

Regulation of Highly Cytokinergic IgE-Induced Mast Cell Adhesion by Src, Syk, Tec, and Protein Kinase C Family Kinases¹

Jiro Kitaura,* Koji Eto,[†] Tatsuya Kinoshita,* Yuko Kawakami,* Michael Leitges,[‡] Clifford A. Lowell,[§] and Toshiaki Kawakami^{2*}

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 β₇ 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.

Mast cells are the major effector cell type in IgE-mediated immediate hypersensitivity. Traditionally, it is thought that mast cells bound to Ag-specific FcεRI, so-called sensitized mast cells, encounter multivalent Ag, and then IgE-bound receptors are aggregated or cross-linked, leading to cell activation (1). Activated mast cells secrete preformed and newly synthesized proinflammatory mediators involved in inflammatory processes of allergy, such as histamine, proteases, lipids, cytokines, and chemokines. The mouse FcεRI consists of an IgE-binding α subunit, a signal-amplifying β subunit, and disulfide-bonded two molecules of signal-generating γ subunit (2). FcεRI aggregation leads to the activation of β subunit-bound Lyn, a prominent Src family protein-tyrosine kinase (PTK)³ in mast cells (3). Lyn phosphorylates the tyrosine residues in the ITAMs of the β and γ subunits (4). Phosphorylated ITAMs of β and γ subunits, respectively, recruit Lyn and Syk, a member of another PTK family with

two tandemly arranged Src homology 2 domains, leading to the activation of these PTKs. Active Lyn and Syk phosphorylate numerous cellular target proteins, leading to the activation of multiple signaling pathways, including PI3K and phospholipase C (PLC)-γ. An alternative pathway involving Fyn (another Src family PTK) was shown to activate PI3K (5). PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate into two second messengers, i.e., diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃). IP₃ triggers Ca²⁺ release from intracellular stores and then Ca²⁺ influx. Increased DAG and Ca²⁺ lead to the activation of protein kinase C (PKC) (reviewed in Ref. 6). Both PKC and Ca²⁺ are required for optimal degranulation (7).

Cell-cell and cell-extracellular matrix (ECM) interactions mediated by integrins play a critical role in cell proliferation, differentiation, survival, apoptosis, and function. Activation of leukocytes by agonists that bind to diverse classes of receptors triggers ligand recognition by integrins. This process is termed inside-out signaling. Activation of mast cells by FcεRI aggregation and stem cell factor (SCF) induces adhesion to fibronectin (FN) (8) predominantly via integrin α₅β₁ (9–11). Upon FcεRI aggregation and SCF stimulation, FN-adherent cells exhibit stronger effector functions such as histamine release and cytokine production than non-adherent cells (10). Adherent cells exhibit more intense tyrosine phosphorylation of certain proteins, including focal adhesion kinase p125^{FAK}, than nonadherent cells (12, 13). FcεRI- and SCF-induced mast cell adhesion involves several signaling pathways: PI3K and PLC-γ are required for adhesion induced by both stimuli (14). Lyn and Ca²⁺ influx seem to be required for FcεRI aggregation- but not SCF-induced adhesion, and PKC may also be involved in FcεRI aggregation- but not SCF-induced adhesion (11).

We and others (15, 16) recently demonstrated that monomeric IgE can promote mast cell survival. This observation, together with earlier studies showing that IgE in the absence of cross-linking reagents (such as Ag or anti-IgE) can increase the surface expression of FcεRI (17–19), has transformed the traditional view

*Division of Cell Biology, La Jolla Institute for Allergy and Immunology, San Diego, CA 92121; [†]Division of Vascular Biology, Department of Cell Biology, Scripps Research Institute, La Jolla, CA 92037; [‡]Max Planck Institute for Experimental Endocrinology, Hannover, Germany; and [§]Department of Laboratory Medicine, University of California, San Francisco, CA 94143-0134

Received for publication November 10, 2004. Accepted for publication January 11, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This study was supported by Public Health Service Grants AI50209 and AI/GM38348 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 K.E.).

² To whom all correspondence should be addressed to Dr. Toshiaki Kawakami, Division of Cell Biology, La Jolla Institute for Allergy and Immunology, 10355 Science Center Drive, San Diego, CA 92121. E-mail address: toshi@liai.org

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 monomeric 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 $\alpha_5\beta_1$ 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 monomeric HC, but not PC, IgEs can induce β_1 and β_7 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, PKC ϵ , and PKC θ 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- ϵ -206, abbreviated hereafter as 206) and H1 DNP- ϵ -26 mAbs (23) were purified as described previously (21). Anti-dansyl, anti-trinitrophenyl (TNP) (IgE-3), anti-TNP (C48-2), and anti-TNP (C38-2) mouse IgE mAbs were purchased from BD Pharmingen. Anti-DNP IgE (SPE-7) was 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. Some experiments that demanded large amounts of IgE were performed with less pure preparations. The latter preparations were contaminated typically with 5–15% IgE aggregates, although IgG or other Ig isotypes were detected by SDS-PAGE/Coomassie brilliant blue staining. However, these aggregates did not contribute significantly to the extent of mast cell adhesion (i.e., <20% increase over that induced by highly purified monomeric IgE preparations). Function-blocking anti-integrin mAbs were purchased from BD Pharmingen: anti-integrin α_4 , anti-integrin α_5 , anti-integrin β_1 , anti-integrin β_2 and anti-integrin β_7 . Following are the sources of other commercial Abs: anti-phospho-Btk (Tyr²²³), anti-phospho-Src family (Tyr⁴¹⁶), anti-phospho-PLC- γ_2 (Tyr¹²¹⁷), anti-phospho-p44/42 MAPK (Thr²⁰²/Tyr²⁰⁴), anti-phospho-p38 MAPK (Thr¹⁸⁰/Tyr¹⁸²), and anti-phospho-Akt (Ser⁴⁷³) were purchased from Cell Signaling Technology; anti-phospho-tyrosine mAb 4G10 were obtained from Upstate Cell Signaling Solutions; anti-p38, anti-Akt, anti-Lyn, anti-Syk, anti-JNK1, and anti-PLC- γ_2 were obtained from Santa Cruz Biotechnology; anti-ERK was purchased from Zymed Laboratories; and FITC-conjugated anti-mouse IgG was obtained from Jackson ImmunoResearch Laboratories.

Cells

Bone marrow cells from wild-type and mutant mice were cultured in IL-3-containing medium for 4–6 wk to generate immature mast cells (bone marrow-derived mast cells (BMMCs)) with >95% purity (c-Kit⁺Fc ϵ RI⁺ by flow cytometry). The following mutant mice were used: *lyn*^{-/-} (24), *fyn*^{-/-} (25), *lyn/fyn*^{-/-} (26), *hck*^{-/-} (27), *syk*^{-/-} (28), *btk*^{-/-} (29), *PKC β* ^{-/-} (30), *PKC ϵ* ^{-/-} (31), and *PKC θ* ^{-/-} (32). Animal experiments were approved by the Animal Care Committee of the La Jolla Institute for Allergy and Immunology and performed in accordance with the guidelines of the National Institutes of Health.

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 μ 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 μ g/ml 206 IgE, washed twice and incubated with DNP₂₃-HSA, and resuspended at 5×10^5 cells/ml in assay medium. For the other stimulations, naive BMMCs were used. The cells in 100 μ 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 μ l of trypsin for 20 min to measure the number of adherent cells using trypan 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 μ g/ml Abs for 30 min at 37°C before the addition of the cells and Abs to the plate.

Confocal microscopy

BMMCs (2.5×10^5 cells in 0.5 ml) were seeded into coverslips placed in 12-well plates coated with 20 μ 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, then stained with anti-phosphotyrosine mAb (PY 20) (BD Pharmingen) for 60 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- α (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 μ g/ml aprotinin, 10 μ g/ml leupeptin, 25 μ M *p*-nitrophenyl *p*'-guanidinobenzoate, 1 μ M pepstatin, 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 μ Ci of [γ -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 Fc ϵ RI 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 MnCl₂ (a direct activator of integrins). Incubation of BMMCs with 5 μ 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

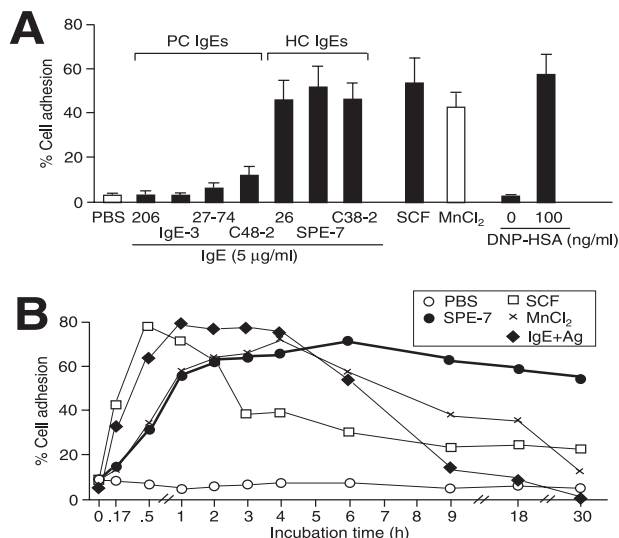


FIGURE 1. HC IgEs induce mast cell adhesion on FN-coated plates in uniquely slow but sustained kinetics. BMMCs from wild-type mice with a mixed genetic background of 129 and B6 strains were incubated with 5 μ g/ml of various monoclonal IgEs, 100 ng/ml SCF, or 1 mM MnCl₂ for 60 min (A) or the indicated periods (B). In the case of IgE+Ag stimulation, BMMCs were first sensitized by an overnight culture with 0.5 μ g/ml 206 IgE and then stimulated with 100 ng/ml DNP₂₃-HSA for 60 min (A) or the indicated periods (B). Adherent cells were counted.

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 β_1 and β_7 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 $\alpha_5\beta_1$ (9–11, 22). As shown previously (9, 35), integrins β_1 , β_2 , and β_7 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 anti-integrin Abs before stimulation with HC IgE. We confirmed that HC IgE-induced adhesion is inhibited dramatically by anti-integrin β_1 (Fig. 2B). However, it was also inhibited by anti-integrin β_7 , albeit to a lesser extent. Preincubation with a combination of anti-integrin β_1 and anti-integrin β_7 completely inhibited HC IgE-induced adhesion. These results indicate that HC IgE induces FN-mediated cell adhesion mainly through

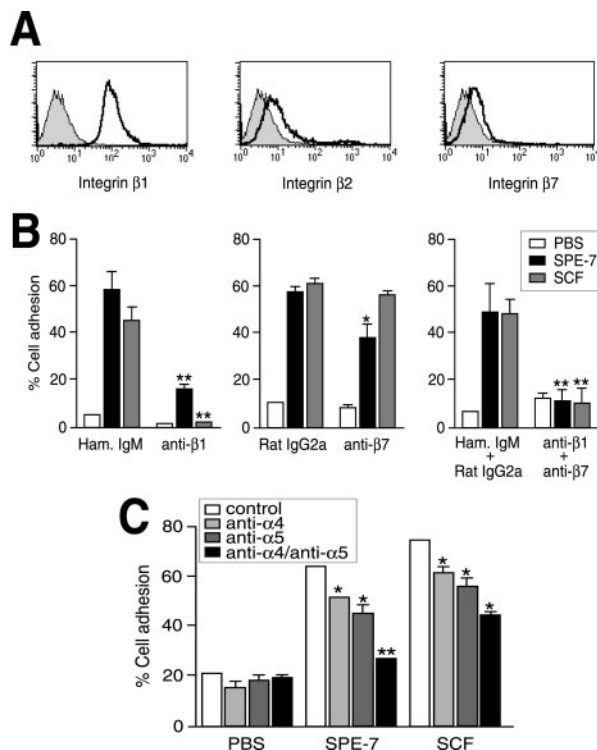


FIGURE 2. Expression of β integrins and the involvement of integrins in HC IgE-induced mast cell adhesion. A, Expression of β integrins was measured by flow cytometry. B and C, Wild-type BMMCs were preincubated with function-blocking Abs against specific integrin subunits for 30 min before being seeded into FN-coated plates containing 5 μ g/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.

integrin β_1 and, to a lesser extent, through integrin β_7 . Consistent with the previous studies on the role of $\alpha_5\beta_1$ (9), anti-integrin α_5 also inhibited HC IgE-induced BMMC adhesion to FN substantially but incompletely (Fig. 2C). Anti-integrin α_4 inhibited BMMC adhesion to a lesser extent, and a combination of anti- α_4 and anti- α_5 inhibited HC IgE-induced adhesion almost to basal levels.

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

Fc ϵ RI 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 (Fig. 3C). IgE+Ag-induced adhesion was reduced slightly at low Ag concentrations in Lyn-deficient cells, and this reduction was more pronounced in Lyn/Fyn-doubly deficient cells (Fig. 3D). By contrast, deficiencies of these Src family kinases had no or little effect on SCF-induced adhesion.

Syk, which requires Lyn but not Fyn for its activation in mast cells (5, 39, 40), is known to be essential for almost all known aspects of mast cell activation induced by Fc ϵ RI aggregation (41–43). HC IgE-induced adhesion was not only abrogated but also reduced below basal levels in Syk-deficient cells, and these cells

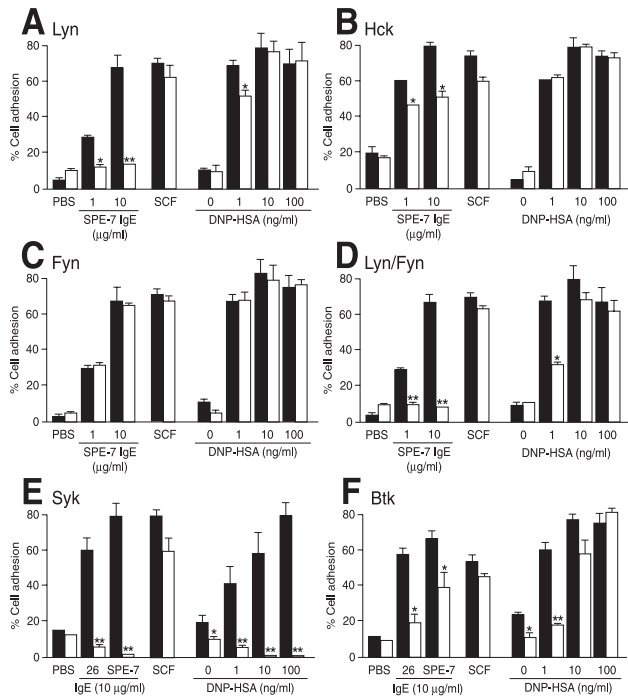


FIGURE 3. HC IgE-induced mast cell adhesion requires Lyn and Syk. Mutant mast cells deficient in Src, Syk, or Tec family members (indicated by their names), and the respective control cells were incubated with the indicated concentrations of SPE-7 IgE or 100 ng/ml SCF on FN-coated plates for 60 min before counting adherent cells. 206 IgE-sensitized cells were stimulated by the indicated concentrations of DNP₂₃-HSA for 60 min. The filled and empty columns represent wild-type and mutant cells, respectively. * and **, $p < 0.05$ and $p < 0.01$ (vs wild-type cells; 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- γ 2 (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, similar to cells deficient in Src or Syk family kinases, SCF-stimulated, Btk-deficient cells adhered as efficiently as wild-type cells.

PKC β is under the control of Lyn, Syk, and Btk through PLC- γ 1/2 (46) and plays a critical role in degranulation and cytokine production in Fc ϵ RI-cross-linked mast cells (46, 49–51). PKC β 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 PKC β 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.

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

In contrast with PKC θ -deficient mast cells, HC IgE-induced adhesion was not reduced but rather increased at a suboptimal dose

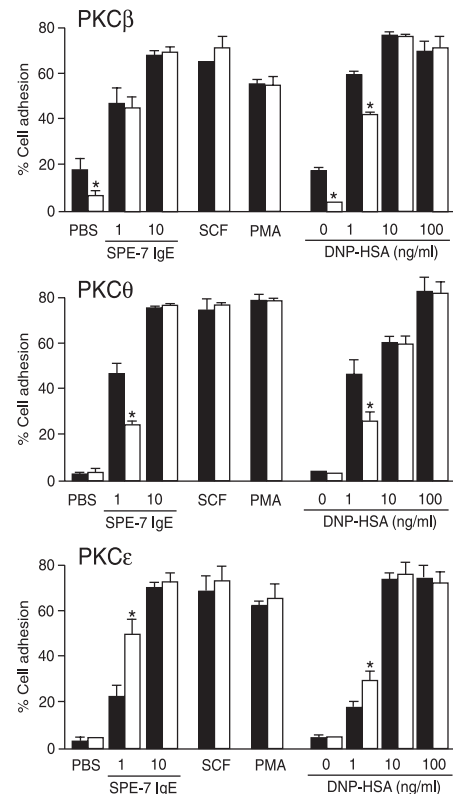


FIGURE 4. PKC θ plays a contributory role and PKC ϵ plays a negative regulatory role in HC IgE-induced mast cell adhesion. Mutant and control cells were stimulated as described for Fig. 3. In addition, these cells were also stimulated by 100 nM PMA for 60 min. * and **, $p < 0.05$ and $p < 0.01$ (vs wild-type cells; Student's t test), respectively.

of 1 µg/ml in PKC ϵ -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 Fc ϵ RI, c-Kit, or integrin β_1 (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 β_1 (and β_7)-dependent adhesion requires Src (Lyn and Hck) and Syk family kinases. Btk and PKC θ play a minor role in these adhesions, whereas PKC ϵ plays a negative regulatory role.

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

Fc ϵ RI 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. *Syk*^{-/-} BMMCs 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 FcεRI, 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

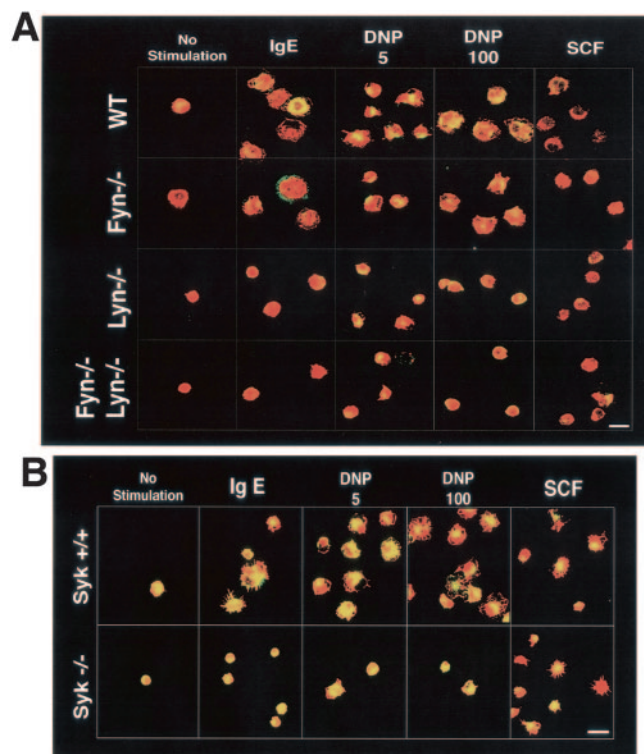


FIGURE 5. HC IgE-induced mast cell spreading on FN-coated plates is dependent on Lyn and Syk. *A*, BMMCs from *fyn*^{-/-}, *lyn*^{-/-}, *fyn*^{-/-} *lyn*^{-/-}, and wild-type control mice were plated on FN for 30 min with no stimulation or stimulation with 5 μg/ml SPE-7 IgE, IgE+Ag (5 or 100 ng/ml DNP₂₃-HSA), or 100 ng/ml SCF. The cells were then fixed, permeabilized, stained for F-actin (red) and phosphotyrosine (green), and analyzed by confocal microscopy. Results shown are representative of three independent experiments. Bar, 10 μm. *B*, BMMCs from *syk*^{-/-} and wild-type mice were analyzed similarly.

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, FcεRI, 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/

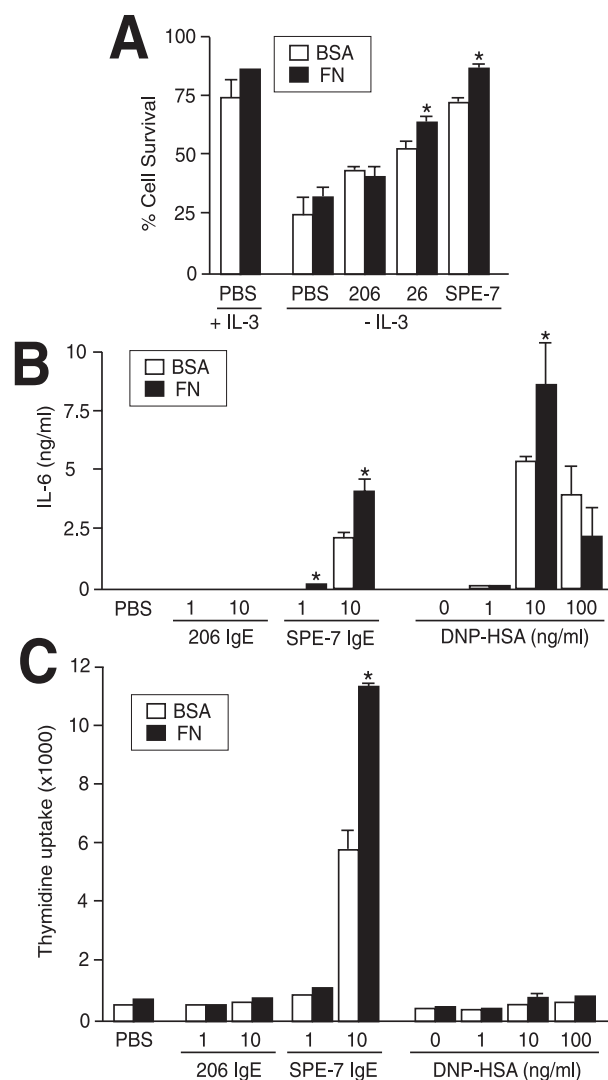


FIGURE 6. Adhesion to FN increases HC IgE-induced survival, IL-6 production and DNA synthesis. *A*, Wild-type BMMCs were left unstimulated (PBS) or stimulated by IgEs or IgE+Ag with or without IL-3 in FN- or BSA-coated plates for 72 h. Survival of adherent and nonadherent cells was quantified by flow cytometry after staining with annexin V and propidium iodide. *B*, Wild-type BMMCs were stimulated similarly with 206 or SPE-7 IgE or by IgE+Ag in the presence of IL-3 for 20 h. IL-6 in culture supernatants was quantified by ELISA. *C*, Wild-type BMMCs in adhesion medium were stimulated similarly in FN- or BSA-coated plates for 48 h in the presence of [³H]thymidine for the last 12 h. The radioactivity incorporated into acid-insoluble compartments was counted by liquid scintillation. *, *p* < 0.05 (vs the cells incubated in BSA-coated plates; Student's *t* test).

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 first performed immunoblotting analysis of tyrosine-phosphorylated proteins in HC IgE- and IgE+Ag-stimulated cells. When BMMCs 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 similar to that by IgE+Ag, tyrosine phosphorylation peaked at ~1 h in HC IgE-stimulated cells on FN-coated plates and then started to decline (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 Tyr²²³ (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 isoforms. Because Ca²⁺ was shown to be critical for HC IgE-induced mast cell adhesion (22), we estimated the activity of PLC- γ 2, a major PLC- γ isoform in mast cells. PLC- γ 2 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- γ 2 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 β_1 integrin, as shown recently (22). In addition, our study has identified β_7 integrin as another mediator of mast cell adhesion. Importantly, we have identified Lyn and Syk as requisite proximal PTKs and Hck, Btk, and PKC θ 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

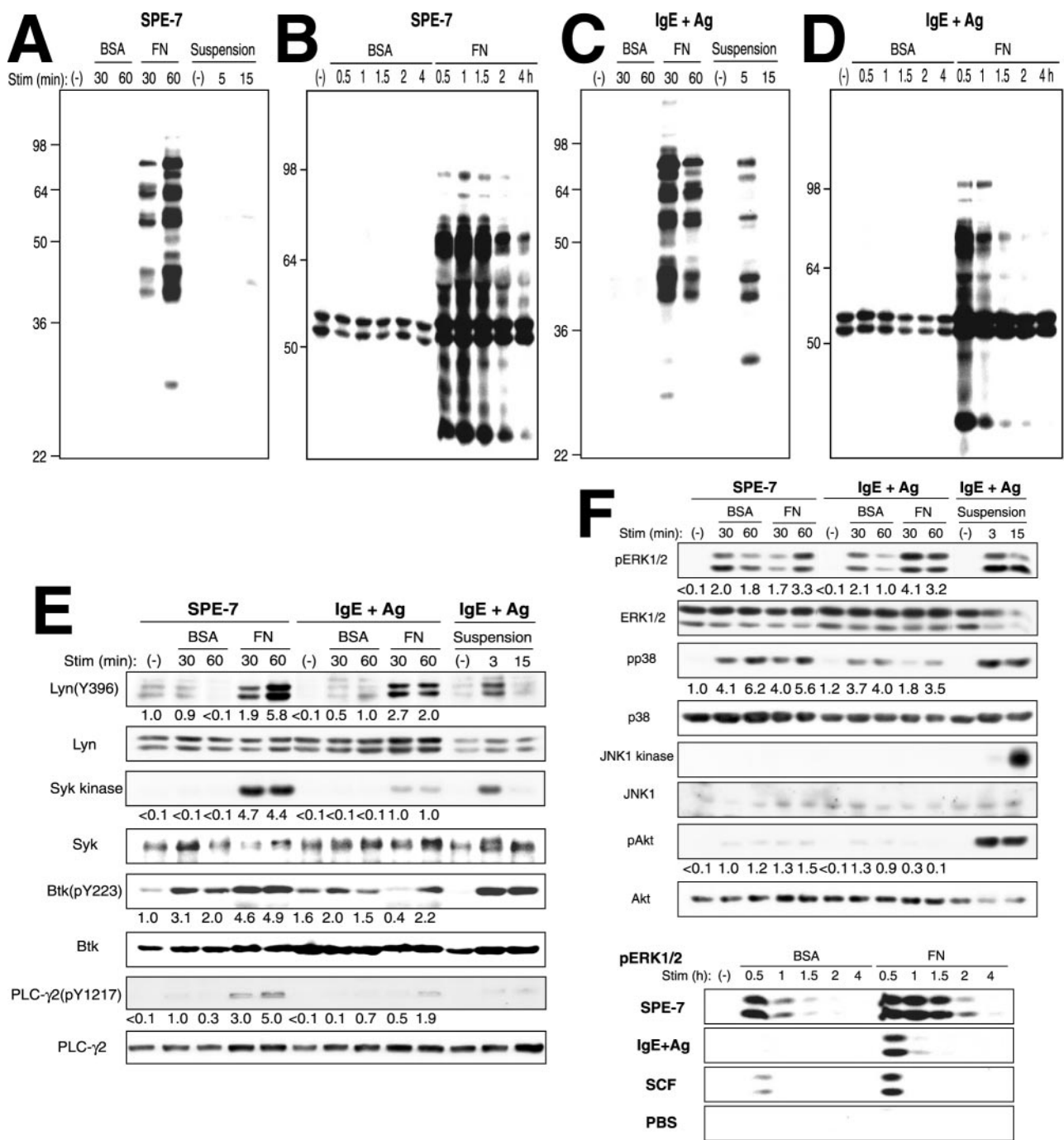


FIGURE 7. Signal transduction triggered by HC IgE vs IgE+Ag in adherent and nonadherent mast cells. *A–F*, Wild-type BMMCs were left unstimulated (PBS) or stimulated by SPE-7 IgE or IgE+Ag in FN-coated or BSA-coated plates for the indicated periods. Cell lysates were analyzed by immunoblotting with anti-phospho-tyrosine mAb 4G10 (*A–D*) or the indicated Abs. *A*, *C*, *E*, and *F*, In some cases, suspended cells were stimulated by IgE+Ag in Tyrode’s solution. Cell lysates were analyzed by immunoblotting with the indicated Abs. When phospho-specific Abs were used for the first probing, the same blots were stripped and reprobed with the respective nonphospho-specific Abs. *E* and *F*, In vitro kinase assays were conducted on immune complexes of Syk and JNK1. Intensities of the phosphorylated bands induced by SPE-7 IgE and IgE+Ag were measured by densitometry. For Lyn and ERKs, only results with p53^{lyn} and ERK1 are shown. IgE+Ag samples (*three rightmost lanes in E and F*) were analyzed by gels separate from the other samples, which were analyzed by single gels. So, the apparent reduction in Lyn amounts in “IgE+Ag” samples does not reflect real amounts. Results representative of two (*A–E*) or three (*F*) experiments are shown.

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 β_1 integrin-initiated outside-in and Fc ϵ RI-initiated inside-out signals use PTKs of the Src family. Importantly, Src family PTKs directly interact with the integrin β cytoplasmic domains: integrin β_{1A} 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 β_1 -binding Lyn or Hck, but not integrin β_1 -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 β_1 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 Tyr³⁹⁶ 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: Fc ϵ RI cross-linking increases PI3K activity in anti-LAT, anti-Fc ϵ RI β , 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 Fc ϵ RI-cross-linked cells: osmiophilic patches termed the primary signaling domain contain Fc ϵ RI, 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 Fc ϵ RI 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 Fc ϵ RI 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, Fc ϵ RI-induced secretion of cytokines is enhanced

in Lyn-deficient mast cells, indicative of a role in negative regulation possibly via the Fc γ RIIB1 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 $\alpha_5\beta_1$ (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_2 phosphorylation is regulated by Btk and Syk (47, 48), the Btk-dependent signaling component for HC IgE-induced adhesion may be mediated by PLC- γ_2 . 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 PKC θ and PKC ϵ as the positive and negative regulators, respectively, of HC IgE-induced adhesion. As BMMCs express PKC α , PKC β I, and PKC β II among the conventional isoforms, it is speculated that PKC α , which is known to be activated by adhesion via integrin $\alpha_5\beta_1$ (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.

References

- Metzger, H. 1992. The receptor with high affinity for IgE. *Immunol. Rev.* 125:37.
- Kinet, J. P. 1999. The high-affinity IgE receptor (FcεRI): from physiology to pathology. *Annu. Rev. Immunol.* 17:931.
- Eiseman, E., and J. B. Bolen. 1992. Engagement of the high-affinity IgE receptor activates src protein-related tyrosine kinases. *Nature* 355:78.
- Jouvin, M. H., M. Adamczewski, R. Numerof, O. Letourneur, A. Valle, and J. P. Kinet. 1994. Differential control of the tyrosine kinases Lyn and Syk by the two signaling chains of the high affinity immunoglobulin E receptor. *J. Biol. Chem.* 269:5918.
- Parravicini, V., M. Gadina, M. Kovarova, S. Odom, C. Gonzalez-Espinosa, Y. Furumoto, S. Saitoh, L. E. Samelson, J. J. O'Shea, and J. Rivera. 2002. Fyn kinase initiates complementary signals required for IgE-dependent mast cell degranulation. *Nat. Immunol.* 3:741.
- Nishizuka, Y. 1992. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* 258:607.
- Ozawa, K., Z. Szallasi, M. G. Kazanietz, P. M. Blumberg, H. Mischak, J. F. Mushinski, and M. A. Beaven. 1993. Ca²⁺-dependent and Ca²⁺-independent isozymes of protein kinase C mediate exocytosis in antigen-stimulated rat basophilic RBL-2H3 cells: reconstitution of secretory responses with Ca²⁺ and purified isozymes in washed permeabilized cells. *J. Biol. Chem.* 268:1749.
- Dastyk, J., J. J. Costa, H. L. Thompson, and D. D. Metcalfe. 1991. Mast cell adhesion to fibronectin. *Immunology* 73:478.
- Kinashi, T., and T. A. Springer. 1994. Steel factor and c-kit regulate cell-matrix adhesion. *Blood* 83:1033.
- Ra, C., M. Yasuda, H. Yagita, and K. Okumura. 1994. Fibronectin receptor integrins are involved in mast cell activation. *J. Allergy Clin. Immunol.* 94:625.
- Fehlner-Gardiner, C. C., S. Uniyal, C. G. von Ballestrem, and B. M. Chan. 1996. Differential utilization of VLA-4 (α₄β₁) and -5 (α₅β₁) integrins during the development of mouse bone marrow-derived mast cells. *Differentiation* 60:317.
- Hamawy, M. M., S. E. Mergenhagen, and R. P. Siraganian. 1993. Cell adherence to fibronectin and the aggregation of the high affinity immunoglobulin E receptor synergistically regulate tyrosine phosphorylation of 105–115-kDa proteins. *J. Biol. Chem.* 268:5227.
- Hamawy, M. M., S. E. Mergenhagen, and R. P. Siraganian. 1993. Tyrosine phosphorylation of pp125^{FAK} by the aggregation of high affinity immunoglobulin E receptors requires cell adherence. *J. Biol. Chem.* 268:6851.
- Kinashi, T., J. A. Escobedo, L. T. Williams, K. Takatsu, and T. A. Springer. 1995. Receptor tyrosine kinase stimulates cell-matrix adhesion by phosphatidylinositol 3 kinase and phospholipase C-γ1 pathways. *Blood* 86:2086.
- Asai, K., J. Kitaura, Y. Kawakami, N. Yamagata, M. Tsai, D. P. Carbone, F. T. Liu, S. J. Galli, and T. Kawakami. 2001. Regulation of mast cell survival by IgE. *Immunology* 14:791.
- Kalesnikoff, J., M. Huber, V. Lam, J. E. Damen, J. Zhang, R. P. Siraganian, and G. Krystal. 2001. Monomeric IgE stimulates signaling pathways in mast cells that lead to cytokine production and cell survival. *Immunology* 14:801.
- Furuichi, K., J. Rivera, and C. Isersky. 1985. The receptor for immunoglobulin E on rat basophilic leukemia cells: effect of ligand binding on receptor expression. *Proc. Natl. Acad. Sci. USA* 82:1522.
- Hsu, C., and D. MacGlashan, Jr. 1996. IgE antibody up-regulates high affinity IgE binding on murine bone marrow-derived mast cells. *Immunol. Lett.* 52:129.
- Yamaguchi, M., C. S. Lantz, H. C. Oettgen, I. M. Katona, T. Fleming, I. Miyajima, J. P. Kinet, and S. J. Galli. 1997. IgE enhances mouse mast cell FcεRI expression in vitro and in vivo: evidence for a novel amplification mechanism in IgE-dependent reactions. *J. Exp. Med.* 185:663.
- Kawakami, T., and S. J. Galli. 2002. Regulation of mast-cell and basophil function and survival by IgE. *Nat. Rev. Immunol.* 2:773.
- Kitaura, J., J. Song, M. Tsai, K. Asai, M. Maeda-Yamamoto, A. Mocsai, Y. Kawakami, F. T. Liu, C. A. Lowell, B. G. Barisas, S. J. Galli, and T. Kawakami. 2003. Evidence that IgE molecules mediate a spectrum of effects on mast cell survival and activation via aggregation of the FcεRI. *Proc. Natl. Acad. Sci. USA* 100:12911.
- Lam, V., J. Kalesnikoff, C. W. Lee, V. Hernandez-Hansen, B. S. Wilson, J. M. Oliver, and G. Krystal. 2003. IgE alone stimulates mast cell adhesion to fibronectin via pathways similar to those used by IgE + antigen but distinct from those used by Steel factor. *Blood* 102:1405.
- Liu, F. T., J. W. Bohn, E. L. Ferry, H. Yamamoto, C. A. Molinaro, L. A. Sherman, N. R. Klinman, and D. H. Katz. 1980. Monoclonal dinitrophenyl-specific murine IgE antibody: preparation, isolation, and characterization. *J. Immunol.* 124:2728.
- Chan, V. W., F. Meng, P. Soriano, A. L. DeFranco, and C. A. Lowell. 1997. Characterization of the B lymphocyte populations in Lyn-deficient mice and the role of Lyn in signal initiation and down-regulation. *Immunity* 7:69.
- Stein, P. L., H. M. Lee, S. Rich, and P. Soriano. 1992. pp59^{lyn} mutant mice display differential signaling in thymocytes and peripheral T cells. *Cell* 70:741.
- Yu, C. C., T. S. Yen, C. A. Lowell, and A. L. DeFranco. 2001. Lupus-like kidney disease in mice deficient in the Src family tyrosine kinases Lyn and Fyn. *Curr. Biol.* 11:34.
- Lowell, C. A., P. Soriano, and H. E. Varmus. 1994. Functional overlap in the src gene family: inactivation of hck and fgr impairs natural immunity. *Genes Dev.* 8:387.
- Turner, M., P. J. Mee, P. S. Costello, O. Williams, A. A. Price, L. P. Duddy, M. T. Furlong, R. L. Geahlen, and V. L. Tybulewicz. 1995. Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. *Nature* 378:298.
- Khan, W. N., F. W. Alt, R. M. Gerstein, B. A. Malynn, I. Larsson, G. Rathbun, L. Davidson, S. Muller, A. B. Kantor, L. A. Herzenberg, et al. 1995. Defective B cell development and function in Btk-deficient mice. *Immunity* 3:283.
- Leitges, M., C. Schmedt, R. Guinamard, J. Davoust, S. Schaal, S. Stabel, and A. Tarakhovskiy. 1996. Immunodeficiency in protein kinase Cβ-deficient mice. *Science* 273:788.
- Khasar, S. G., Y. H. Lin, A. Martin, J. Dadgar, T. McMahon, D. Wang, B. Hundle, K. O. Aley, W. Isenberg, G. McCarter, et al. 1999. A novel nociceptor signaling pathway revealed in protein kinase Cε mutant mice. *Neuron* 24:253.
- Sun, Z., C. W. Arendt, W. Ellmeier, E. M. Schaeffer, M. J. Sunshine, L. Gandhi, J. Annes, D. Petrzilka, A. Kupfer, P. L. Schwartzberg, and D. R. Littman. 2000. PKC-θ is required for TCR-induced NF-κB activation in mature but not immature T lymphocytes. *Nature* 404:402.
- Obergfell, A., K. Eto, A. Mocsai, C. Buensuceso, S. L. Moores, J. S. Brugge, C. A. Lowell, and S. J. Shattil. 2002. Coordinate interactions of Csk, Src, and Syk kinases with α_{IIb}β₃ initiate integrin signaling to the cytoskeleton. *J. Cell Biol.* 157:265.
- Eto, K., R. Murphy, S. W. Kerrigan, A. Bertoni, H. Stuhlmann, T. Nakano, A. D. Leavitt, and S. J. Shattil. 2002. Megakaryocytes derived from embryonic stem cells implicate CalDAG-GEFI in integrin signaling. *Proc. Natl. Acad. Sci. USA* 99:12819.
- Dastyk, J., and D. D. Metcalfe. 1994. Stem cell factor induces mast cell adhesion to fibronectin. *J. Immunol.* 152:213.
- Kinashi, T., T. Asaoka, R. Setoguchi, and K. Takatsu. 1999. Affinity modulation of very late antigen-5 through phosphatidylinositol 3-kinase in mast cells. *J. Immunol.* 162:2850.
- Hutchcroft, J. E., R. L. Geahlen, G. G. Deanin, and J. M. Oliver. 1992. FcεRI-mediated tyrosine phosphorylation and activation of the 72-kDa protein-tyrosine kinase, PTK72, in RBL-2H3 rat tumor mast cells. *Proc. Natl. Acad. Sci. USA* 89:9107.
- Kawakami, Y., L. Yao, T. Miura, S. Tsukada, O. N. Witte, and T. Kawakami. 1994. Tyrosine phosphorylation and activation of Bruton tyrosine kinase upon FcεRI cross-linking. *Mol. Cell. Biol.* 14:5108.
- El-Hillal, O., T. Kurosaki, H. Yamamura, J. P. Kinet, and A. M. Scharenberg. 1997. syk kinase activation by a src kinase-initiated activation loop phosphorylation chain reaction. *Proc. Natl. Acad. Sci. USA* 94:1919.
- Kawakami, Y., J. Kitaura, A. B. Satterthwaite, R. M. Kato, K. Asai, S. E. Hartman, M. Maeda-Yamamoto, C. A. Lowell, D. J. Rawlings, O. N. Witte, and T. Kawakami. 2000. Redundant and opposing functions of two tyrosine kinases, Btk and Lyn, in mast cell activation. *J. Immunol.* 165:1210.
- Rivera, V. M., and J. S. Brugge. 1995. Clustering of Syk is sufficient to induce tyrosine phosphorylation and release of allergic mediators from rat basophilic leukemia cells. *Mol. Cell. Biol.* 15:1582.
- Zhang, J., E. H. Berenstein, R. L. Evans, and R. P. Siraganian. 1996. Transfection of Syk protein tyrosine kinase reconstitutes high affinity IgE receptor-mediated degranulation in a Syk-negative variant of rat basophilic leukemia RBL-2H3 cells. *J. Exp. Med.* 184:71.
- Costello, P. S., M. Turner, A. E. Walters, C. N. Cunningham, P. H. Bauer, J. Downward, and V. L. Tybulewicz. 1996. Critical role for the tyrosine kinase Syk in signalling through the high affinity IgE receptor of mast cells. *Oncogene* 13:2595.
- Rawlings, D. J., A. M. Scharenberg, H. Park, M. I. Wahl, S. Lin, R. M. Kato, A. C. Fluckiger, O. N. Witte, and J. P. Kinet. 1996. Activation of BTK by a phosphorylation mechanism initiated by SRC family kinases. *Science* 271:822.

45. Kurosaki, T., and M. Kurosaki. 1997. Transphosphorylation of Bruton's tyrosine kinase on tyrosine 551 is critical for B cell antigen receptor function. *J. Biol. Chem.* 272:15595.
46. Kawakami, Y., J. Kitaura, S. E. Hartman, C. A. Lowell, R. P. Siraganian, and T. Kawakami. 2000. Regulation of protein kinase C β I by two protein-tyrosine kinases, Btk and Syk. *Proc. Natl. Acad. Sci. USA* 97:7423.
47. Fluckiger, A. C., Z. Li, R. M. Kato, M. I. Wahl, H. D. Ochs, R. Longnecker, J. P. Kinet, O. N. Witte, A. M. Scharenberg, and D. J. Rawlings. 1998. Btk/Tec kinases regulate sustained increases in intracellular Ca²⁺ following B-cell receptor activation. *EMBO J.* 17:1973.
48. Scharenberg, A. M., O. El-Hillal, D. A. Fruman, L. O. Beitz, Z. Li, S. Lin, I. Gout, L. C. Cantley, D. J. Rawlings, and J. P. Kinet. 1998. Phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P3)/Tec kinase-dependent calcium signaling pathway: a target for SHIP-mediated inhibitory signals. *EMBO J.* 17:1961.
49. Nechushtan, H., M. Leitges, C. Cohen, G. Kay, and E. Razin. 2000. Inhibition of degranulation and interleukin-6 production in mast cells derived from mice deficient in protein kinase C β . *Blood* 95:1752.
50. Kawakami, Y., J. Kitaura, L. Yao, R. W. McHenry, A. C. Newton, S. Kang, R. M. Kato, M. Leitges, D. J. Rawlings, and T. Kawakami. 2003. A Ras activation pathway dependent on Syk phosphorylation of protein kinase C. *Proc. Natl. Acad. Sci. USA* 100:9470.
51. Kawakami, Y., H. Nishimoto, J. Kitaura, M. Maeda-Yamamoto, R. M. Kato, D. R. Littman, M. Leitges, D. J. Rawlings, and T. Kawakami. 2004. Protein kinase C β II regulates Akt phosphorylation on Ser-473 in a cell type- and stimulus-specific fashion. *J. Biol. Chem.* 279:47720.
52. Liu, Y., C. Graham, V. Parravicini, M. J. Brown, J. Rivera, and S. Shaw. 2001. Protein kinase C θ is expressed in mast cells and is functionally involved in Fc ϵ receptor I signaling. *J. Leukocyte Biol.* 69:831.
53. Pfeiffer, J. R., and J. M. Oliver. 1994. Tyrosine kinase-dependent assembly of actin plaques linking Fc ϵ R1 cross-linking to increased cell substrate adhesion in RBL-2H3 tumor mast cells. *J. Immunol.* 152:270.
54. Kitaura, J., W. Xiao, M. Maeda-Yamamoto, Y. Kawakami, C. A. Lowell, and T. Kawakami. 2004. Early divergence of Fc ϵ receptor I signals for receptor up-regulation and internalization from degranulation, cytokine production, and survival. *J. Immunol.* 173:4317.
55. Nishizumi, H., and T. Yamamoto. 1997. Impaired tyrosine phosphorylation and Ca²⁺ mobilization, but not degranulation, in lyn-deficient bone marrow-derived mast cells. *J. Immunol.* 158:2350.
56. Xia, Z., M. Dickens, J. Raingeaud, R. J. Davis, and M. E. Greenberg. 1995. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* 270:1326.
57. Yamasaki, S., E. Ishikawa, M. Kohno, and T. Saito. 2004. The quantity and duration of Fc γ R signals determine mast cell degranulation and survival. *Blood* 103:3093.
58. Franke, T. F., D. R. Kaplan, and L. C. Cantley. 1997. PI3K: downstream AKTion blocks apoptosis. *Cell* 88:435.
59. Hemmings, B. A. 1997. Akt signaling: linking membrane events to life and death decisions. *Science* 275:628.
60. Marte, B. M., and J. Downward. 1997. PKB/Akt: connecting phosphoinositide 3-kinase to cell survival and beyond. *Trends Biochem. Sci.* 22:355.
61. Hynes, R. O. 2002. Integrins: bidirectional, allosteric signaling machines. *Cell* 110:673.
62. Arias-Salgado, E. G., S. Lizano, S. Sarkar, J. S. Brugge, M. H. Ginsberg, and S. J. Shattil. 2003. Src kinase activation by direct interaction with the integrin β cytoplasmic domain. *Proc. Natl. Acad. Sci. USA* 100:13298.
63. Serve, H., N. S. Yee, G. Stella, L. Sepp-Lorenzino, J. C. Tan, and P. Besmer. 1995. Differential roles of PI3-kinase and Kit tyrosine 821 in Kit receptor-mediated proliferation, survival and cell adhesion in mast cells. *EMBO J.* 14:473.
64. Dastyg, J., D. Taub, M. C. Hardison, and D. D. Metcalfe. 1998. Tyrosine kinase-deficient W^v c-kit induces mast cell adhesion and chemotaxis. *Am. J. Physiol.* 275:C1291.
65. Wilson, B. S., J. R. Pfeiffer, Z. Surviladze, E. A. Gaudet, and J. M. Oliver. 2001. High resolution mapping of mast cell membranes reveals primary and secondary domains of Fc ϵ R1 and LAT. *J. Cell Biol.* 154:645.
66. Hibbs, M. L., D. M. Tarlinton, J. Armes, D. Grail, G. Hodgson, R. Maglito, S. A. Stackel, and A. R. Dunn. 1995. Multiple defects in the immune system of Lyn-deficient mice, culminating in autoimmune disease. *Cell* 83:301.
67. Malbec, O., D. C. Fong, M. Turner, V. L. Tybulewicz, J. C. Cambier, W. H. Fridman, and M. Daeron. 1998. Fc ϵ receptor I-associated lyn-dependent phosphorylation of Fc γ receptor IIB during negative regulation of mast cell activation. *J. Immunol.* 160:1647.
68. Odom, S., G. Gomez, M. Kovarova, Y. Furumoto, J. J. Ryan, H. V. Wright, C. Gonzalez-Espinosa, M. L. Hibbs, K. W. Harder, and J. Rivera. 2004. Negative regulation of immunoglobulin E-dependent allergic responses by Lyn kinase. *J. Exp. Med.* 199:1491.
69. Pereira, S., and C. Lowell. 2003. The Lyn tyrosine kinase negatively regulates neutrophil integrin signaling. *J. Immunol.* 171:1319.
70. Lev, S., D. Givol, and Y. Yarden. 1991. A specific combination of substrates is involved in signal transduction by the kit-encoded receptor. *EMBO J.* 10:647.
71. Mostafavi-Pour, Z., J. A. Askari, S. J. Parkinson, P. J. Parker, T. T. Ng, and M. J. Humphries. 2003. Integrin-specific signaling pathways controlling focal adhesion formation and cell migration. *J. Cell Biol.* 161:155.
72. Kawakami, Y., T. Miura, R. Bissonnette, D. Hata, W. N. Khan, T. Kitamura, M. Maeda-Yamamoto, S. E. Hartman, L. Yao, F. W. Alt, and T. Kawakami. 1997. Bruton's tyrosine kinase regulates apoptosis and JNK/SAPK kinase activity. *Proc. Natl. Acad. Sci. USA* 94:3938.
73. Smurthwaite, L., S. N. Walker, D. R. Wilson, D. S. Birch, T. G. Merrett, S. R. Durham, and H. J. Gould. 2001. Persistent IgE synthesis in the nasal mucosa of hay fever patients. *Eur. J. Immunol.* 31:3422.
74. Coker, H. A., S. R. Durham, and H. J. Gould. 2003. Local somatic hypermutation and class switch recombination in the nasal mucosa of allergic rhinitis patients. *J. Immunol.* 171:5602.