCA CCA TGT-3', reverse 5'- CCA AAG GAC CAC ACG TCC GA-3'; rat Hck forward 5'- GGA TGG GAT GTG TGA AGT CCA-3', reverse 5'- CGA ACA GAA AGT GAG TAG CT-3'; rat Yes forward 5'- CAC TAA AGC CAG GTA CAA TGA-3', reverse 5'- CCA AAT GAC CAC ACA TCT GA-3'; rat GAPDH forward 5'-GTGGAGTCTACTGGCGTC TTC-3', reverse 5'-CCAAGGCTGTGGGCAAGGTCA-3'.

Cloning of Lyn, Fyn, Fgr, c-Src, and Yes from RBL-2H3 cells

The SFKs were cloned into pCMV Vector (Stratagene, La Jolla, CA) by PCR amplification using following primers: 5'-TCCCCGCGGCACCGC GAGCGAGAAATATG-3' and 5'-CCGCTCGAGTGGCTGCT GCTGATACTGC-3' for LynB; 5'-GGAATTCGAGCTTGGATAATGGGCTGTG-3' and 5'-G CGTCGACTCACA GGTTTTCACCGGGCTG-3' for Fyn; 5'-GGAATTCGGAATGGGCTGTGTGTTCTG C-3' and 5'-CCG CTCGAGGTCAGGCTATGTCTGGTCTCC-3' for Fgr; 5'-GG AATTCATGGGCAGCAACAAGAGCAAG-3' and 5'-CCGC TCGAGCACACAGTTCCTATAGGTTCT-3' for c-Src; 5'-TT CCCGCGGATGGGCTGCATTAAAAGTAAAG-3' and 5'-G CGTCGACTTATAA ATTTTCTCCTGG TTGG-3' for Yes. Sequence and expression were confirmed by sequencing and Western blot analysis.

Transient transfection of cells with Src-family kinases, cell stimulation, and immunoblotting

RBL-2H3 cells were grown as monolayers in minimal essential medium with Earle's salts, supplemented with 200 mM glutamine, 10,000 units/ml penicillin, 10,000 units/ml streptomycin, and 15% fetal bovine serum. Cells were transiently transfected with each DNA preparation (1 μg DNA per 10^6 cells unless stated otherwise) by electroporation (AMAXA, program T-11). Successful transfection was confirmed by Western blotting. Cells were used within 48 hr of transfection. Transfected cells (~1.0 x 10^6 cells/6-well plates) were washed with fresh growth medium at 4 hr after transfection and incubated with 50 ng/ml IgE for 3 hr. The cells were washed and medium was replaced with a PIPES-buffered medium (25 mM PIPES, pH7.2, 159 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 5.6 mM glucose, and 0.1% fatty acid-free fraction V from bovine serum). Cells were stimulated with 25 ng/ml DNP-BSA for 7 min or as indicated, chilled with ice to terminate stimulation, and then washed twice with ice-cold PBS. Cells were lysed in 0.15 ml with ice-cold lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Nonidet p-40, 10% glycerol, 60 mM octyl β-glucoside, 10 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 2.5 mM nitrophenylphosphate, 0.7 μg/ml pepstatin, and protease inhibitor cocktail tablet). Lysates were kept on ice for 30 min and then centrifuged 15,000 x g for 15 min at 4°C. The supernatant was dissolved in 2x Laemmli buffer. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes (Schleicher and Schuell, BA85), and incubated with the specific antibody at 1:1000 dilution. The immunoreactive proteins were detected by use of horse-radish peroxidase-coupled secondary antibodies and Enhanced Chemiluminescence according to the manufacturer's instructions (Amersham Pharmacia Biotech).

Measurement of degranulation

Secretion of granules was determined by measurement of release of the granule marker, β-hexosaminidase, by use of a colorimetric assay in which release of p-nitrophenol from p-nitrophenyl-N-acetyl-b-D-glucosaminide was measured. Values were expressed as the percent of intracellular β -hexosaminidase that was released into the medium.

Location of Lyn, Fyn, Fgr, and c-Src in RBL-2H3 mast cells

RBL-2H3 cells were suspended in complete growth medium, transferred to Lab-Tek chambered coverslips (Nalge Nunc International, Naperville, IL), and then incubated overnight at 37°C. The cultures were washed three times

with phosphate buffered saline (PBS). Cultures were fixed in 4% formaldehyde in PBS for 10 min, washed, and permeabilized with 0.5% Triton X-100 for 15 min. The fixed cells were washed again before incubation for 60 min with a blocking reagent, 1% bovine serum albumin in PBS. The coverslips were incubated for 2 hr with a solution of the primary antibody in 1% bovine serum albumin in PBS, washed, and then incubated with rhodamine-conjugated secondary antibody for 45 min. The coverslips were washed and mounts prepared by use of the Prolonged Antifade Kit (Molecular Probes, Eugene, OR). Confocal images were taken in a Bio-Rad MRC 1024 confocal laser scanning microscope with an Apochromat 60× objective.

Results

Src family kinase inhibitor, PP2, suppresses degranulation in mast cells

It has been well established that SFKs such as Lyn and Fyn are critical for activation of mast cells. As shown in Fig. 1, PP2, a specific SFK inhibitor, inhibited degranulation in Ag or thapsigargin-stimulated RBL-2H3 cells. This result confirmed that SFKs are important for the activation of mast cells.

Identification of endogenous Src family kinases

Although it is well established that Lyn is important for mast cell activation, the expression and roles of other SFKs in mast cells are not clear and controversial. Therefore, it is very interesting to note what kinds of SFKs are expressed endogenously in the cells. At first, analysis by RT-PCR revealed that RBL-2H3 cells express abundant message for Lyn, Fgr, c-Src, and Yes, and less abundant message for Fyn (Fig. 2A). The message for Hck was not detected in the cells even with two different kinds of primers. However, we can not exclude the possibility that the different primers for various SFKs make different signals. Next, those SFKs were identified using commercially available antibodies. Consistent with the results from RT-PCR, various SFKs were detected in the cells (Fig. 2B). Following the studies, location of each SFK was measured by confocal microscopy. Lyn and Fyn are reported as being located on plasma membrane [12,21]. Less is known about other SFKs in the cells. As shown in Fig. 2C., Lyn and Fgr kinases were located on plasma membrane, and c-Src and Yes were located on the intracellular organelles. However, we could not identify the endogenous expression of Fyn by this confocal microscopy because the expression level was very low in RBL-2H3 cells (Fig. 2). However, it was shown that overexpressed Fyn was located on plasma membrane (data not shown). In summary, those results strongly suggested that RBL-2H3 cells contain endogenous

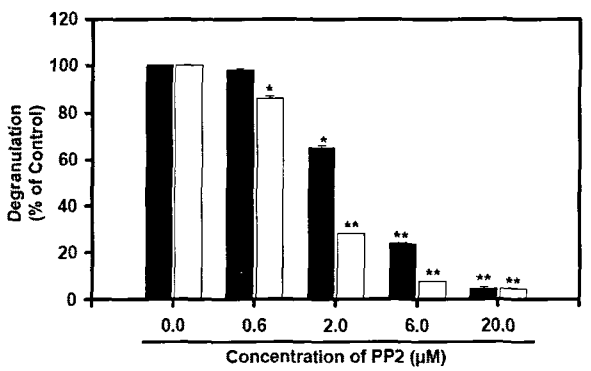

Fig. 1. Degranulation is suppressed by the Src family kinase inhibitor, PP2. RBL-2H3 cells were primed with DNP-specific IgE. The indicated concentration of PP2 were added 10 min before stimulation with 25 ng of DNP-BSA/ml (■) or 150 nM thapsigargin (□) for 15 min. Degranulation was determined by the release of the granule marker, β-hexosaminidase. Values are expressed as percentages of Ag-induced release without PP2 and are the means ± s.e.m. of values from three independent expreiments.

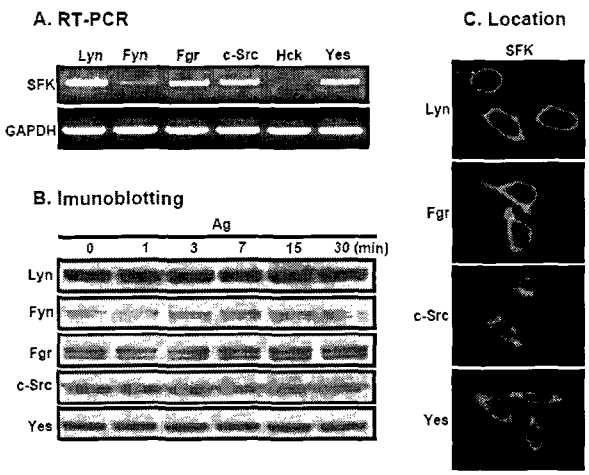

Fig. 2. Determination of endogenous Src family kinases by RT-PCR, immunoblotting, and confocal microscopy. (A) The presence of mRNA for multiple Src family kinases was determined by RT-PCR. (B) Immunoblots of lysates of RBL-2H3 cells were prepared for detection of endogenous Src kinases. (C) In addition, the cells were examined by confocal microscopy to determine the intracellular distribution of the various Src family kinases.

Lyn, Fgr, c-Src, Yes and low level of Fyn.

Roles of Src family kinases on degranulation

It is generally accepted that upon FcεRI cross-linking, Fc εRI-associated SFK Lyn becomes activated and phosphorylates the β- and γ-chain in ITAMs [18]. Also, a recent report showed that another SFK, Fyn, also is important for FcεRI-initiated signaling and biological responses [13]. However, it is controversial whether Lyn positively or negatively regulates FcεRI-mediated activation of mast cells [11,13,18] and less is known about the roles of other SFKs such as Fgr, c-Src, and Yes in the cells. Therefore it is very interesting to study about the roles of SFKs, specially for Fgr, c-Src, and Yes. As shown in Fig. 3, overexpression of Lyn significantly inhibited degranulation. In contrast, Fyn and Fgr strongly stimulated degranulation in the cells. It is the first report that Fgr positively regulates the activation of mast cells. Further studies are necessary to identify the mechanism.

Discussion

The activation of IgE-mediated mast cell signaling is initiated by the interaction of FcεRI with the SFK Lyn and subsequently on the downstream activation of Syk and other tyrosine kinases [13]. However, amplification of the full array of activating signals is thought to require the assembly of two distinct clusters of signaling molecules at the plasma membrane [14]. One is assembled around the linker for activation of T cells (LAT) and the other around the Grb2-associated binder-2 (Gab2). The LAT cluster of proteins enables propagation of Syk-mediated signals. These signals include the phosphatidylinositol (PI) 3-kinase-dependent phosphorylation of Tec kinases, Btk and Itk, which in turn phosphorylate and activate PLCγ1 and PLCγ 2 to promote a calcium signal through the generation of inositol 1,4,5-trisphosphate [15]. The Gab2 cluster consists of Fyn and the Src homology 2 domain-containing protein tyrosine phosphatase, SHP-2, in addition to PI 3-kinase. Less is known about the function of this cluster but it appears to facilitate PI 3-kinase-dependent phosphorylation of the survival factor Akt by the phosphoinositide-dependent kinase (PDK) and the activation of PKC [6,13]. These clusters appear to localize in distinct but also different regions of the plasma membrane [22].

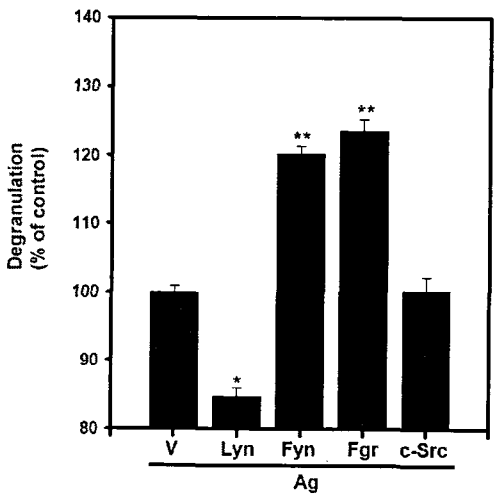

Fig. 3. Role of Src family kinases on degranulation of mast cells. Cells were made to express Src family kinases, Lyn, Fyn, Fgr, or c-Src, primed with DNP-specific IgE, and stimulated with antigen for 15 min to assess the effects on degranulation determined by the release of the granule marker, β-hexosaminidase. Results are expressed as percentage of the values from the vector-transfected cells and are the mean ± s.e.m. of values from three experiments.

In a widely accepted model [18], it is believed that receptor aggregation leads to the activation of β subunit-associated Lyn and subsequently Lyn phosphorylates tyrosine residues in the ITAM motifs in the cytoplasmic regions of β and γ subunits to amplify the Ag-induced signaling in mast cells. However, recent studies demonstrated that Lyn plays a negative regulatory role in the cells [12], particularly in B cell signaling [24]. Thus, the role of Lyn in mast cells has been controversial [7,8,11,13]. More recently, it was demonstrated that Lyn positively regulates degranulation on low intensity stimulation of FcεRI, whereas it works as a negative regulator by high intensity stimulation in mast cells [23]. Consistent with the previous report [23], our results showed that Lyn negatively regulated degranulation by a high intensity antigen stimulation in mast cells (Fig. 3). However, further study is necessary to identify the initial role of Lyn in the cells. As shown in Fig. 2, because multiple SFKs were expressed in RBL-2H3 mast cells, a reasonable candidate for the positive regulator should be Fyn or other SFKs such as Fgr, and c-Src. Paravicini et al. [13] showed that Fyn deficiency impairs degranulaiton and that the Fyn kinase-dependent pathway does not require Lyn or LAT for its initiation. Consistent with the previous report [13], overexpression of Fyn positively regulated FcεRI-mediated degranulation in mast

cells. Furthermore, our findings showed that a significant amount of Fgr was expressed in RBL-2H3 mast cells (Fig. 2) and in bone marrow-derived mast cells (BMMC, data not shown). Overexpression of Fgr kinase strongly stimulated degranulation in FcεRI-mediated mast cells (Fig. 3). Less is known about the role of Fgr kinase in FcεRI-mediated signaling in mast cells. Fgr is most highly expressed mature blood granulocytes and monocytes as well as tissue macrophages. Although Fgr negatively regulates Fcγ receptor-mediated phagocytosis in macrophages [5], it appears to play a positive role in integrin or chemokine mediated responses in macrophages [17], eosinophils [4,19], and neutrophils [10]. If this finding is applicable to antigen-stimulated mast cells, the possibility exists that Fgr kinsae has an important role for the activation of early signaling molecules such as Syk, LAT, SLP-76, and others. Further studies on the role of Fgr kinase are in progress in the cells. In summary, we showed that isoforms of SFKs, Lyn, Fyn, Fgr, c-Src, and Yes are differentially expressed and located in RBL-2H3 mast cells. Overexpression of Fyn and Fgr, not Lyn and c-Src, stimulated Ag-induced degranulation in the cells. The findings strongly suggest for the first time that each of SFK isoform can regulate differentially FcεRI-mediated signaling in the RBL-2H3 mast cells.

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초록 : 비만세포의 탈과립에 대한 다양한 Src-family kinase의 역할

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흰쥐유래의 비만세포인 RBL-2H3 세포는 다양한 Src-family kinase를 발현한다. 현재까지의 연구결과에 의하면 비만세포의 초기 활성화에 Lyn kinase가 중요한 역할을 한다고 알려져 왔다. 그러나 그 세포에서 발현되는 다양한 다른 Src-family kinase의 역할은 불분명하다. 본 연구에서는 비만세포에서 다양한 Src-family kinase가 세포 내 다른 곳에서 다양하게 발현되고 있다는 사실을 RT-PCR, immunoblotting 그리고 confocal microscopy 기법을 이용하여 증명하였다. 그 결과 Lyn 및 Fgr kinase는 세포막에 위치하고 c-Src 및 Yes kinase는 세포 내 과립에 존재하는 것을 알 수 있었다. 모든 Src-family kinase를 클로닝하고 과발현하여 탈과립 대한 영향을 평가하였다. 그 결과 Fyn과 Fgr kinase는 비만세포에서 항원 유도의 탈과립을 증가시켰으며 반면 Lyn kinsae는 탈과립을 억제시키는 것을 확인할 수 있었다. 이러한 결과는 비만세포 초기 신호전달계에서 Fgr가 중요한 역할을 할 가능성을 제시한다.