Regulation of Highly Cytokinergic IgE-Induced Mast Cell Adhesion by Src, Syk, Tec, and Protein Kinase C Family Kinases

Jiro Kitaura, Koji Eto, Tatsuya Kinoshita, Yuko Kawakami, Michael Leitges, Clifford A. Lowell and Toshiaki Kawakami


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*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Mast cells play a critical role in IgE-dependent immediate hypersensitivity. Recent studies have shown that, contrary to the traditional view, binding of monomeric IgE to FcεRI results in a number of biological outcomes in mast cells, including survival. However, IgE molecules display heterogeneity in inducing cytokine production; highly cytokinergic (HC) IgEs cause extensive FcεRI aggregation, which leads to potent enhancement of survival and other activation events, whereas poorly cytokinergic (PC) IgEs can do so inefficiently. The present study demonstrates that HC, but not PC, IgEs can efficiently induce adhesion and spreading of mouse mast cells on fibronectin-coated plates in slow and sustained kinetics. HC IgE-induced adhesion through β1 and β2 integrins promotes survival, IL-6 production, and DNA synthesis. Importantly, we have identified Lyn and Syk as requisite tyrosine kinases and Hck, Btk, and protein kinase C θ as contributory kinases in HC IgE-induced adhesion and spreading, whereas protein kinase C ε plays a negative role. Consistent with these results, Lyn, Syk, and Btk are activated in HC IgE-stimulated cells in a slower but more sustained manner, compared with cells stimulated with IgE and Ag. Thus, binding of HC IgEs to FcεRI induces adhesion of mast cells to fibronectin by modulating cellular activation signals in a unique fashion. The Journal of Immunology, 2005, 174: 4495–4504.

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1 This study was supported by Public Health Service Grants AI50209 and AI/GM33848 from the National Institutes of Health (to T.K.). Part of this study was performed during a tenure of fellowship from the American Heart Association (to T.K.).

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3 Abbreviations used in this paper: PTK, protein-tyrosine kinase; PLC, phospholipase C; DAG, diacylglycerol; IP3, inositol 1,4,5-trisphosphate; ECM, extracellular matrix; PKC, protein kinase C; SCF, stem cell factor; FN, fibronectin; HC, highly cytokinergic; PC, poorly cytokinergic; TNP, trinitrophenyl; BMMC, bone marrow-derived mast cell.
of IgE-mast cell binding as a sensitization step into the new one that monoclonic IgE can induce activation and survival of mast cells (20). Indeed, all monoclonal IgE molecules tested so far not only promote survival, but different IgE molecules also induce varied levels of activation. At one extreme end of spectrum, some IgE molecules, termed highly cytokinergic (HC) IgEs, induce the production and secretion of various cytokines and other activation events, including degranulation, whereas other IgE molecules, termed poorly cytokinergic (PC), do so very inefficiently (21).

A recent study showed that three HC IgE molecules can trigger mast cell adhesion to FN with sustained kinetics and mainly via the $\alpha_\beta_1$ integrin, and adhesion to FN enhances IgE-induced signaling, leading to the increased survival and cytokine production (22). In this study, we have extended these previous observations and showed that mononeric HC, but not PC, IgEs can induce $\beta_1$ and $\beta_2$ integrin-mediated mast cell adhesion and spreading onto FN-coated plates. We have identified Lyn and Syk as requisite signaling molecules for these events. Minor roles for Hck, Btk, PKCe, and PKC$\theta$ for adhesion were also found. Changes in the activation statuses of membrane-proximal PTKs and other signaling molecules were consistent with the slow but sustained process of HC IgE-induced adhesion and spreading.

Materials and Methods

IgEs and Abs

Anti-DNP mouse IgE (H1 DNP-epsilon-206, abbreviated hereafter as 206) and H1 DNP-epsilon-26 mAbs (23) were purified as described previously (21). Antidansyl, anti-trinitrophenyl (TNP) (IgE-3), anti-TNP (C48-2), and anti-TNP (25) mouse IgE mAbs were purchased from Sigma-Aldrich. We used highly purified monomeric IgE preparations (gel filtration-purified as the final purification step) in many key experiments shown in Figs. 1, 2, 5, and 6.

DNP IgE (SPE-7) was purchased from Sigma-Aldrich. We used highly purified monomeric IgE preparations for these events. Minor roles for Hck, Btk, PKC$\alpha$, and PKC$\theta$ for adhesion were also found. Changes in the activation statuses of membrane-proximal PTKs and other signaling molecules were consistent with the slow but sustained process of HC IgE-induced adhesion and spreading.

Results

Highly cytokinergic IgE-induced mast cell adhesion

Given the heterogeneity among IgE molecules in the capacity to induce FceRI aggregation (21), we first tested whether both HC and PC IgEs can induce mast cell adhesion to plastic wells coated with FN. In comparison, mast cells were also stimulated with a PC (206) IgE and Ag (this mode of stimulation hereafter termed IgE+Ag), SCF, or 1 mM MnCl$_{2}$ (a direct activator of integrins). Incubation of BMMCs with 5 $\mu$g/ml of various IgE molecules for 60 min led to either high- or very low-level cell adhesion (Fig. 1A). As shown by Lam et al. (22), efficient adhesion (~50% of input cells) was observed with HC IgEs. However, poor adhesion (<5–10% of input cells) was observed with PC IgEs for up to 3 h of incubation. In contrast with varied levels of cytokine production induced by different HC IgEs, adhesion induced by various HC IgEs was uniformly high. For example, IL-6 production induced by C38-2 or 26 IgE was only one-twentieth or one-seventh, respectively, that by SPE-7 IgE (21), but all of these HC IgEs induced similar levels in adhesion. Similarly, high-level adhesion

Flow cytometry

Flow cytometric analysis of receptor expression and apoptosis was performed as described previously (21).

Adhesion assay

Ninety-six-well microtiter plates (no. 353072; BD Falcon) were coated with 20 $\mu$g/ml bovine FN or BSA (Sigma-Aldrich) in PBS for 12 h at 4°C, blocked with 4% BSA in PBS for 1 h at 37°C, and then washed twice with assay medium (RPMI 1640 medium, 1% BSA, and 20 mM HEPES) just before assay. For IgE+Ag stimulation, BMMCs were sensitized overnight with 0.5 $\mu$g/ml 206 IgE, washed twice and incubated with DNP$_3$-HSA, and resuspended at 5 $\times$ 10$^5$ cells/ml in assay medium. For the other stimulations, naive BMMCs were used. The cells in 100 $\mu$l were added to each FN (or BSA)-coated well followed by the addition of a stimulant. After incubation at 37°C for the indicated periods, assay wells were rinsed with an assay buffer to remove nonadherent cells and finally filled with 100 $\mu$l of trypsin for 20 min to measure the number of adherent cells using trypsin blue staining under microscopy. The degree of adhesion is calculated by dividing the number of the adherent cells by the number of input cells. In assays with Abs, BMMCs were preincubated with 20 $\mu$g/ml Abs for 30 min at 37°C before the addition of the cells and Abs to the plate.

Confocal microscopy

BMMCs (2.5 $\times$ 10$^5$ cells in 0.5 ml) were seeded into coverslips placed in 12-well plates coated with 20 $\mu$g/ml FN. After stimulation at 37°C for 30 or 60 min, coverslips were washed twice with PBS and fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. The cells were washed with PBS, permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature, washed with PBS, and incubated with 10% goat serum in PBS for 1 h at room temperature. The cells were stained with rhodamine-phalloidin (Molecular Probes) for 45 min at room temperature, washed with PBS, and finally stained with goat anti-mouse IgG conjugated with fluorescein (BioSource International) for 60 min at room temperature. Washed cells were analyzed with a Leica fluorescence microscope equipped with a laser scanning confocal system (MRC 1024; Bio-Rad). Images were processed in Photoshop 7.0 (Adobe) as described previously (33, 34).

Measurements of cytokines

Supernatants of BMMCs that had been incubated with IgE were measured by ELISA for IL-6 and TNF-$\alpha$ (BD Pharmingen).

Immunoblotting analysis

Mast cells were lysed in 1% Nonidet P-40-containing lysis buffer (20 mM Tris-HCl (pH 8.0), 0.15 M NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM PMSF, 10 $\mu$g/ml aprotinin, 10 $\mu$g/ml leupeptin, 25 $\mu$g/ml n-pitrophenyl p'-guanidinobenzoate, 1 $\mu$M peptatin, and 0.1% sodium azide). Cell lysates were analyzed by SDS-PAGE, followed by immunoblotting. Proteins reactive with primary Ab were visualized with an HRP-conjugated secondary Ab and ECL reagents (PerkinElmer).

In vitro kinase assays

Syk and JNK1 molecules were immunoprecipitated from HC IgE- or IgE+Ag-stimulated mast cells with respective Abs. Immune complexes were incubated with a substrate (GST-HS1 for Syk and GST-c-Jun for JNK1) in the kinase buffer in the presence of 10 $\mu$Ci of [$\gamma$-32P]ATP. Reaction products were analyzed by SDS-PAGE and autoradiography of dried gels.

Results

HC, but not PC, IgEs induce mast cell adhesion to FN efficiently

Given the heterogeneity among IgE molecules in the capacity to induce FceRI aggregation (21), we first tested whether both HC and PC IgEs can induce mast cell adhesion to plastic wells coated with FN. In comparison, mast cells were also stimulated with a PC (206) IgE and Ag (this mode of stimulation hereafter termed IgE+Ag), SCF, or 1 mM MnCl$_2$ (a direct activator of integrins). Incubation of BMMCs with 5 $\mu$g/ml of various IgE molecules for 60 min led to either high- or very low-level cell adhesion (Fig. 1A). As shown by Lam et al. (22), efficient adhesion (~50% of input cells) was observed with HC IgEs. However, poor adhesion (<5–10% of input cells) was observed with PC IgEs for up to 3 h of incubation. In contrast with varied levels of cytokine production induced by different HC IgEs, adhesion induced by various HC IgEs was uniformly high. For example, IL-6 production induced by C38-2 or 26 IgE was only one-twentieth or one-seventh, respectively, that by SPE-7 IgE (21), but all of these HC IgEs induced similar levels in adhesion. Similarly, high-level adhesion...
was observed when BMMCs were stimulated with IgE+Ag, SCF, or MnCl2 (Fig. 1A), as shown previously (10, 35, 36). So, we decided to further investigate HC IgE-induced mast cell adhesion and compare it to those by other stimuli. In most following experiments, we used SPE-7 IgE as a representative of the HC IgE.

We next compared kinetics of adhesion induced by HC IgE with those by IgE+Ag, SCF, and MnCl2. Compared with IgE+Ag and SCF stimulation, SPE-7 IgE-induced adhesion was substantially slower; this stimulation needed 30 min before 30% adhesion was achieved, and the peak adhesion (~70%) was only seen at 2 h (Fig. 1B). However, high-level adhesion induced by SPE-7 IgE was maintained for a longer time with >50% adhesion seen at the last tested time point (30 h) of the experiment. MnCl2-induced adhesion also exhibited slow kinetics, but high-level adhesion was not maintained. Therefore, the slow but sustained adhesion to FN-coated plates is characteristic of HC IgE-induced mast cell activation, which is distinctly different from that induced by other stimuli.

**HC IgE-induced mast cell adhesion to FN is mediated through β1 and β2 integrins**

Mast cell adhesion to FN-coated plates induced by HC IgEs, IgE+Ag and SCF was shown to be mediated predominantly by the high-affinity state of integrin αβ1 (9–11, 22). As shown previously (9, 35), integrins β1, β2, and βδ are expressed on the surface of BMMCs (Fig. 2A). Expression of these integrins was not changed by stimulation for 1–3 h with HC IgE, SCF, or IgE+Ag (data not shown). We tested whether these integrins are involved in HC IgE-induced adhesion to FN by preincubating BMMCs with function-blocking Abs against specific integrin subunits for 30 min before being seeded into FN-coated plates containing 5 µg/ml SPE-7 IgE or 100 ng/ml SCF or PBS. Cell adhesion was measured 60 min after incubation.

**HC IgE-triggered inside-out signaling involves Src, Syk, Tec, and PKC family kinases**

FcεRI aggregation induces the activation of Src, Syk, and Tec family PTKs in the vicinity of plasma membranes (3, 37, 38). Therefore, we next examined the roles of these PTKs in HC IgE (SPE-7 or 26)-induced mast cell adhesion to FN using mast cells deficient in these kinases. As shown previously (22), Lyn deficiency abrogated HC IgE-induced adhesion (Fig. 3A). Hck deficiency also reduced adhesion by 22–35% (Fig. 3B), whereas Fyn deficiency did not significantly affect HC IgE-induced adhesion but incompletely (Fig. 2C). Anti-integrin α5 inhibited BMMC adhesion to a lesser extent, and a combination of anti-α5 and anti-α8 inhibited HC IgE-induced adhesion almost to basal levels.

**FIGURE 1.** HC IgEs induce mast cell adhesion on FN-coated plates in uniquely slow but sustained kinetics. BMMCs from wild-type mice with a mixed genetic background of 129 and B6 strains were incubated with 5 µg/ml of various monoclonal IgEs, 100 ng/ml SCF, or 1 mM MnCl2 for 60 min (A) or the indicated periods (B). In the case of IgE+Ag stimulation, BMMCs were first sensitized by an overnight culture with 0.5 g/ml of various monoclonal IgEs, 100 ng/ml SCF, or 1 mM MnCl2 for 60 min (A) or the indicated periods (B). Adherent cells were counted.

**FIGURE 2.** Expression of β integrins and the involvement of integrins in HC IgE-induced mast cell adhesion. A, Expression of β integrins was measured by flow cytometry. B and C, Wild-type BMMCs were preincubated with function-blocking Abs against specific integrin subunits for 30 min before being seeded into FN-coated plates containing 5 µg/ml SPE-7 IgE or 100 ng/ml SCF or PBS. Cell adhesion was measured 60 min after incubation. * and **, p < 0.05 and p < 0.01 (vs the control Ab-treated sample by Student's t test), respectively.
did not adhere to FN upon IgE+Ag stimulation (Fig. 3E). In contrast, adhesion induced by SCF or MnCl₂ was almost intact in Syk-deficient cells.

Btk activity is regulated by Lyn and Syk (44–46) and active Btk in concert with Syk phosphorylates and activates PLC-γ2 (47, 48). Btk deficiency also significantly reduced HC IgE-induced adhesion (Fig. 3F). IgE+Ag-induced adhesion was affected strongly by Btk deficiency at low Ag doses. However, unlike cells deficient in Src or Syk family kinases, SCF-stimulated, Btk-deficient cells adhered as efficiently as wild-type cells.

PKCβ is under the control of Lyn, Syk, and Btk through PLC-γ1/2 (46) and plays a critical role in degranulation and cytokine production in FcεRI-cross-linked mast cells (46, 49–51). PKCβ deficiency resulted in lower basal adhesiveness to FN compared with wild-type cells (Fig. 4, upper panel). However, HC IgE-induced adhesion was not significantly affected by PKCβ deficiency. SCF- or PMA-induced adhesion was not affected either. Interestingly, IgE+Ag-induced adhesion was reduced slightly at low concentrations of Ags, a defect shared by Lyn-deficient and Btk-deficient cells.

PKCθ, when overexpressed, can play a role in the activation of ERK, IL-3 gene transcription, and degranulation in response to FcεRI aggregation (52). HC IgE-induced adhesion was reduced at suboptimal IgE concentrations by PKCθ deficiency, whereas SCF- or PMA-induced adhesion was not affected (Fig. 4, middle panel). Similar to cells deficient in Lyn, Btk, or PKCθ, IgE+Ag-induced adhesion was reduced at low concentrations of Ags in PKCθ-deficient cells.

In contrast with PKCθ-deficient mast cells, HC IgE-induced adhesion was not reduced but rather increased at a suboptimal dose of 1 μg/ml in PKCθ-deficient cells (Fig. 4, lower panel). Similarly, IgE+Ag-induced adhesion also tended to be increased at low Ag concentrations. Therefore, different members of the PKC family play redundant as well as opposing functions in HC IgE- and IgE+Ag-induced adhesion.

In the above experiments, none of the gene-targeted mast cells used exhibited changes in surface expression of FcεRI, c-Kit, or integrin β₇ (data not shown). Therefore, the changes in FN adhesiveness in mutant cells seem to reflect the changes in integrin affinity/avidity. We did not see any significant differences between SPE-7 and 26 IgEs with any of the kinase-deficient cells. Our data collectively suggest that HC IgE-induced integrin β₇ (and β₆)–dependent adhesion requires Src (Lyn and Hck) and Syk family kinases. Btk and PKCθ play a minor role in these adhesions, whereas PKCε plays a negative regulatory role.

HC IgE-induced mast cell spreading is dependent on Src and Syk family kinases

FcεRI aggregation induces actin polymerization and reorganization of the cytoskeleton leading to morphological changes characterized by actin plaques, membrane ruffling (lamellipodia), and filopodia (53). We examined morphological changes in FN-adherent mast cells induced by HC IgE, IgE+Ag, and SCF by visualizing F-actin and tyrosine-phosphorylated proteins under a confocal microscope. Unstimulated BMMCs displayed a round shape with few lamellipodia or filopodia. Upon HC IgE stimulation, cells became spread with numerous filopodia and lamellipodia, in which F-actin was colocalized with tyrosine-phosphorylated proteins (Fig. 5). IgE+Ag and SCF also induced a morphology characterized by the presence of abundant lamellipodia and some filopodia.
Consistent with the data shown in Fig. 3, Fyn-deficient cells exhibited morphologies indistinguishable from wild-type cells. By contrast, HC IgE-stimulated, Lyn-deficient cells had less abundant filopodia than similarly stimulated wild-type cells (Fig. 5A). Lyn/Fyn-doubly deficient cells had even fewer filopodia and were less spread upon HC IgE stimulation. Lyn deficiency also caused severe defects in spreading induced by IgE+Ag. However, SCF-stimulated, Lyn-deficient and Lyn/Fyn-deficient cells exhibited abundant filopodia and lamellipodia. Lyn/Fyn-doubly deficient cells stimulated by HC IgE or IgE+Ag exhibited a morphology similar to unstimulated wild-type cells, whereas, upon SCF stimulation, syk−/− cells exhibited the filopodia-rich shape similar to wild-type cells (Fig. 5B). These results collectively indicate that both Lyn and Syk, but not Fyn, are required for HC IgE-induced mast cell spreading on FN-coated plates, similar to adhesion. Syk was also necessary for IgE+Ag-induced spreading similar to adhesion. In addition, IgE+Ag-induced spreading was also dependent on Lyn even under high Ag conditions where Lyn was dispensable for adhesion.

**Adhesion to FN enhances HC IgE-induced survival, cytokine production, and DNA synthesis**

Both HC IgE and IgE+Ag appear to induce aggregation of FceRI, leading to activation and/or survival (21, 54). As previously shown (22), stronger survival effects of HC, but not PC, IgEs were seen on FN-adherent cells upon growth factor withdrawal, compared with nonadherent cells (Fig. 6A). We also observed an increased secretion of IL-6 from HC IgE- and IgE+Ag-stimulated BMMCs on FN-coated plates compared with similarly stimulated cells on BSA-coated plates (Fig. 6B). In contrast with Lam et al. (22), enhanced DNA synthesis was seen in HC IgE-stimulated cells on FN (Fig. 6C).

**Activation of PTKs by HC IgE vs IgE+Ag**

It is reasonable to assume that HC IgE and IgE+Ag induce similar, although not necessarily identical, intracellular signals because both stimuli engage the same receptor, FceRI, leading to receptor aggregation. However, some differences in their consequences have been noticed: survival effects were observed by a wide range of IgE concentrations without suppression by very high (>50 μg/
ml IgE concentrations (15), whereas only a narrow range of weak-to-moderate stimulation by IgE+Ag could induce survival (54); degranulation induced by HC IgE has a short lag period before its initiation whereas that by IgE+Ag is very fast (21); furthermore, HC IgE-induced adhesion is slow but sustained, whereas IgE+Ag-induced adhesion is quick and short-lived (Fig. 1B). In addition, we have found that Lyn and Hck are necessary for HC IgE-induced adhesion, whereas these PTKs are dispensable for IgE+Ag-induced adhesion (Fig. 3). These observations suggest that there are qualitative as well as quantitative differences in signaling induced by HC IgE and IgE+Ag, whereas no observable signaling events were induced by PC IgEs (15). To gain insight into signaling mechanisms underlying the different adhesive and other outcomes by HC IgE and IgE+Ag, we performed immunoblotting analysis of tyrosine-phosphorylated proteins in HC IgE- and IgE+Ag-stimulated cells. When BMIMCs were stimulated by IgE+Ag on BSA-coated plates (to which the cells did not adhere), tyrosine phosphorylation of cellular proteins peaked at ~1–3 min (data not shown) and then returned to basal levels within 30 min, which was exactly the same as that in IgE+Ag-stimulated cells in suspension (Figs. 7, C and D). However, when cells were stimulated by IgE+Ag on FN-coated plates, tyrosine phosphorylation of cellular proteins peaked at ~30 min and then returned to basal levels within 2 h of stimulation (Fig. 7, C and D). Although the pattern of tyrosine phosphorylation was (Fig. 7, A and B). However, unlike IgE+Ag stimulation, HC IgE-induced phosphorylation in FN-adherent cells was observed easily at 4 h, the last time point of measurement (Fig. 7B). These analyses revealed firstly that a peak in tyrosine phosphorylation precedes that in adhesion: tyrosine phosphorylation peaked ~1 h and adhesion reached the peak level ~2 h in SPE-7 IgE-treated cells, whereas tyrosine phosphorylation peaked ~30 min and adhesion reached the peak level ~1 h in IgE+Ag-stimulated cells. Secondly, it was shown that adhesion is sustained long after tyrosine phosphorylation wanes and returns to baseline. Parenthetically, no tyrosine phosphorylation was observed in PC IgE-stimulated cells on either FN- or BSA-coated plates (data not shown).

Lyn is a major PTK that phosphorylates numerous proteins in IgE+Ag-stimulated mast cells (40, 55). Therefore, we compared kinase activity of Lyn and Lyn-dependent PTKs, Syk and Btk, in HC IgE- and IgE+Ag-stimulated mast cells. Lyn activation, as measured by immunoblotting for phosphorylation on Tyr396 in the activation loop, also exhibited slower kinetics but an increased magnitude upon stimulation by HC IgE on FN-coated plates than that by IgE+Ag (Fig. 7E). Similar to the kinetics of general tyrosine phosphorylation, Lyn phosphorylation also peaked ~60 and 30 min in SPE-7 IgE- and IgE+Ag-stimulated adherent cells, respectively. This is consistent with the role of Lyn in tyrosine phosphorylation of many cellular proteins. Despite the importance of Hck in HC IgE-induced adhesion, low expression of Hck hampered similar analysis (data not shown). Enhanced activation by HC IgE vs IgE+Ag was even more remarkable in Syk kinase activity (Fig. 7E and data not shown). The same tendency, albeit to a lesser degree, was seen in Btk activity, as measured for phosphorylation on Tyr742 (autophosphorylation site). Activation of Lyn, Syk, and Btk was much more transient in both HC IgE- and IgE+Ag-stimulated nonadherent cells (Fig. 7E and data not shown). Parenthetically, the presence of FN that leached out into cell lysates precluded efficient immunoprecipitation of some proteins, particularly Hck, Fyn, and Lyn, making it difficult to accurately measure these kinase activities in vitro (data not shown). However, slower and more sustained activation of Lyn, together with stronger activation of Syk and Btk, could explain the slower and sustained adhesion of HC IgE-stimulated mast cells on FN, compared with IgE+Ag-stimulated cells. The differences in the magnitude of activity of these PTKs, particularly Syk, between adherent and nonadherent cells could account for the increased survival, IL-6 production, and DNA synthesis in adherent cells.

**Downstream signaling events induced by HC IgE vs IgE+Ag**

PLC-γ hydrolyzes phosphatidylinositol 4,5-bisphosphate into DAG and IP₃. IP₃ induces Ca²⁺ release from intracellular stores, and DAG (with or without Ca²⁺) activates several PKC isofoms. Because Ca²⁺ was shown to be critical for HC IgE-induced mast cell adhesion (22), we estimated the activity of PLC-γ, a major PLC-γ isofom in mast cells. PLC-γ2 phosphorylation on Tyr1217 was more pronounced in adherent cells than in nonadherent cells upon HC IgE and IgE+Ag stimulation (Fig. 7E), in line with the previous studies that Lyn is required for Ca²⁺ responses (5, 22, 40, 55), and PLC-γ2 phosphorylation is under a concerted regulation of Syk and Btk (47, 48).

Mast cell survival is increased by adhesion to FN (10). ERKs of the MAPK family are implicated as a critical signal transducer for survival in various cell types (56), including HC IgE-stimulated mast cells (57). Akt (= protein kinase B) is another serine/threonine kinase crucial for survival in many cell types (reviewed in Refs. 58–60). By contrast, JNK and p38, two other MAPK subfamilies, are involved in apoptosis under certain conditions (56). Therefore, we compared activities of these kinases in adherent vs nonadherent cells upon HC IgE and IgE+Ag stimulation. Consistent with their presumed role in HC IgE-induced survival (57), activation of ERK1 and ERK2, as evaluated by phosphorylation at their activation loops, was more prolonged in adherent cells than in nonadherent cells upon HC IgE stimulation (Fig. 7F, upper and lower panels). By contrast, ERK activation was transient in IgE+Ag-stimulated cells under conditions that did not enhance survival, just like that in SCF-stimulated cells (Fig. 7F, lower panel). These results, although consistent with the role for ERKs in IgE-mediated survival, do not prove that HC IgE-dependent increment in survival in adherent cells over nonadherent cells is due to the prolonged ERK activation. By contrast, p38 activities were comparable in adherent and nonadherent cells. Activation of JNK1 and Akt was strongly induced by IgE+Ag, but their activities returned to baseline within 30 min, and no difference in their activities was observed between adherent and nonadherent cells (Fig. 7F). Activities of JNK1 and Akt in HC IgE-stimulated cells were much lower than in IgE+Ag-stimulated cells and indistinguishable between adherent and nonadherent cells. Consistent with no observed tyrosine phosphorylation, activation of MAPKs or Akt was not observed in PC IgE-stimulated cells on FN- or BSA-coated plates (data not shown). These results suggest that the prolonged activation of ERKs, but not JNK, p38, or Akt, is involved in HC IgE-induced survival.

**Discussion**

The present study demonstrates that HC IgEs can induce adhesion and spreading of mouse mast cells on FN-coated plates in slow and sustained kinetics, whereas PC IgEs cannot do so efficiently. Similar to IgE+Ag- and SCF-induced adhesion (14, 36), HC IgE-induced adhesion was mediated mainly through β₁ integrin, as shown recently (22). In addition, our study has identified β₂ integrin as another mediator of mast cell adhesion. Importantly, we have identified Lyn and Syk as requisite proximal PTKs and Hck, Btk, and PKCθ as contributory kinases in HC IgE-induced adhesion. Consistent with these results, analysis of activation status of signaling proteins has revealed that Lyn phosphorylation on Tyr396...
is induced in a slower but more sustained fashion in HC IgE-stimulated adherent cells than in IgE+Ag-stimulated adherent cells. Lyn-dependent Syk and Btk were more strongly activated in HC IgE-stimulated cells than in IgE+Ag-stimulated cells. Activation of Lyn, Syk, and Btk was much more transient in nonadherent cells than in adherent cells. These signaling data support the data on the increased survival, IL-6 production/secretion, and DNA synthesis in HC IgE-stimulated adherent cells, compared with similarly stimulated nonadherent cells.

Mast cells do not efficiently adhere and spread on FN without activation through FcεRI or c-Kit. Both stimuli use β1 integrin as the major adhesion receptor. The engagement of integrins by ECM...
ligands triggers outside-in signals that collaborate with immunoreceptor-initiated signals (including inside-out signals that activate integrins) to determine cell fate and function (61). Both β1 integrin-initiated outside-in and FcεRI-initiated inside-out signals use PTKs of the Src family. Importantly, Src family PTKs directly interact with the integrin β cytoplasmic domains: integrin β1 binds to Lyn, Hck, and c-Yes but not c-Src, Fyn, or c-Fgr (62).

These binding data and integrin-clustering experiments suggest that integrin-Src family PTK interactions initiate Src PTK activation following integrin clustering (outside-in signaling). Interestingly, deficiencies in integrin β1-binding Lyn or Hck, but not integrin β1-nonbinding Fyn, result in defects in HC IgE-induced mast cell adhesion. Although Lyn and Hck may not work only by binding to the integrin, it is tempting to speculate that Src family PTKs bound to a integrin β1 subunit are used in HC IgE-recruited, inside-out signaling as well. If this is the case, inside-out signaling and outside-in signaling can be well synchronized at the level of Src family PTKs during the slow, sustained signaling process in HC IgE-stimulated cells. However, inside-out signals generated by IgE+Ag stimulation, which induces a strong but transient activation of the PTKs, might not be synchronized with outside-in signals as efficiently as in HC IgE-stimulated cells. This possibility is supported by the stronger Lyn phosphorylation on Tyr106 in HC IgE-stimulated adherent cells, compared with IgE+Ag-stimulated adherent cells (Fig. 7E). The difference between HC IgE- and IgE+Ag-stimulated adherent cells was even more remarkable in the kinase activity of Syk, a Lyn substrate (Fig. 7E).

Although HC IgE-induced adhesion data nicely correlate the requirement of Lyn and Hck with integrin-Src family PTK interactions, it was somewhat unexpected that Fyn deficiency did not lead to a defect in HC IgE- or IgE+Ag-induced adhesion. Thus, earlier studies showed that PI3K is required for HC IgE-, IgE+Ag-, and SCF-induced adhesion (22, 36, 63, 64), and Parravicini et al. (5) proposed that Fyn regulates PI3K activity through Gab2 phosphorylation. However, in our unpublished study using Fyn-deficient mice that had been extensively backcrossed into the C57Bl/6 strain, we could not see such drastic defects in degranulation, cytokine production, or several signaling events as described by Parravicini et al. (5), raising the possibility that the differences between the two studies might be attributable to genetic background. Interestingly, earlier studies suggested multiple distributions of PI3K: FcεRI cross-linking increases PI3K activity in anti-LAT, anti-FceRIβ, and anti-Gab2 immune complexes (65).

Therefore, the PI3K pathway under the control of Lyn (and Hck) might be required for mast cell adhesion. In this regard, immunoelectron microscopic analysis identified two types of cell surface areas rich in signaling molecules in FcεRI-cross-linked cells: osmiophilic patches termed the primary signaling domain contain FcεRI, Gab2, and PI3K among others, and another type termed the secondary signaling domain contains LAT and PI3K (65).

Mast cell adhesion and spreading are intimately related events. As expected from adhesion data, mast cell spreading induced by HC IgE requires Lyn and Syk but not Fyn. Interestingly, however, IgE+Ag-induced spreading is dependent not only on Syk but also on Lyn, in contrast with IgE+Ag-induced adhesion in which Lyn is dispensable. Therefore, the requirement for Lyn in inside-out signaling seems to depend on how FcεRI is stimulated.

This and previous studies demonstrate that Lyn is a positive regulator of HC IgE-induced mast cell adhesion to FN. Other Lyn-mediated positive regulatory aspects of mast cell activation through FcεRI have been shown in a reduction in passive cutaneous anaphylactic responses in Lyn-deficient mice (66) and the delayed Ca2+ responses to IgE+Ag in Lyn-deficient mast cells (40, 55). However, FcεRI-induced secretion of cytokines is enhanced in Lyn-deficient mast cells, indicative of a role in negative regulation possibly via the FcγRIIB1 receptor (40, 55, 67). Enhanced degranulation was also reported in some, but not all, studies of Lyn-deficient mast cells, and a recent study showed that this is due to the increased Fyn activity and leads to an enhanced anaphylactic response in young Lyn-deficient mice (68). Interestingly, Lyn plays a negative regulatory role in adhesion of neutrophils to FN and other ECM proteins and ICAM-1 (69). This may suggest the importance of the cell context in determining the positive vs negative roles of Lyn in adhesion and other cellular functions.

Extracellular Ca2+ has been shown to be required for HC IgE-, IgE+Ag-, and SCF-induced increase in the avidity of α1β1 (22).

Upstream of Ca2+ responses, pharmacological experiments indicate that PLC-γ is also required for HC IgE-, IgE+Ag-, and SCF-induced adhesion (22). These previous findings were underpinned by our results: upstream PTK regulators of PLC-γ activity and Ca2+ response, i.e., Lyn, Syk, and Btk, are all required for optimal adhesion stimulated by HC IgE (Fig. 3). By contrast, deficiencies of these PTKs did not result in defects in SCF-induced adhesion, suggesting that c-Kit, but not Lyn, Syk, or Btk, phosphorylates and activates PLC-γ in SCF-stimulated cells (70). Because PLC-γ phosphorylation is regulated by Btk and Syk (47, 48), the Btk-dependent signaling component for HC IgE-induced adhesion may be mediated by PLC-γ2. However, a Btk-independent component(s) must exist, given that HC IgE-induced adhesion was defective only partially in Btk-deficient cells despite the complete abrogation of adhesion in Syk-deficient cells. Potentially, other Tec family PTKs such as Tec and Itk might be involved in this process.

Downstream of PLC-γ and Ca2+, roles of PKC isoforms in HC IgE-induced adhesion seem complex. Pharmacological studies suggest that conventional PKC isoforms play a positive role in HC IgE- and IgE+Ag-induced adhesion and that some PKC isoform(s), which can be inhibited by a pan-specific PKC inhibitor, plays a negative regulatory role in HC IgE-induced adhesion (22).

Our present study has identified PKCθ and PKCe as the positive and negative regulators, respectively, of HC IgE-induced adhesion. As BMMCs express PKCθ, PKCβ1, and PKCβII among the conventional isoforms, it is speculated that PKCs, which is known to be activated by adhesion via integrin α1β1 (71), may play a positive regulatory function in HC IgE-induced adhesion.

ERKs are implicated in HC IgE-induced survival (57). Our results demonstrate the prolonged activation of these kinases in HC IgE-stimulated adherent cells compared with similarly stimulated nonadherent cells (Fig. 7F), supporting this possibility. The prolonged ERK activation is also consistent with the prolonged and enhanced Syk activity in HC IgE- and IgE+Ag-stimulated adherent cells, compared with similarly stimulated nonadherent cells. Thus, Syk is known to positively regulate ERKs (43). The enhanced Syk activity, particularly in HC IgE-stimulated cells, is compatible with the essential role for Syk in IgE-induced survival (21).

By contrast, our analysis suggests that JNK1, p38, or Akt might not be involved in differential regulation of adhesion, survival, or other activation events by HC IgE vs IgE+Ag. Because activities of these kinases, particularly JNK1, were shown previously to be under the control of Syk and Btk (43, 72), signaling pathways required for adhesion to FN seem to branch out at the level of these PTKs or upstream of them.

The present study demonstrates that mast cell adhesion can be induced by HC, but not PC, IgEs in a uniquely slow and sustained fashion. Recent studies have shown that IgE can be produced not only in lymphoid organs but also in inflammatory mucosal tissues in allergic diseases (73, 74). Therefore, it is tempting to suggest that HC IgE-induced, Lyn- and Syk-dependent mast cell adhesion...
is involved in the recruitment of mast cells to such inflammation sites with high IgE concentrations. Such a possibility warrants additional investigation.

FcεRI stimulation induces secretion of a plethora of chemical, lipid, peptide, and protein mediators, including cytokines and chemokines. Many of them have the capacity to affect adhesion, spreading, and migration of mast cells, as well as other cell types. Our present study focused on overall roles of various kinases on adhesion and spreading. However, it is likely that some of these kinases are involved in adhesion and spreading induced by the mediators secreted by FcεRI stimulation. This is quite likely for HC IgE-induced mast cell adhesion given the prolonged time course of this form of mast cell adhesion. Therefore, this possibility warrants additional investigation.

Acknowledgments

We thank Masato Takeuchi for participating in the initial phase of this study and Wasif Khan, Robert O. Messing, and Dan R. Littman for donating mutant mice. We are greatly indebted to Sanford J. Shattil for allowing K. Eto to work on this project and for reading this manuscript very carefully.

Disclosures

The authors have no financial conflict of interest.

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