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Original Article

Association of IgG Fc receptor II with tyrosine kinases in the human basophilic leukemia cell line KU812F

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ABSTRACT

Background: We previously reported that cross-linking of IgG Fc receptor II (FcγRII) induces intracellular calcium mobilization, but not histamine release in human basophils. To clarify functional activities of FcγRII on human basophils, we analyzed the FcγRII-mediated signaling events in the human basophilic leukemia cell line KU812F.

Methods: Flow cytometric methods were used to investigate the effect on intracellular calcium mobilization of cross-linking of FcγRII. KU812F cells were preincubated with anti-FcγRII monoclonal antibody (IV.3). After the addition of various concentrations of the tyrosine kinase inhibitor genistein or buffer alone, cells were stimulated with goat antimouse IgG $F(ab')_2$ (GAM) and analyzed with the flow cytometer. Next, in order to test the signaling events after cross-linking of FcγRII, we examined tyrosine kinase activity. The time-course of tyrosine phosphorylation after cross-linking of FcγRII and the effect of genistein on this tyrosine phosphorylation were tested by immunoblotting. Immunoprecipitation was also performed to identify the type of tyrosine kinase associated with signal transduction of FcγRII.

Results: The tyrosine kinase inhibitor genistein inhibited intracellular calcium mobilization caused by cross-linking of FcγRII in a dose-dependent manner. Rapid tyrosine phosphorylation after FcγRII cross-linking was shown by immunoblot analysis and this phosphorylation was inhibited by genistein. Furthermore, tyrosine phosphorylation of Lyn and Syk was observed upon cross-linking of FcγRII.

Conclusions: Tyrosine phosphorylation is necessary for the signaling pathway through FcγRII and tyrosine phosphorylation of Lyn and Syk, at least, is actively involved in this signal transduction.

Key words: basophilic leukemia cell line, IgG Fc receptor II, intracellular calcium mobilization, signal transduction, tyrosine kinase.

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INTRODUCTION

Human basophils are known to play an important role in allergic diseases, such as bronchial asthma and allergic rhinitis. Cross-linking of high-affinity IgE Fc receptor (FcεRI) on basophils evokes histamine release and synthesis of cysteinyl leukotrienes.[1] The IgG Fc receptors (FcγR) are broadly expressed on hematopoietic cells and are classified into three major classes: I, II and III.[2–4] We have reported previously that a low-affinity receptor, namely FcγRII, is expressed on human basophils and cross-linking of FcγRII induces intracellular calcium mobilization.[5,6]

To clarify the functional activities of FcγRII on human basophils, we examined FcγRII-mediated signaling events using the human basophilic leukemia cell line KU812F. The KU812F cell line is a human immature basophilic cell line that has been used in studies of basophil biology.[7–10] Because tyrosine phosphorylation is one of the earliest biological events observed following cross-linking of FcγRII, we examined tyrosine phosphorylation following FcγRII stimulation to study the effect of FcγRII on human basophils. It is most likely that the Src family or the Syk family tyrosine kinases may be involved in FcγRII-mediated signaling.[11] These tyrosine kinases have been shown to associate physically with transmembrane receptors and play a role in the transduction of signals by a member of the immunologically functional membrane receptors.[12,13] Here, we demonstrate that FcγRII-mediated signal transduction in KU812F cells requires involvement of tyrosine kinases, especially Lyn and Syk, in an initiating phase.

METHODS

Cell preparations and cultures

Cells from the human basophilic leukemia cell line KU812F were obtained from RIKEN Cell Bank (Tsukuba, Japan) and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS).

Intracellular calcium measurements

Intracellular calcium measurements were performed according to the method described previously.[6] Briefly, KU812F cells were resuspended in Hanks' balanced salt solution (HBSS), without calcium and magnesium, containing 0.01 mol/L EDTA (Sigma, St Louis, MO, USA) and 0.1% bovine serum albumin (BSA; Sigma). These cells (1×10^6 /mL) were treated with the $F(ab')_2$ of anti-FcγRII monoclonal antibody (IV.3; 10 μg/mL; Medarex, Princeton, NJ, USA) for 30 min at 4°C, washed twice and loaded with 2 μmol/L fluo-3/AM (Dojindo, Kumamoto, Japan) at room temperature for 40 min. Cells were washed twice and incubated with HBSS containing calcium and magnesium in the absence or presence of the tyrosine kinase inhibitor genistein (Sigma) for 30 min at 37°C. Then, cells were stimulated with 20 μg/mL goat antimouse IgG $F(ab')_2$ (GAM; Biosource International Tago, Camarillo, CA, USA) and analyzed with a FACSCalibur flow cytometer (Beckton Dickinson Immunocytometry Systems, San Jose, CA, USA) using CELLQuest software (Beckton Dickinson Immunocytometry Systems). Fluorescence was monitored through a 525 nm band pass filter using a linear scale. At each time point, 5000 cells were analyzed with appropriate forward and side scatter gating and mean fluorescence intensity was calculated.

Immunoblotting

The KU812F cells were adjusted to 1×10^7 /mL and preincubated in the presence of IV.3 (10 μg/mL) for 30 min at 4°C. Cells were stimulated with GAM (20 μg/mL) for 1, 3, 5 and 10 min at 37°C. Then, cells were lysed in sample buffer (50 mmol/L Tris, pH 6.8, 500 mmol/L dithiothreitol (DTT), 10% sodium dodecyl sulfate (SDS), 5% bromophenol blue (BPB), 0.5 mol/L EDTA, 10% glycerol). Lysates were denatured by boiling and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) and immunoblotted with anti-phosphotyrosine monoclonal antibody (4G10; Upstate Biotechnology, Lake Placid, NY, USA). The phosphotyrosine was detected by horseradish peroxidase-conjugated protein A and an enhanced chemilluminescence detection system (Amersham Biosciences, Buckinghamshire, UK).

To study the effect of genistein on tyrosine phosphorylation induced by FcγRII cross-linking, KU812F cells (1×10^7 /mL) were treated with IV.3 (10 μg/mL) for 30 min at 4°C and incubated with HBSS containing calcium and magnesium in the absence or presence of genistein (50–200 μmol/L) for 30 min at room temperature. Cells (1×10^7 /mL) were then stimulated with GAM (20 μg/mL) for 3 min at 37°C and lysed in sample buffer. Lysates were boiled, subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The phosphotyrosine was immunoblotted with 4G10 and detected as described above.

Immunoprecipitation

The KU812F cells were preincubated with IV.3 (10 μg/mL) for 30 min at 4°C and stimulated with GAM (20 μg/mL) for 3 min at 37°C. Cells were lysed at 1×10^7 /mL in 1% Nonidet P (NP)-40 (1% NP-40, 10 mmol/L Tris·HCl, pH 8.0, 150 mmol/L NaCl, 0.5 mmol/L EDTA, 10 mmol/L NaF, 1 mmol/L phenylmethylsulphonyl fluoride) for 30 min on ice. The lysates were centrifuged and cleared with protein A Agarose (Pierce, Rockford, IL, USA). Aliquots of the cleared lysates were incubated with anti-Lyn (Upstate Biotechnology), anti-Syk (Upstate Biotechnology) and anti-phospholipase C-γ1 (PLC-γ1) (Upstate Biotechnology) antisera. Then, immune complexes were precipitated with protein A Agarose. The immunoprecipitates were washed five times with lysis buffer containing inhibitor (1% NP-40 containing 10 mg/mL each aprotinin, leupeptin and chymostatin) and suspended in sample buffer. Samples were denatured by boiling, separated on a 6% SDS-PAGE gel, transferred to nitrocellulose, immunoblotted with 4G10 and detected as described for immunoblotting. After being deprobed, each membrane was reprobed with anti-Lyn, anti-Syk and anti-PLC-γ1 antisera to clarify that each line included even immunoprecipitates.

Results

Effect of genistein on intracellular calcium mobilization induced by FcγRII cross-linking

Treatment of KU812F cells with a specific antibody to FcγRII (IV.3) alone caused no mobilization of intracellular calcium (data not shown). However, cross-linking of FcγRII with $F(ab')_2$ of goat antimouse IgG (GAM) induced a rapid mobilization of intracellular calcium (Fig. 1). The tyrosine kinase inhibitor genistein inhibited FcγRII-mediated intracellular calcium mobilization in a dose-dependent manner. The increase in

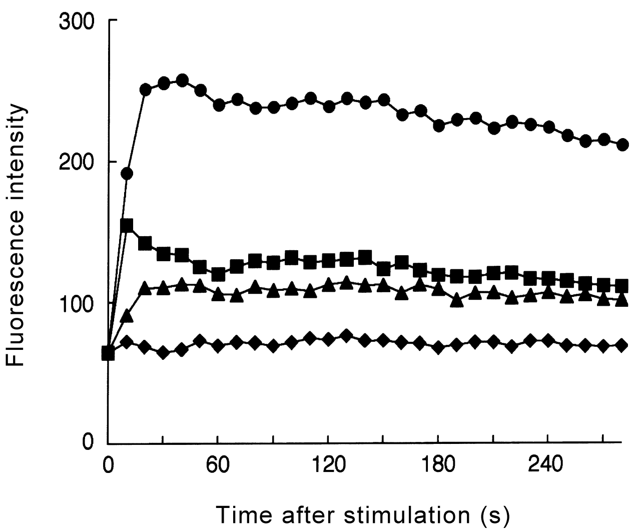

Fig. 1 Inhibitory effect of genistein (●, none; ■, 50 μmol/L; ▲, 100 μmol/L; ◆, 200 μmol/L) on intracellular calcium mobilization induced by FcγRII cross-linking. KU812F cells were treated with anti-FcγRII monoclonal antibody (IV.3) and were preincubated in the absence or presence of genistein at different concentrations. Cells were then stimulated with goat antimouse IgG $F(ab')_2$ (GAM; 20 μg/mL). Data are representative of seven independent experiments.

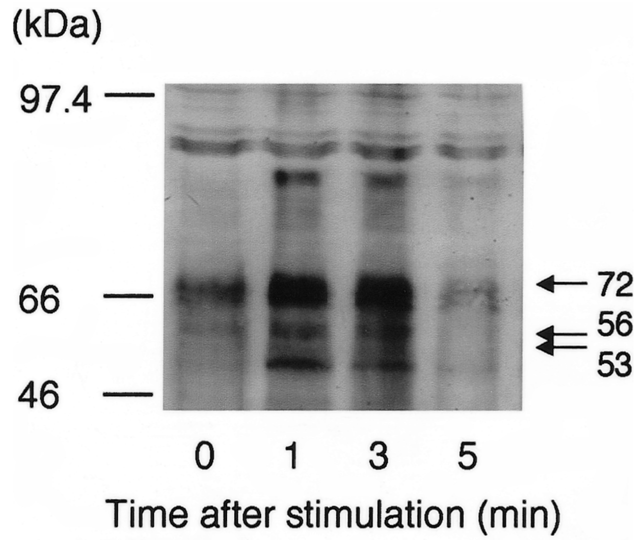

Fig. 2 Kinetics of tyrosine phosphorylation after stimulation with goat antimouse IgG $F(ab')_2$ (GAM). The KU812F cells (1×10^7 /mL per lane) were incubated with anti-FcγRII, IV.3 (10 μg/mL) and stimulated with GAM (20 μg/mL) for the periods indicated. Tyrosine phosphorylation was determined by immunoblotting as descrived in Methods. Anti-phosphotyrosine monoclonal antibody (4G10) was used as a probe. Arrows indicate the position of 53, 56 and 72 kDa, respectively. Data are representative of five independent experiments.

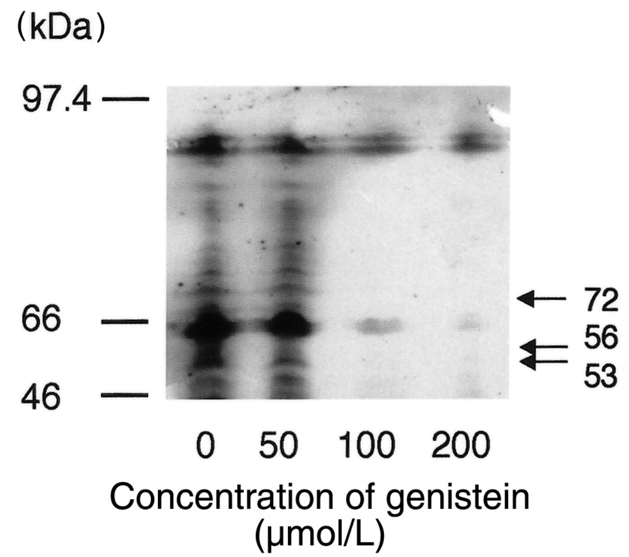

Fig. 3 Inhibitory effect of genistein on tyrosine phosphorylation induced by FcγRII cross-linking. KU812F cells (1×10^7 /mL) were treated with IV.3 (10 μg/mL) and incubated in the absence or presence of genistein (50–200 μmol/L). Cells were then stimulated with goat antimouse IgG $F(ab')_2$ (GAM; 20 μg/mL) for 3 min. Tyrosine phosphorylation was determined by immunoblotting as described in Methods. Arrows indicate the position of 53, 56 and 72 kDa, respectively. Data are representative of five independent experiments.

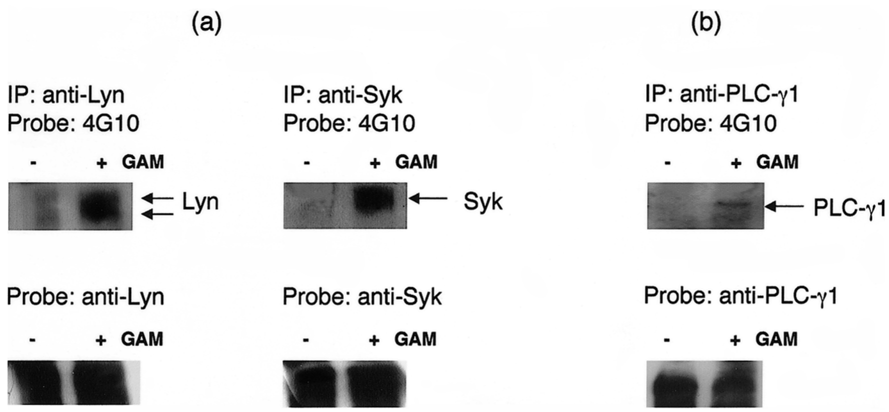

Fig. 4 Tyrosine kinases and phospholipase C-γ1 (PLC-γ1) associated with signal pathway activated by FcγRII cross-linking. (a) Tyrosine kinases (Lyn, Syk) and (b) PLC-γ1 were immunoprecipitated from activated KU812F cells using anti-Lyn, anti-Syk and anti-PLC-γ1 antisera, respectively. After sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrotransfer, precipitates were immunoblotted with anti-phosphotyrosine (4G10). To verify equal loading, blots were also reprobed with antibodies against the proteins themselves. The positions of Lyn (53 and 56 kDa), Syk (72 kDa) and PLC-γ1 (135 kDa) are indicated. Data are representative of three independent experiments. IP, immunoprecipitation; GAM, goat antimouse IgG $F(ab')_2$.

intracellular calcium after cross-linking of FcγRII was completely blocked by 200 μmol/L genistein.

Tyrosine phosphorylation induced by FcγRII cross-linking in KU812F cells

Although much is known about the functional aspects of Fc receptors (FcR), signaling events occurring after FcR cross-linking are not well understood. The above results suggest the involvement of tyrosine kinase in the signaling events induced by FcγRII cross-linking. We investigated the tyrosine phosphorylation of cellular proteins following FcγRII stimulation. When we added GAM to cross-link FcγRII, tyrosine phosphorylation occurred within 1 min (Fig. 2). Phosphorylated proteins in the 53, 56 and 72 kDa ranges were detected. This phosphorylation lasted 3 min and declined after 5 min. Treatment with IV.3 alone was unable to stimulate tyrosine phosphorylation (data not shown).

Effect of genistein on tyrosine phosphorylation induced by FcγRII cross-linking

We examined the effect of genistein on tyrosine phosphorylation that was induced by FcγRII cross-linking. Genistein inhibited the phosphorylation of multiple proteins, including 53, 56 and 72 kDa proteins, in a dose-dependent manner (Fig. 3).

Tyrosine kinases associated with the signal pathway activated by FcγRII cross-linking in KU812F cells

Recent studies have demonstrated that multiple substrates, including Src and Syk family tyrosine kinases, are involved in the signal transduction mediated by many FcR.[11,14] We investigated whether Lyn (53, 56 kDa) and Syk (72 kDa), which belong to the Src and Syk families, respectively, are activated by FcγRII cross-linking. Tyrosine phosphorylation of each substrate was detected only when it was activated by FcγRII cross-linking (Fig. 4a). Because tyrosine phosphorylation of PLC-γ1 caused the release of intracellular Ca^{2+} stores,[15] we also examined whether PLC-γ1 was activated. Immunoblotting using 4G10 clearly showed the tyrosine phosphorylation of PLC-γ1 induced by FcγRII cross-linking (Fig. 4b).

Discussion

We have reported previously that both KU812F cells and human peripheral blood basophils induce intracellular signaling events, such as calcium mobilization, following FcγRII cross-linking.[6] The data presented here clearly show that tyrosine phosphorylation is necessary for the signaling pathway via FcγRII and tyrosine phosphorylation of Lyn, Syk and PLC-γ1, at least, is actively involved in this signal transduction.

Engagement of FcR on hematopoietic cells results in the transduction of a signal leading to activation of tyrosine kinases, elevation of intracellular calcium levels, release of inflammatory mediators and transcription of cytokine genes.[4,16–18] It is agreed that human FcγR can be divided into three major classes (I, II and III) on the basis of differences in their structures and affinities for IgG. Furthermore, FcγRII can be divided into three subclasses: A, B and C.[19] Recent studies have shown that the intracellular domain of FcγRII contains a conserved amino acid motif; type IIA and IIC contain the immunoreceptor tyrosine-based activation motif (ITAM)[4,16,20] and type IIB contains the immunoreceptor tyrosine-based inhibition motif (ITIM) or the AENTITY motif.[21] More recently, it has been reported that cross-linking of FcγRII downregulates the signal of FcεRI on mouse B cells.[22] Moreover, many reports have indicated that FcγRIIB may play an important role in immune regulation.[23] To examine whether tyrosine phosphorylation is necessary for elevation of intracellular calcium levels induced by cross-linking of FcγRII, calcium mobilization was examined in the presence of genistein. Genistein[24] is a derivative of isoflavones and a tyrosine kinase inhibitor. Genistein inhibited the elevation of intracellular calcium levels induced by cross-linking of FcγRII in a dose-dependent manner. To confirm tyrosine phosphorylation following FcγRII stimulation, we next performed immunoblot analysis using antiphosphotyrosine monoclonal antibody (4G10) as a probe. Rapid tyrosine phosphorylation was observed and this phosphorylation was inhibited by genistein. These data suggest that the involvement of tyrosine kinases is required to initiate FcγRII-mediated signal transduction in human basophils.

Lyn is a Src family tyrosine kinase containing Src homology (SH) 1–3 domains and Syk is a Syk family tyrosine kinase containing two SH2 domains.[25] These SH2 domains, present in tyrosine kinases and many other molecules, bind to specific phosphorylated tyrosine residues in the assembly of signaling complexes.[25,26] In different cell systems, FcγRIIA and FcεRI activate common sets of tyrosine kinases, such as Lyn and Syk through ITAM.[12–14,26–29] Syk SH2 preferentially interacts with the γ-subunits, whereas Lyn SH2 binds the β-subunit of FcεRI.[26] We have demonstrated that cross-linking of FcγRII induces activation of Lyn, Syk and PLC-γ1 in KU812F cells. Phospholipase C-γ1 is known to be a substrate for Syk[30] and one of the several PLC isoforms that convert phosphatidyl inositol 4,5-bisphosphate to diacylglycerol and inositol trisphosphate, leading to the activation of protein kinase C and the release of intracellular Ca^{2+} stores, respectively.[15] Thus, FcγRII stimulation may activate KU812F cells to induce production and/or release of inflammatory mediators. However, cross-linking of FcγRII on KU812F cells that express FcγRIIA mRNA as well as FcγRIIB mRNA could not provoke histamine release, as reported previously.[6] Moreover, cross-linking of FcγRII on KU812F cells failed to induce superoxide anion production (data not shown).

In summary, our study has shown that FcγRII-mediated signal transduction in KU812F cells requires involvement of tyrosine kinases, especially Lyn and Syk, in an initiating phase. These findings may be useful for further analyses of FcγRII-mediated signal transduction and functions in human basophils.

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