Identification of the FcεRI-activated tyrosine kinases Lyn, Syk, and Zap-70 in human basophils

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Abstract:

Background: In human blood basophils, cross-linking the high-affinity IgEreceptor FcεRI with multivalent antigen activates a signaling pathway leading to Ca^{2+} mobilization, actin polymerization, shape changes, secretion, and cytokine production. **Methods and Results:** The role of tyrosine kinases in human FcεRI signaling was explored by using human basophils isolated by Percoll gradient centrifugation followed by negative and/or positive selection with antibody-coated magnetic beads. FcεRI cross-linking of more than 95% pure basophil preparations activates the protein-tyrosine kinases Lyn and Syk, previously linked to FcεRI-coupled rodent mast cell activation, as well as Zap-70, previously implicated in T-cell receptor signaling, and causes the tyrosine phosphorylation of multiple proteins. The presence of Lyn, Syk, and Zap-70 in basophils was confirmed by Western blotting in lysates of highly purified basophils and independently by confocal fluorescence microscopy in cells labeled simultaneously with kinase-specific antibodies and with the basophil-specific antibody 2D7. Comparable amounts of Lyn and Syk were found in basophils and B cells, whereas T cells appear to have greater amounts of Zap-70 than basophils. The tyrosine kinase inhibitor piceatannol spares IgE-mediated Lyn activation but inhibits IgE-induced Syk and Zap-70 activation as well as overall protein tyrosine phosphorylation and secretion. Overall protein-tyrosine phosphorylation increases steadily over a range of anti-IgE concentrations that are low to optimal for secretion. However, tyrosine phosphorylation continues to increase at high anti-IgE concentrations that elicit very little secretion (the characteristic high-dose inhibition of secretion). **Conclusions:** Our data demonstrate the association of anti-IgE–stimulated, protein-tyrosine phosphorylation by a cascade of tyrosine kinases, including Zap-70 as well as Lyn and Syk, with the initiation of FcεRI-mediated signaling in human basophils.

Keywords: IgE receptor | IgE | basophils | allergy | signal transduction | tyrosine kinases

Article:

Abbreviations
Anti-PY: Antiphosphotyrosine antibodies
BSA: Bovine serum albumin
EDTA: Ethylenediamine tetraacetic acid
FcεRI: High-affinity IgE receptor
FITC: Fluorescein isothiocyanate
HBSS: Hanks' buffered saline solution
ITAM: Immunoreceptor tyrosine activation motif
mAb: Monoclonal antibody
PBS: Phosphate-buffered saline
PKC: Protein kinase C
SDS-PAGE: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TCR: T-cell receptor
RT-PCR: Reverse transcription–polymerase chain reaction

The high-affinity IgE receptor FcεRI is expressed on primary and cultured basophils and mast cells as well as on human monocytes,[1] eosinophils,[2] dendritic cells,[3, 4] and Langerhans cells.[5] Cross-linking this receptor with multivalent antigen is the initial event leading to allergy, asthma, and other immediate hypersensitivity reactions. Despite its clear clinical importance, studies on early events linking FcεRI cross-linking to downstream signaling pathways have been largely restricted to rodent mast cell lines. In particular, work in the RBL-2H3 rat mast cell model has shown that FcεRI-mediated cell activation is initiated by the activation of a series of receptor-associated tyrosine kinases, including Lyn and Syk,[6, 7, 8] and is propagated by the tyrosine phosphorylation and activation of substrates that include the receptor's own β and γ subunits,[9, 10] two phospholipase C γ isoforms,[11, 12, 13] the 85 kd regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase),[14, 15] Bruton's kinase,[16] and others including the GTP/GDP exchange protein Vav[17] and the adaptor protein Grb2,[18]both implicated in the activation of *ras* and related GTPases. These early events in turn result in Ca^{2+} mobilization, GTPase activation, and the activation of multiple downstream enzymes including protein kinase C (PKC) isoforms and Erk and Jnk MAP kinase family members.[19, 20, 21] A variety of cellular responses ensue. Biologic responses that occur within seconds to minutes of FcεRI cross-linking include secretion, F-actin assembly, membrane ruffling, and the assembly of actin plaques mediating increased cell-substrate adhesion.[22] Increased cytokine gene expression is typically detected within 1 to 4 hours after receptor activation.

In human basophils, it has been demonstrated that FcεRI cross-linking induces Ca^{2+} mobilization from both cytoplasmic stores and from influx,[23, 24] activates PKC,[25, 26] and leads to secretion, actin polymerization,[27] shape changes,[28, 29] and cytokine production.[30, 31] An extensive literature describes the kinetics of IgE-mediated secretion in these cells,[32, 33, 34, 35, 36] demonstrates the substantial variability between donors in receptor density and receptor-mediated responses,[37, 38, 39, 40] and shows that coligation of independently expressed cytokine receptors can prime basophils for enhanced responses to FcεRI cross-linking.[41, 42, 43] It has been established that human basophils can be activated through G-protein– coupled receptors that bind chemotactic peptides and chemokines as well as by FcεRI cross-linking that presumably activates a tyrosine kinase–coupled signaling pathway.[44, 45, 46, 47, 48, 49] However, the biochemical

events that initiate and propagate the signaling cascade have not been well characterized in human basophils.

The purpose of this study was to identify the FcεRI-activated protein-tyrosine kinases of normal human basophils and to define the relation between FcεRI-mediated protein phosphorylation and degranulation. Our results identify Zap-70, previously only associated with the T-cell receptor, to be among the anti-IgE–activated kinases in human basophils; they implicate the activation of protein-tyrosine kinases in the initiation of the FcεRI-coupled signaling cascade; and they show that the protein-tyrosine phosphorylation response can be uncoupled from secretion at high concentrations of anti-IgE cross-linker.

METHODS

Antibodies

The basophil-specific mouse monoclonal antibody (mAb) 2D7[50] was a gift from Dr L. Schwartz (Medical College of Virginia, Richmond). mAb 2D7 was fluorescein isothiocyanate (FITC)-labeled with the FluoReporter FITC Protein Labeling Kit (Molecular Probes, Eugene, Ore.) at a 1:60 molar ratio of FITC to antibody. Affinity-purified rabbit polyclonal antiphosphotyrosine antibodies (anti-PY) were prepared by G. Deanin and J. Potter (University of New Mexico).[51] The IgG mouse mAb 29C6 against the FcεRI (IgE binding) subunit[52] was a gift from Dr R. Chizzonite (Hoffman-La Roche, Nutley, NJ). Human IgE prepared from human myeloma plasma was obtained from Cortex Biochem, San Leandro, Calif. Goat anti-human IgE antibodies were isolated from serum by affinity chromatography and were obtained from Biosource, Camarillo, Calif. Rabbit polyclonal anti-Syk antibodies were a gift of Dr. R. Geahlen, Purdue University.[53] Rabbit polyclonal antibodies to Lyn and to Zap-70 were obtained from Santa Cruz Biotechnology, Santa Cruz, Calif. Horseradish peroxidase–rabbit immunoglobulin antibody were purchased from Amersham Corp. (Arlington Heights, Ill.). Anti–Zap-70 antibodies were not cross-reactive with the closely related Syk gene product, as determined by the manufacturer and in our own laboratory (Fig. 2, *C*).

Cell purification

Venous blood (100 to 450 mL) was collected from normal donors who had given informed consent as approved by the Human Studies Committee at the University of New Mexico. Heparin (0.01% vol/vol) was added as anticoagulant. None of the donors were atopic by history. For larger volumes of blood (>300 mL) the whole blood was first centrifuged at 2000 *g* for 5 minutes and the buffy coat obtained. Whole blood or buffy coat was mixed 1:1 with modified 1× Hanks' buffered saline solution (HBSS, Gibco-BRL, Grand Island, N.Y.), layered over discontinuous Percoll gradients, and centrifuged at 750 *g* for 15 minutes with no brake, resulting in a Percoll-enriched basophil population.[54] Purities from this initial step ranged from 20% to 73%. For experiments requiring a highly purified basophil population, the negative selection technique of Bjerke et al.[55] was used with some modifications. Briefly, the Percoll-enriched basophils were incubated with mouse antibodies against contaminating cells; CD3 or CD4 and CD8 (T cells), CD14 (monocytes), CD16 (neutrophils and natural killer cells), CD19 and CD22 (B cells), and anti-glycophorin AB (red blood cells) (all CD antibodies from Sigma Chemical, St.

Louis, Mo.) in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) for 30 minutes. The cells were washed with 20 ml of PBS/BSA at 800 *g*, and the pellet was resuspended in a 1:10 dilution of goat anti-mouse IgG microbeads (Miltenyi Biotec, Auburn, Calif.) and incubated for 15 minutes. The cells were then washed as above, resuspended in 200 μL of PBS/BSA, and applied to a MidiMacs separation column. For increased purities in some experiments, positive selection with antibody to the chain of the IgE receptor (29C6; 2 μg/mL) was used in combination with the MidiMacs beads followed by cell sorting of these cells with the basophils removed from other cells on the basis of their forward and side-scatter characteristics.[54] Sorted cells contained more than 99% basophils with no other cells detected in these preparations as determined by Wright's stain. All negative and positive selection procedures were performed at 4° C to prevent activation of the cells.

To compare the amounts of Lyn, Syk, and Zap-70 in basophils versus B cells (Lyn and Syk) or T cells (Zap-70) and to obtain B cells to immunoprecipitate Syk, the monocyte/lymphocyte fraction obtained after Percoll-gradient centrifugation described above was washed twice with PBS/BSA followed by incubation with mouse antibodies to CD19 and CD22 (B cells) or a combination of CD3, CD4, and CD8 (T cells) for 30 minutes at 4° C. The cells were isolated with goat anti-mouse IgG microbeads and MidiMacs separation columns as described above. Wright-stained analysis of cytospin preparations revealed a homogenous cell population in all experiments.

Immune complex kinase assays

Highly purified (≥95%) basophils were isolated by use of the negative selection method described above. Suspensions of basophils (2.0 to 7.8 × 10^5 cells/condition, depending on cell yield) were primed for 1 hour with human IgE (10 μg/mL), washed with $HBSS^-$ (HBSS without Ca^{2+} or Mg^{2+}),[56] and equal aliquots incubated with prewarmed $HBSS^+$ (HBSS with 1 mmol/L $CaCl_2$ and 1 mmol/L $MgCl_2$) with (activated) or without (nonactivated) 5 μg/mL of goat anti-human IgE for 5 minutes at 37° C. Supernatants were collected for histamine release analysis as described below. In some experiments, piceatannol (10 μg/mL) was present during the last 30 minutes of priming and during activation. Cell activation was stopped by the addition of ice-cold PBS containing 0.01 mol/L ethylenediamine tetraacetic acid (EDTA) (PBS/EDTA) and centrifugation. All procedures were performed in duplicate. In one set of samples, the pellets were lysed in 400 μL of 50 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 1% Brij, 1 mmol/L sodium orthovanadate, and 1 μg/mL each of antipain and leupeptin (lysis buffer) and incubated for 10 minutes on ice. Immune complexes were generated by incubating clarified supernatants with anti-Syk, anti-Lyn, anti–Zap-70, or anti-PY antibodies; the antibodies were preadsorbed to protein A-Sepharose beads (Pharmacia, Uppsala, Sweden) as described.[57] Kinase activity was measured by adding 40 μL of kinase buffer (25 mmol/l HEPES, 10 mmol/L $MnCl_2$, pH 7.5) containing 10 μCi of [γ-^{32}P]ATP (Amersham, 3000 Ci/mmol) to the washed immune complexes and transferred to a 30° C heat block for 15 minutes. Precipitates were washed three times with 1 mL of ice-cold kinase buffer, followed by the addition of 40 μL of Laemmli sample buffer containing 5% 2-mercaptoethanol and proteins separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel (5.0 to 8.0 × 10^5 cell equivalents/lane). Equal numbers of cell equivalents from activated and nonactivated samples were applied to the gel for each of the kinase experiments. Molecular weight markers (Gibco-

BRL, 10 kd ladder) were included in each gel. The gels were dried and the ^{32}P-labeled proteins were visualized by autoradiography. The duplicate samples were separated by SDS-PAGE, followed by blotting with antikinase antibodies (see below) to ensure that activation does not affect the immunoprecipitation of kinases.

Western blotting

Highly purified basophils (96% to >99% pure) were obtained by negative selection followed by positive selection and cell sorting as described above. Cell pellets were resuspended at a concentration of 5×10^6 cells/mL in lysis buffer, vortexed, and held on ice for 10 minutes. Insoluble material was removed by centrifugation at 10,000 *g* for 5 minutes. The lysate was mixed 1:1 (vol/vol) with Laemmli sample buffer [2% SDS (wt/vol), 10% (vol/vol) glycerol, 60 mmol/L Tris (pH 6.8), and 0.1% bromophenol blue], heated at 95° C for 5 minutes, and applied to a 10% polyacrylamide gel (1×10^5 cell equivalents/lane or 20 μg/mL). Molecular weight markers (Gibco-BRL) were included in each gel. After SDS-PAGE, proteins were transferred to nitrocellulose with a semidry apparatus at 400 mA for 1 hour. Membranes were blocked for 1 hour with Blotto (5% nonfat milk and 0.05 mol/L Tween-20 in PBS) and washed three times with Tris-buffered saline containing 0.05% (vol/vol) Tween 20, pH 7.4 (TTBS). Membrane strips were incubated overnight at 4° C with 1 μg/mL each of anti-Syk, anti-Lyn, or anti–Zap-70 antibodies in PBS/BSA or with normal rabbit serum as a negative control (1:500 dilution). Membranes were washed three times with TTBS, incubated with ^{125}I-labeled donkey anti-rabbit IgG (0.5 μCi/ml; Amersham, UK) in PBS/BSA for 1 hour at RT, washed six times with TTBS, then dried and analyzed by autoradiography. In some experiments, Western blotting was performed with antikinase antibodies and peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence detection reagents (Amersham Corp.) to increase sensitivity.

To determine whether anti–Zap-70 antibodies cross-react with Syk, anti-Syk immunoprecipitates were generated from CD19/CD22-purified human B-cell lysates, separated by SDS-PAGE, and immunoblots probed with either anti-Syk or anti–Zap-70 antibodies. Briefly, lysate from 1×10^6 B cells was clarified by microcentrifugation (10,000 *g* for 5 minutes at 4° C) and incubated with protein A-Sepharose beads preadsorbed with anti-Syk antibodies (1 μg) or nonspecific rabbit immunoglobulin (1 μg). After 2 hours, beads were centrifuged at 10,000 *g* for 1 minute, washed, and resuspended in 50 μL of 2× sample buffer. Beads were boiled and samples containing 10 μg of protein per lane were used for SDS-PAGE (7.5% polyacrylamide gel) followed by transfer to nitrocellulose. Western blotting was performed as described above with the use of antibodies to Syk and Zap-70.

To control for possible differences in the amounts of kinases immunoprecipitated from activated versus nonactivated cells, basophils (2.0 to 7.8×10^5 cells/condition) were primed and activated as above. After lysis of equal numbers of unstimulated basophils, immune complexes were generated with antikinase antibodies, separated on a 10% gel, and transferred to nitrocellulose. As a negative control, nonspecific rabbit IgG was substituted for the kinase antibodies during immunoprecipitation with activated lysates (data not shown).

Immunofluorescence labeling and confocal microscopy

For the simultaneous immunolocalization of tyrosine kinases and the 2D7 basophil marker, Percoll-enriched basophils (35% purity) were settled onto poly-L-lysine–treated coverslips for 1 hour. Cells were fixed in 2% paraformaldehyde in PBS for 20 minutes followed by permeabilization with saponin (0.1% vol/vol final) for 30 minutes. The fixed cells were incubated overnight at 4° C in a humid chamber with the basophil-specific mAb antibody FITC-2D7, together with rabbit polyclonal antibodies specific for Lyn, Syk, or Zap-70 (5 μg/mL final for each antibody). As a negative control, normal rabbit serum (Sigma, St. Louis, Mo) was substituted for the kinase antibodies. After overnight incubation, the slides were washed three times with PBS and incubated for 1 hour with a 1/500 dilution of rhodamine-conjugated donkey anti-rabbit IgG antibody (Jackson Labs, West Grove, Pa). After further washing with PBS, the coverslips were mounted onto glass slides with a fluorescence-enhancing medium (Vectashield, Vector Labs, Burlingame, Calif). Confocal imaging was performed with a Bio-Rad MRC-600 Confocal Imaging System interfaced with an inverted optical microscope (Nikon Diaphot). A krypton-argon laser (15 mW) was used as a light source, with a 568 nm excitor filter and YHS (rhodamine) filter block. In double-labeling experiments, no rhodamine emission signal was detected in the fluorescein channel.

Antiphosphotyrosine flow cytometry

Percoll-enriched basophil populations (30% to 58% pure) were primed with 10 μg/mL human IgE for at least 1 hour followed by 10% vol/vol human AB serum for 30 minutes. In some experiments, piceatannol (10 μg/mL) was present during the last 30 minutes of priming and during activation. The cells were washed once with PBS/BSA and once with $HBSS^{=}$. Cells (1×10^5 cells/100 μL) were activated for varying times in suspension by incubation in prewarmed $HBSS^{+}$ plus indicated concentrations of goat anti-human IgE. In some experiments, the Fc portion of human IgG (Fc blocker; 1 μg/mL, Jackson Labs, West Grove, Pa.) was added before the goat anti-human IgE activation. Reactions were terminated by the addition of 900 μL of ice-cold PBS/EDTA and centrifugation for 5 minutes at 800 *g* for 5 minutes. Supernatants were stored at –20° C for histamine determination. Cell pellets were immediately resuspended in fixative (2% vol/vol paraformaldehyde in PBS, pH 7.4) for 20 minutes followed by the addition of saponin (0.02% vol/vol final) for 15 minutes at room temperature. Fixed cells were washed twice with PBS/BSA and incubated sequentially for 30 minutes at RT in PBS/BSA containing 1 μg/mL anti-PY and with FITC-conjugated goat anti-rabbit antibody (1:50 in PBS/BSA; Cappell, Durham, N.C.) as previously described.[58] Washed cells were resuspended in 0.4 mL of PBS and mean channel fluorescence determined for at least 10,000 cells with a Coulter Epics Elite flow cytometer. Basophils were identified on the basis of their forward and side-scatter pattern as described previously.[54]

Histamine release

Histamine in cell pellets and supernatants was measured by radioimmunoassay (AMAC-Immunotech, Westbrook, Maine). For measurement of total histamine, 1×10^5 cells were lysed by three freeze-thaw cycles in PBS/EDTA (1 mL) followed by centrifugation at 10,000 *g* for 5 minutes to remove debris; the supernatant was collected for analysis. Spontaneous and anti-IgE–stimulated release was measured by incubating cells in parallel with or without anti-IgE and collecting the supernatants after centrifuging as above. IgE-mediated release was corrected for

spontaneous release over the time course of incubation and expressed as percentage of total histamine present in cells held on ice.

Statistics

Statistical comparison of the piceatannol-treated cells with nontreated cells was carried out with the paired *t* test with Welch's correction. The accepted level of significance was $P < .02$.

RESULTS

Human basophils contain anti-IgE–activated tyrosine kinases, including Zap-70

By analogy with previous studies in RBL-2H3 cells, we expected that FcεRI cross-linking would activate protein-tyrosine kinases in human basophils. This hypothesis was tested by measuring the in vitro tyrosine phosphorylating activities of antiphosphotyrosine (anti-PY) immune complexes prepared from highly purified (by negative selection) resting and anti-IgE–activated cells. The results of these initial experiments are given in Fig. 1, *A*.

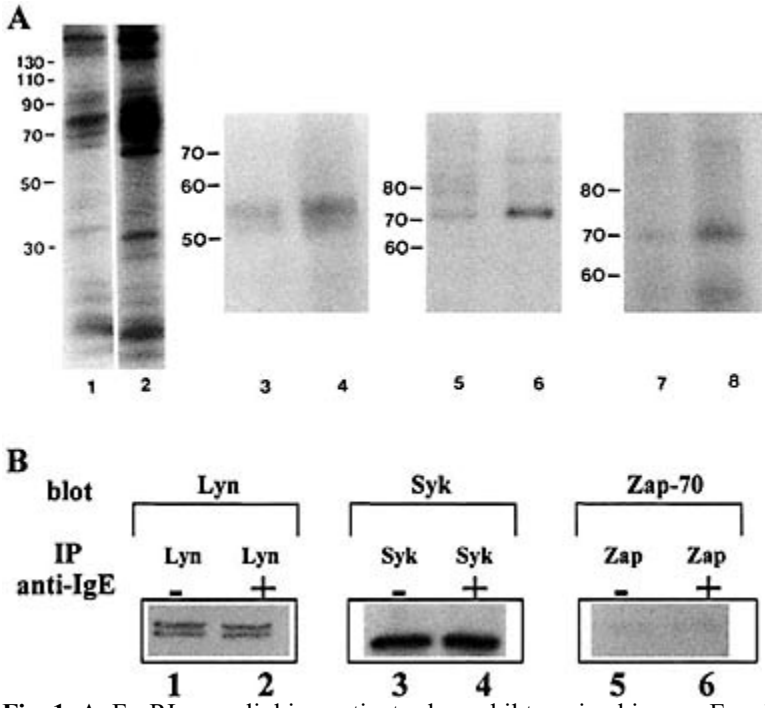

Fig. 1. A, FcεRI cross-linking activates basophil tyrosine kinases. Equal numbers of highly purified normal human basophils (95% to 98%) were obtained by negative selection, primed with human IgE, and incubated in $HBSS^+$ for 5 minutes at 37° C with (*lanes 2*, *4*, *6* and 8,) or without (*lanes 1*, *3*, *5* and *7*) anti-human IgE (5 μg/mL). Cells were lysed and the clarified supernatants immunoprecipitated with anti-PY (*1*, *2*), anti-Lyn (*3*, *4*), anti-Syk (*5*, *6*), or anti–Zap-70 antibodies (*7*, *8*). Kinase activities in immunoprecipitates were measured as described in the Methods section. Results shown are representative of five different experiments, each done in duplicate. Molecular mass markers (kd) are indicated. **B,** Anti-IgE activation does not affect the amount of kinase immunoprecipitated from basophils. Equal numbers of highly purified (by negative selection), IgE-primed basophils (from the same experiment in **A**) were incubated with or without anti-IgE as above, lysed, and immunoprecipitated *(IP)*with anti-Lyn (*1*, *2*), anti-Syk (*3*, *4*), anti–Zap-70 (*5*, *6*), or irrelevant rabbit IgG antibodies (data not shown). Blots were probed with the same antibodies as described in the Methods section followed by horseradish peroxidase–conjugated secondary antibodies and enhanced chemiluminescence.

There was substantial kinase activity associated with anti-PY immunoprecipitates prepared from lysates of highly purified resting basophils *(lane 1*). This activity was increased in immune complexes from lysates of cells that had been activated by 5-minute incubation with anti-human IgE (*lane 2*). The kinases and kinase substrates in these anti-PY immune complexes included proteins comigrating in the 50 to 60 kd range of Src kinase family members, the 70 kd range of Syk family members, and in the range of the FcεRI β and γ subunits (32 and approximately 12 kd).

To identify specific kinases involved in this stimulated response, further immune complex kinase assays were performed with immune complexes generated by antibodies to Lyn, Syk (tyrosine kinases previously identified in RBL-2H3 cells), and Zap-70 (a Syk family member found in T cells but not previously implicated in FcεRI-coupled signaling). Kinase assays performed with anti-Lyn immunoprecipitates from resting cells show modest basal phosphorylation of 56 kd and 53 kd proteins corresponding to the two Lyn isoforms found in hematopoietic cells (*lane 3*). The phosphorylation of the 56 kd Lyn isoform was substantially increased in immune complexes generated from lysates of anti-IgE–activated cells (*lane 4*). Similarly, in vitro kinase assays with anti-Syk immune complexes from lysates of unstimulated cells revealed a 72 kd phosphoprotein corresponding to Syk as well as several coprecipitating phosphoproteins (*lane 5*). The phosphorylation of Syk as well as several of the coprecipitating proteins was enhanced when the anti-Syk immune complexes were prepared from lysates of anti-IgE–stimulated cells (*lane 6*). Because phosphorylation assays in anti-PY immunoprecipitates revealed a protein band at 70 kd, we also performed anti–Zap-70 immune complex kinase assays. There was little or no kinase activity associated with anti–Zap-70 immune complexes from resting cells (*lane 7*). However, anti–Zap-70 immunoprecipitates from activated cells showed autophosphorylation of a 70 kd phosphoprotein, corresponding to Zap-70 that has previously been identified only in T cells (*lane 8*). The mean stimulated histamine release from the experiments in Fig. 1, *A* was 12% in 5 minutes ($n = 8$).

To ensure that equal amounts of kinases are immunoprecipitated from nonactivated and activated cells, identical lysates of resting and activated purified basophils from the above experiments were incubated with antibodies to Lyn, Syk, Zap-70, or with an irrelevant rabbit IgG and the resulting immune complexes separated by SDS-PAGE, transferred to nitrocellulose, and Western blotted with antibodies to Lyn, Syk, or Zap-70. Proteins were detected with the use of peroxidase-conjugated secondary antibodies and enhanced chemiluminescence. The results are presented in Fig. 1, *B*. Very similar amounts of Lyn (*lanes 1, 2*), Syk (*lanes 3, 4*), and Zap-70 *(lanes 5, 6*) were immunoprecipitated from resting (*lanes 1, 3, 5*) and activated cells (*lanes 2, 4, 6*). The specificity of the kinase immunoprecipitation and detection was demonstrated by substituting an irrelevant rabbit IgG (rIgG) for the kinase antibodies (data not shown).

These findings indicate the presence of at least three FcεRI-associated tyrosine kinases, Lyn, Syk, and Zap-70, as well as multiple kinase substrates, in highly purified basophil cell suspensions. Because Zap-70, in particular, has previously been detected only in T cells, further studies were performed to ensure that these anti-IgE–activated kinases were not constituents of contaminating cells (<5% of these highly purified preparations) that responded during the 5-minute assay to substances released or synthesized by activated basophils.

Western blotting analysis of highly purified human basophils

Basophil preparations of more than 96% purity were generated by positive selection, and their total detergent-soluble proteins were separated by SDS-PAGE under reducing conditions, followed by Western blotting. The blots were probed with antikinase antibodies or with normal rabbit serum (as a control), followed by ^{125}I-conjugated anti-rabbit antibodies. As shown in, *A*, blotting lysates of positively selected basophils with antibodies to Lyn revealed two bands with apparent molecular masses of 53 and 56 kd (Fig. 2, *A*; *lane 1*), whereas antibodies to Syk revealed a band at 72 kd (Fig. 2, *A; lane 2*).

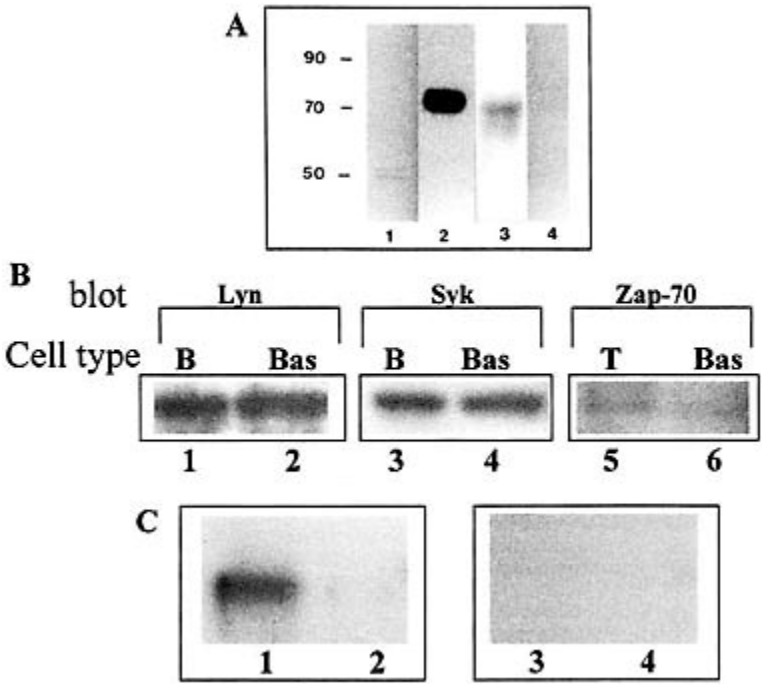

Fig. 2. A, Identification of Lyn, Syk, and Zap-70 in basophils by Western blot analysis. Equal aliquots of an extract of positively selected, highly purified basophils (96% purity) were subjected to SDS-PAGE under reducing conditions in a 10% polyacrylamide gel. Sample was electrophoretically blotted onto nitrocellulose and incubated with antibodies to Lyn (*lane 1*), Syk (*lane 2*), or Zap-70 (*lane 3*) followed by ^{125}I-rabbit antibodies and autoradiography. Substitution of antikinase antibodies with normal rabbit serum resulted in no staining (*lane 4*). **B,** Comparison of kinase levels in basophils *(Bas)* versus B cells (Lyn and Syk) and T cells (Zap-70). Lysates of FACS-sorted, highly purified basophil (>99% purity; 20 μg/lane) were separated by 10% SDS-PAGE and analyzed by Western blot analysis with antibodies to Lyn (*lanes 1, 2*), Syk (*lanes 3, 4*), and Zap-70 (*lanes 5, 6*). Equal amounts of protein (20 μg/lane) from B cells (*lanes 1*, *3*) and T cells (*lane 5*) were run in parallel to basophil lysates (*lanes 2*, *4*, *6*) for comparison. **C,** To examine any cross-reactivity of the Zap-70 antibodies to Syk, Syk was immunoprecipitated from CD19/CD22-selected B-cell lysates and immunoprecipitates separated on a 7.5% gel, immunoblotted, and stained with either antibodies to Syk (*lane 1* and *2*) or Zap-70 (*lane 3* and *4*) followed by ^{125}I-rabbit antibodies and autoradiography. Zap-70 antibodies showed no cross-reactivity with Syk. Substitution of anti-Syk antibody with nonspecific rabbit immunoglobulin during the immunoprecipitation revealed no nonspecific binding (*lanes 2* and *4*).

Blotting with the anti–Zap-70 antibody revealed a band with an apparent molecular mass of 70 kd (Fig. 2, *A; lane 3*). There were no radiolabeled bands in lanes that were incubated with normal rabbit serum followed by ^{125}I-conjugated anti-rabbit antibodies (Fig. 2, *A*; *lane 4*). Reverse

transcription–polymerase chain reaction (RT-PCR) experiments ($n = 2$) also revealed the presence of Zap-70 mRNA in highly purified basophils (>99%) (data not shown). These experiments strongly support the evidence obtained with negatively selected basophils (Fig. 1) that human basophils contain Zap-70 as well as Lyn and Syk.

We examined the relative amounts of Lyn, Syk, and Zap-70 in basophils versus B cells (Lyn and Syk) and T cells (Zap-70) by using Western blot analysis. To rule out the possibility of kinase-containing contaminating cells, we used negative selection followed by positive selection with anti–FcεRI-20 to obtain more than 95% basophils, which were then subjected to cell sorting; this resulted in a more than 99% pure basophil population. Lysates (20 μg/lane) from B or T cells as well as basophils were separated on 10% gels and Western blotted with kinase-specific antibodies. Basophils and B cells contain similar amounts of Lyn and Syk (Fig. 2, *B*; *lanes 1* vs *2* [Lyn] and *3* vs *4* [Syk]). T cells appear to have greater amounts of Zap-70 than do basophils (*lanes 5* vs *6*). This was confirmed by PhosphorImager analysis, which indicated approximately twofold more Zap-70 in T cells than in basophils (data not shown).

Because Syk is 60% identical to Zap-70 in human beings,[59] we considered the possibility that antibodies to Zap-70 could cross-react with Syk. Anti-Syk immune or nonspecific rabbit immunoglobulin complexes generated from lysates of human B cells were resolved by SDS-PAGE, transferred to nitrocellulose, and the blots probed with anti-Syk and anti-Zap-70 antibodies. As expected, anti-Syk antibody revealed a band at 72 kd (Fig. 2, *C*; *lane 1)*. Consistent with the manufacturer's report, anti–Zap-70 antibody showed no cross-reactivity with Syk (Fig. 2, *C*; *lane 3*). No Syk or Zap-70 protein was immunoprecipitated when nonspecific rabbit immunoglobulin was substituted for the anti-Syk antibody (Fig. 2, *C*; *lanes 2, 4*).

Immunofluorescence localization of Lyn, Syk, and Zap-70 in human basophils

Confocal microscopy was used to further establish the presence of Zap-70 in human basophils as well as to confirm the presence of Lyn and Syk in these cells. Percoll-enriched, normal human basophils were allowed to settle onto poly-L-lysine–coated slides and incubated with FITC-conjugated 2D7 mAb, which specifically recognizes a granule constituent found in human basophils and not in other cells in peripheral blood.[50] Kinases were identified in the same cells by simultaneous labeling with rabbit anti-kinase primary antibodies followed by rhodamine anti-rabbit IgG. Results are shown in Fig. 3. mAb 2D7–positive cells (basophils) show fluorescence labeling in association with granules (Fig. 3, *A, C, E,*and *G*).

Anti-2D7–positive cells were also reactive with antibodies to Lyn (*B*), Syk (*D*), and Zap-70 (*F*) but showed no labeling with normal rabbit serum (*H*). Staining with all three kinase antibodies was concentrated at the cell periphery in a somewhat irregular distribution that did not overlap the distribution of granules revealed by the 2D7 labeling. Previous studies in other cell types have similarly localized these kinases to or near the plasma membrane, although usually in a more uniform distribution than we observe in basophils.[60, 61] In partially purified cell preparations, many more cells were stained with the Lyn, Syk, and/or Zap-70 antibodies than with mAb 2D7, consistent with the presence of these kinases in contaminating cells as well as in basophils. These studies independently confirm the presence in human basophils of at least three protein-tyrosine kinases, Lyn, Syk, and Zap-70.

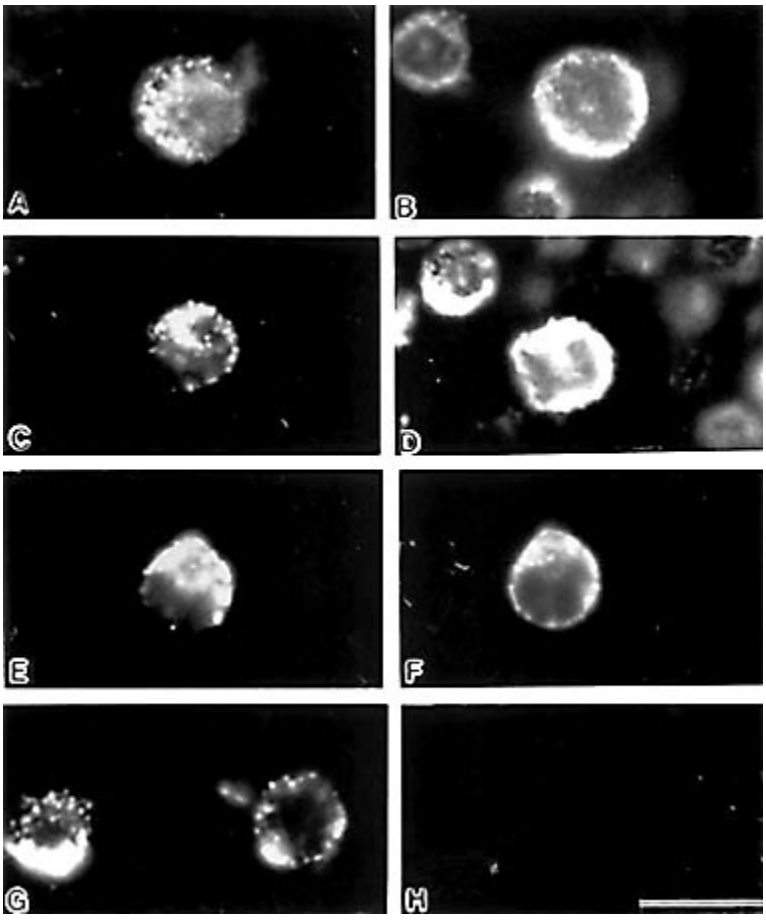

Fig. 3. Identification and localization of Lyn, Syk, and Zap-70 in human basophils by confocal immunofluorescence microscopy. Percoll-enriched human basophils were settled onto poly-L-lysine coverslips and labeled with a mixture of FITC–mouse monoclonal IgG to a basophil-specific antigen (FITC-2D7; **A, C, E, G**) and rabbit anti-Lyn **(B)**, anti-Syk **(D)**, or anti–Zap-70 **(F)** IgG, followed by rhodamine-conjugated donkey anti-rabbit IgG. Basophils identified by fluorescent granule staining also stained with Lyn, Syk, and Zap-70 antibodies as detected by rhodamine staining of the same cells. Basophils incubated with normal rabbit serum showed no staining with rhodamine-conjugated anti-rabbit antibodies **(H)**. Bars, 10 μm.

Piceatannol inhibits IgE-mediated human basophil degranulation

In RBL-2H3 cells, low concentrations of the tyrosine kinase inhibitor piceatannol have little or no effect on Lyn activation and receptor subunit phosphorylation but abolish Syk activation and tyrosine phosphorylation of essentially all other proteins and all downstream responses.[57] The effect of piceatannol on anti-IgE–induced histamine release in human basophils is shown in Fig. 4, *A*.

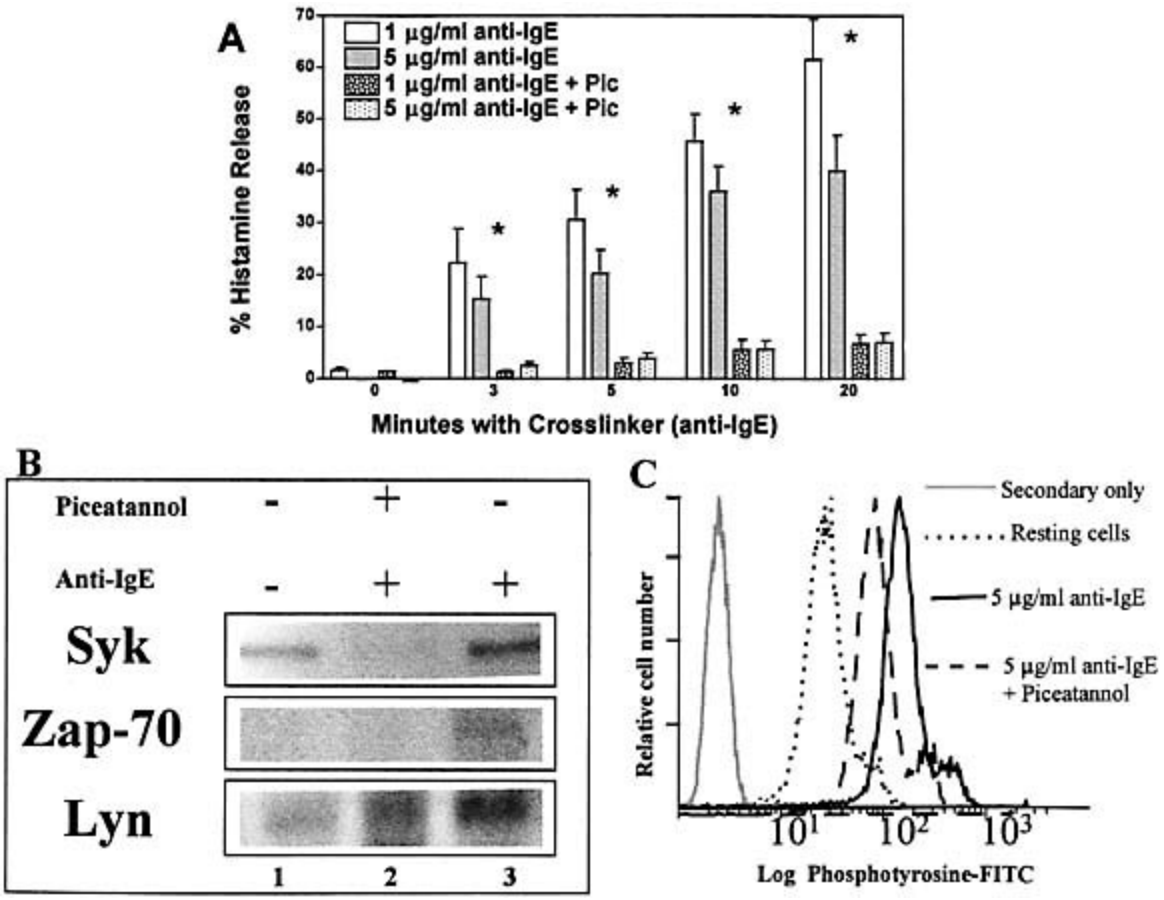

Fig. 4. A, Piceatannol inhibits IgE-mediated histamine release. Percoll-enriched basophils (40% to 50% purity) from three different donors were IgE-primed and incubated for different times with two concentrations of anti-human IgE without (*open boxes*) or with a 30-minute preincubation with 10 µg/mL piceatannol (*dot-filled boxes*). Percent histamine release was measured as described in the Methods section. Results are represented as standard error of the mean of three separate experiments (± SEM*; n*= 3). Spontaneous histamine release (≤6%) was unaffected by piceatannol and is subtracted from the values shown. *Asterisks* designate results for each anti-IgE concentration that was significantly reduced compared with cells not preincubated with piceatannol ($P < .02$). **B,** Piceatannol inhibits Syk and Zap-70 kinase activities. Negatively selected, highly purified basophils (≥95% pure) were IgE-primed and either not activated (*lane 1*) or activated with 5 µg/mL of antihuman IgE for 5 minutes with (*lanes 2*) or without (*lane* 3) a 30-minute preincubation with 10 µg/ml of piceatannol. Reactions were terminated by addition of ice-cold PBS/EDTA and centrifugation. Cells were lysed and their supernatants immunoprecipitated with anti-Syk, anti–Zap-70, or anti-Lyn antibodies. Kinase activities in the immunoprecipitates were measured as described in the Methods section. Results shown are representative of three different experiments. Molecular mass markers (kd) are indicated. **C,** Piceatannol reduces overall cellular tyrosine phosphorylation. Duplicate samples of Percoll-enriched basophils (50% to 70% pure) were IgE-primed and incubated with (*solid black line*) or without (*dotted line*) anti-human IgE alone or after a 30-minute preincubation with 10 µg/mL piceatannol (*dashed line*). Cells were fixed and labeled with rabbit anti-PY antibody followed by FITC anti-rabbit IgG. Mean fluorescence intensity per cell was measured for 10,000 cells/sample by flow cytometry as described in the Methods section. Results shown are in comparison of cells stained with the secondary antibody only (*solid gray line*).

In basophil-enriched preparations (40% to 50% purity), piceatannol pretreatment inhibited anti-IgE–induced histamine release greater than 10-fold (± SEM*; n* = 3; $p < 0.02$). This effect could be seen at both 1 and 5 µg/ml of anti-human IgE and at each time point measured.

The effects of piceatannol on FcεRI-induced Lyn, Syk, and Zap-70 activation were examined with the use of immune complex kinase assays. Highly purified basophils (≥95%) were activated with anti-human IgE with or without preincubation with piceatannol. Cells were lysed and anti-Syk, anti-Lyn, and anti–Zap-70 immune complexes were tested for autophosphorylation, an index of kinase activity. The results of anti-Syk in vitro kinase assays in Fig. 4, *B* confirm the activation of Syk by FcεRI cross-linking and show that piceatannol reduces Syk autophosphorylation in anti-IgE–stimulated cells to below basal levels. Preincubation with piceatannol also reduces Zap-70 autophosphorylation in FcεRI–cross-linked cells to basal levels or lower, as shown in Fig. 4, *B*. In contrast, piceatannol has little or no effect on anti-IgE–induced Lyn autophosphorylation (Fig. 4, *B*). These data link activation of a kinase cascade to FcεRI-mediated basophil activation.

The effect of piceatannol on total levels of antigen-activated tyrosine phosphorylation was measured by flow cytometry of antiphosphotyrosine-labeled cells.[62] Total cellular tyrosine phosphorylation is reduced but does not fall to basal levels in basophils preincubated with piceatannol before anti-IgE activation (Fig. 4, *C*). These results are consistent with evidence above (Fig. 4, *B*) that piceatannol is a selective kinase inhibitor, blocking the activation of Syk and Zap-70 but permitting the continued phosphorylation and activation of Lyn.

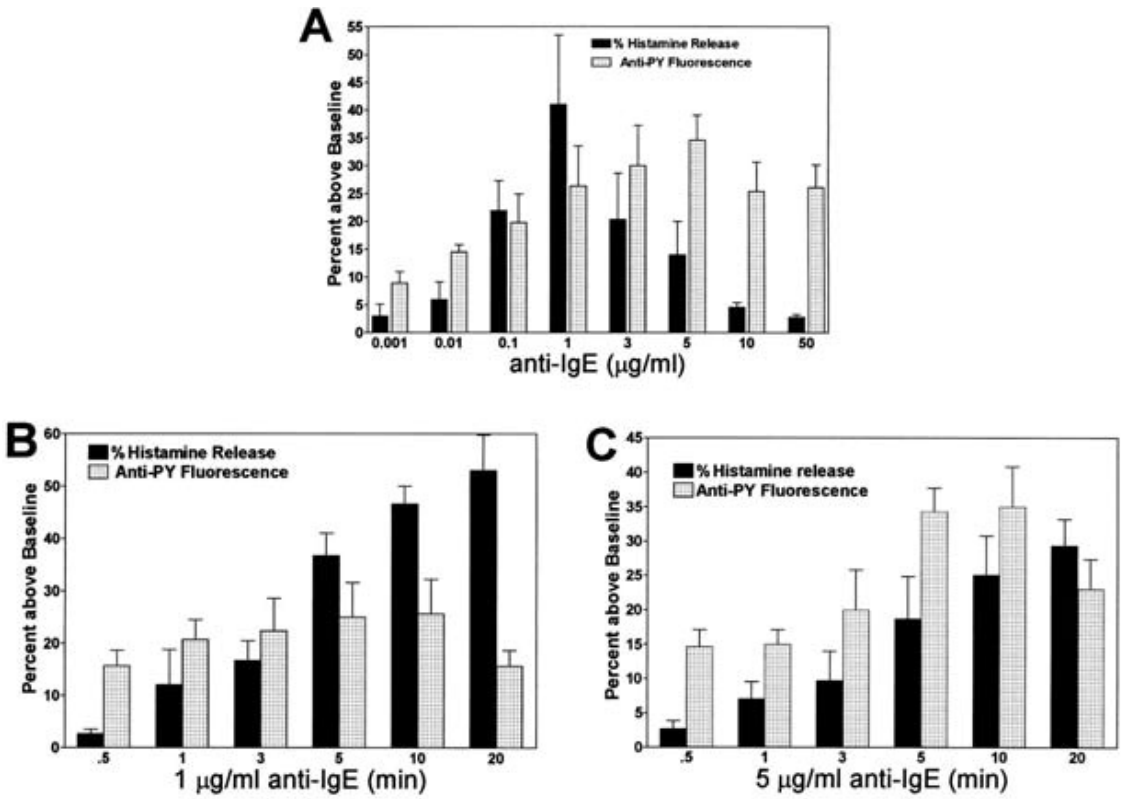

Fig. 5. Relation between overall level of tyrosine phosphorylation and secretion. **A,** Percoll-enriched basophils (30% to 58% pure) were primed with human IgE and incubated in suspension for 10 minutes with varying concentrations of anti-human IgE. IgE-primed cells were incubated with either 1 μg/mL **(B)** or 5 μg/mL **(C)** of anti-human IgE for varying times. Cells were fixed and labeled with rabbit anti-PY antibody followed by FITC anti-rabbit IgG. Mean fluorescence intensity per cell was measured for 10,000 cells/sample by flow cytometry as described in the Methods section. Data are expressed as a percentage above the mean fluorescence intensity in parallel unstimulated samples (mean ± SD; $n = 3$).

Concentration dependence of IgE-mediated protein-tyrosine phosphorylation and histamine release

The relation of anti-IgE–induced kinase activation to signaling responses was further explored with a flow cytometric assay that quantitates fluorescence in antiphosphotyrosine-labeled cells. Histamine release was measured in supernatants of the same cells. The results in Fig. 5, *A* compare the cross-linker concentration dependence of the two responses.

When incubation time is 10 minutes, the addition of increasing amounts of anti-human IgE (0.001 to 1 μg/mL) stimulates histamine release. However, histamine release declines rapidly in response to concentrations of cross-linker that are higher than 1 μg/mL anti-IgE. This is the well-established high-dose inhibition of secretion.[63, 64] It occurs whether or not the cells are preincubated with a human Fc-blocking peptide (data not shown), indicating that the high-dose inhibition of secretion is not caused by the goat anti-IgE molecules binding FcγR on basophils and delivering a negative signal. In contrast, tyrosine phosphorylation continues to increase to 5 μg/mL anti-IgE and remains elevated as anti-IgE concentrations increase to 50 μg/mL (Fig. 5, *A*).

Time course studies with 1 μg/mL and 5 μg/mL of anti-IgE are illustrated in Fig. 5, *B* and *C*. At both concentrations, elevated levels of total cellular tyrosine phosphorylation were detectable within 30 seconds of activation, reached a maximum after 5 to 10 minutes, and then declined. In contrast, histamine release was not significant until 3 minutes after stimulus, and secretion continued for at least 20 minutes (Fig. 5, *B* and *C*). The lower cross-linker concentration (1 μg/mL) consistently resulted in higher histamine release values, whereas the higher cross-linker concentration (5 μg/mL) consistently gave higher overall tyrosine phosphorylation responses. Thus anti-IgE–induced tyrosine phosphorylation response is required for secretion, but the level of overall tyrosine phosphorylation does not predict the extent of secretion except in a narrow range of low cross-linker concentrations.

DISCUSSION

We have established that human basophils contain two FcεRI-associated, protein-tyrosine kinases, Lyn and Syk, previously reported to be essential signal transducing components in rodent FcεRI-signaling models. By Western blotting and image analysis, our data demonstrate the presence of two Lyn kinase isoforms, of 56 and 53 kd, in human basophils in amounts comparable to those found in B cells. This is consistent with previous work demonstrating alternatively spliced forms of Lyn encoding distinct proteins.[65, 66] The presence of Lyn has been demonstrated in other human cell types including macrophages, platelets, and B lymphocytes.[67, 68]

Our data also demonstrate that basophils and B cells have similar amounts of Syk. The presence of Syk in human basophils was expected on the basis of its FcεRI association and antigen-induced activation in RBL-2H3 cells.[7, 8] In addition, Syk has been shown previously to be activated in receptor-coupled signal transduction pathways in B cells,[69] monocytes (HL-60 cells),[70] and platelets.[71] By analogy with published studies in RBL-2H3 cells, we suppose that Syk activation depends on the prior activation of Lyn, resulting in the phosphorylation of

immunoreceptor tyrosine activation motifs (ITAM) in the FcεRI β and γ subunits and the creation of γ-ITAM docking sites for the tandem SH2 domains of Syk.

Unexpectedly, we demonstrated the presence of a third FcεRI-associated tyrosine kinase, Zap-70, in human basophils. Western blotting data and image analysis suggest that the levels of Zap-70 in basophils are somewhat less than that in T cells. Zap-70 was first described in a human leukemia T-cell line in which it was shown to associate with the phosphorylated ζ chain of the T-cell receptor within 15 seconds after receptor stimulation.[72] Although both Zap-70 and Syk are members of the same kinase family and function similarly in their interactions with ITAM-containing receptors, Zap-70 is distinct from Syk as determined by its DNA sequence and tissue distribution.[73, 74] Zap-70 message but not protein was detected by PCR with the use of RNA from RBL-2H3 cells.[74] The presence of Zap-70 in purified populations of human granulocytes has not been examined thoroughly.

The tyrosine kinase inhibitor piceatannol has little or no effect on Lyn activation in human basophils but blocks the activity of both Syk and Zap-70 as measured in in vitro kinase assays. Piceatannol simultaneously abolishes secretion from human basophils. These results imply that the activation of Syk family members by phosphorylation may be required to initiate IgE-mediated secretion from human basophils.

Our studies to date do not distinguish the relative contributions of Syk and Zap-70 to the FcεRI signaling pathway of human basophils. Further work is needed to determine if Zap-70 activates downstream responses that may be the same as or different from the responses to Syk activation. The mechanism of Zap-70 activation in anti-IgE–treated basophils is also unclear. In T cells, Zap-70 interacts with the T-cell receptor (TCR) ζ chain, which we have not been able to detect in human basophils (data not shown). However, there may be an interaction between Zap-70 and the FcεRI γ chain, which is related to the αβ TCR ζ chain and replaces the ζ subunit in δγ T cells. Evidence to support this comes from studies in which GST fusion proteins of the Zap-70 SH2 domains had a similar avidity for the FcεRI γ chain ITAM peptides as it did for the TCR ζ chain ITAM peptides.[75] Also, in COS-1 cells transfected with the FcεRI/FcγRIIIA γ subunit, cotransfection with Zap-70 resulted in an increase in phagocytic signaling mediated by a Zap-70/γ chain interaction.[76] Physiologic studies in Xenopus oocytes also suggest that the γ chain and ζ chain may have interreceptor complementation.[77] Recent work also suggests that Fc-γ and Fc-ζ are interchangeable in their ability to mediate T-cell development and function.[78] Thus it is possible that Zap-70 is activated by interaction with phospho-ITAMs in the FcεRI γ subunit. It is also possible that Zap-70 interacts with the ITAM of the FcεRI β subunit. We have identified proteins at the 70 to 75 kd range that coimmunoprecipitate with antibodies to the FcεRI-β chain in lysates from highly purified, activated basophils, although we have not been able to identify these proteins by using Western blotting (data not shown).

Previous work with human leukemic basophils or basophils derived from bone marrow leukemic basophils suggested that the activation of Syk and the phosphorylation of other substrates occur within 1 minute after FcεRI stimulation.[79] These studies also found a positive correlation between histamine release and tyrosine phosphorylation. However, these studies did not identify either Lyn or Zap-70 activation and did not examine the effects of high concentrations of anti-IgE on histamine release versus total cell phosphorylation in normal peripheral blood basophils.

We found that high concentrations of anti-IgE cross-linker inhibit degranulation as detected by histamine release. Very similar kinetics of histamine release have been reported previously in human basophils[80, 81, 82] and in other FcεRI signaling systems.[83, 84, 85] A previous explanation for these results is that larger FcεRI receptor aggregates formed by multivalent antigen are rapidly desensitized, perhaps by conversion to a detergent-insoluble form, resulting in impaired kinase activation and signal transduction.[32, 84, 86] However, we report that overall protein-tyrosine phosphorylation, a measure of the phosphorylation of kinases and their substrates, remained elevated at anti-IgE concentrations that no longer supported degranulation. These results are inconsistent with the earlier hypotheses linking the interruption of signaling with the inhibition of tyrosine phosphorylation. They are consistent with recent data from Wofsy et al.[86] showing that relatively small aggregates of FcεRI, formed by chemically cross-linked oligomers of IgE, maintained signaling activity for extended incubation periods as determined by protein phosphorylation, although the aggregates formed late in the response were less effective than the aggregates formed early. In a related study, Paolini et al.[87] found that adding monovalent hapten after pronged stimulation with multivalent antigen fails to reverse the phosphorylation of receptor subunits and their coprecipitating proteins, even though it halts secretion.

One hypothesis to explain the apparent dissociation of antigen-induced protein-tyrosine phosphorylation from secretion is that high-dose inhibition of secretion results, at least in part, from events downstream of tyrosine kinase activation. These events could be at the level of signal initiation: Possibly inhibitory sites on kinase substrates are phosphorylated as the intensity of kinase activity increases, resulting in altered enzyme activities. The presence of both stimulatory and inhibitory tyrosine phosphorylation sites is well established for a range of proteins, including Src family members.[88] They could also be at the level of signal propagation. For example, the preferential activation of the SH2-containing inositol phospholipid 5-phosphatase (SHIP) at higher antigen concentrations could prevent the propagation of PI 3-kinase and phospholipase C γ–mediated signals through the accelerated removal of phosphatidylinositol (3,4,5)P_3 (PIP_3) and of Ins(1,4,5)P_3. SHIP was identified as a potential downstream effector of FcεRI signaling on the basis of its ability to bind FcεRI β and γ subunit ITAMs in a yeast tribrid screening assay; it was subsequently shown to associate with several tyrosine-phosphorylated proteins in RBL-2H3 cells.[89]

We conclude that FcεRI-coupled signal transduction in human basophils is initiated by tyrosine kinase activation and modulated by events downstream of receptor-associated kinases. Further studies with highly purified basophils are expected to identify specific effector molecules involved in signal initiation, propagation, and inactivation.

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