

Src homology 2 domain-containing inositol 5' phosphatase is negatively associated with histamine release to human recombinant histamine-releasing factor in human basophils

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Background: The human recombinant histamine-releasing factor (HrHRF) acts as a complete stimulus for histamine release and IL-4 secretion from a subpopulation of highly allergic donor basophils, termed IgE⁺ basophils. Additionally, IgE⁺ basophils release histamine to other secretagogues, IL-3, and deuterium oxide. We hypothesized that IgE⁺ basophils were hyperreleasable.

Objective: Deficiencies in early signal transduction events associated with FcεRI lead to a nonreleasable phenotype, whereas the Src homology 2 domain-containing inositol 5' phosphatase (SHIP) knockout mice have hyperreleasable mast cells. The purpose of this study was to ascertain whether a difference in intracellular signaling molecules could explain the hyperreleasable phenotype of human IgE⁺ basophils.

Methods: Basophils were purified by means of double Percoll gradients and negative selection with magnetic beads. Cell lysates were Western blotted for the tyrosine kinases Lyn and Syk and the phosphatase SHIP. Additionally, histamine release to HrHRF was performed in addition to real-time RT-PCR to investigate mRNA for SHIP.

Results: We show a striking negative correlation between the amount of SHIP protein per cell equivalent, but not Lyn or Syk, and maximum histamine release to HrHRF. This deficiency of SHIP was observed in basophils, but not lymphocytes or monocytes, of these IgE⁺ donors. Additionally, levels of mRNA for SHIP did not differ between IgE⁺ and IgE⁻ donor basophils, which is consistent with a posttranscriptional mechanism of protein regulation. SHIP and phosphatidylinositol 3-kinase reciprocally regulate phosphatidylinositol (3,4,5) triphosphate levels. We also demonstrated that Ly294002, the phosphatidylinositol 3 kinase inhibitor, prevented HrHRF-induced histamine release in IgE⁺ donor basophils.

Conclusion: Taken together, these data suggest that the hyperreleasability of IgE⁺ donors is associated with low levels of SHIP and implicate SHIP as an additional regulator of secretion in human basophils. (*J Allergy Clin Immunol* 2001;108:822-31.)

Key words: Basophil, cytokines, cellular activation, signal transduction

The human recombinant histamine-releasing factor (HrHRF), also known as p23 or translationally controlled tumor protein, was initially described as a complete secretagogue for histamine and IL-4 secretion from basophils of a subset of allergic donors.¹⁻² Initially, it was thought that these donors had a certain type of IgE, termed IgE⁺, that interacted with HrHRF to induce secretion.³ Therefore basophils from HrHRF-responsive donors were termed IgE⁺ basophils. Subsequently, it was demonstrated that HrHRF primes all basophils for histamine release, as well as for IL-4 secretion.⁴ This occurred in basophils that, by definition, did not respond to HrHRF alone, termed IgE⁻ donor basophils. Subsequent data demonstrated that HrHRF did not appear to interact with IgE. First, pharmacologic agents that altered HrHRF-induced histamine release (eg, rottlerin) did not affect anti-IgE-induced release.⁵ Second, rat basophilic leukemia (RBL) cells transfected with the α, β, and γ chains of the human IgE receptor FcεRI did not release histamine to HrHRF, despite sensitization with IgE⁺ molecules.⁶ However, as expected, histamine release was induced by polyclonal anti-IgE in these transfected cells. Third, HrHRF was shown to stimulate eosinophils to produce IL-8 and a calcium response.⁷ This also occurred in the eosinophil cell line AML-3D10, which does not express the α chain of FcεRI and therefore cannot bind IgE with high affinity. Finally, HrHRF has been shown to bind to the Jurkat T-cell line, as well as to inhibit cytokine production from stimulated primary T cells (unpublished data from J. Y. Oh, B. M. Vonakis, and S. M. MacDonald [2000]). The search for a specific HrHRF receptor is ongoing.

Despite the fact that HrHRF did not appear to involve the IgE molecule, we have retained the designation of IgE⁺ and IgE⁻ for consistency with past studies. A molecular basis for the hyperreleasability of a subpopulation of allergic donor basophils remained elusive. The IgE⁺ donors that responded directly to HrHRF also released histamine to IL-3 and deuterium oxide (D₂O).^{8,9} This suggested that these basophils have an element of unexplained hyperreleasability. This concept of releasability is not new in the field of stimulation of human basophils. Two decades ago, Findlay and Lichtenstein¹⁰ described

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Abbreviations used

BMMC:	Bone marrow–derived mast cell
ΔC_T :	Difference in cycle number
D ₂ O:	Deuterium oxide
GST:	Glutathione-S-transferase
HrHRF:	Human recombinant histamine-releasing factor
MFI:	Mean fluorescence intensity
PE:	Phycoerythrin
PI3-K:	Phosphatidylinositol 3 kinase
PIP ₃ :	Phosphatidylinositol (3,4,5) triphosphate
RBC:	Red blood cell
RBL:	Rat basophilic leukemia
SHIP:	Src homology 2 domain–containing inositol 5′ phosphatase
UTR:	Untranslated region

that basophils of patients with asthma, compared with those of control subjects, responded significantly more to D₂O. Their results suggested that there might be a parameter of releasability that must be defined for each separate stimulus and that patients with asthma can be differentiated from other individuals by the response of their basophils to selected stimuli.

There are a number of features of the population response to IgE-mediated stimulation of human basophils. One end of the spectrum is the nonreleaser phenotype. Basophils from these subjects do not release histamine when stimulated through the FcεRI. However, other non-IgE-mediated stimuli, such as the calcium ionophore, the formyl-methionyl-leucyl-phenylalanine peptide, and the phorbol ester, cause secretion from these nonreleasing basophils.¹¹ This suggests that there might be a defect in early signaling events associated with FcεRI. Indeed, Kephley et al¹² published a deficiency of the tyrosine kinase Syk in 3 nonresponding donors. This observation was confirmed and extended to involve the tyrosine kinase Lyn by Lavens-Philips and MacGlashan.¹³ Both of these kinases are known to be critical for initiating FcεRI-mediated signaling.¹⁴ At the other end of the spectrum are donors who are hyperreleasable. By virtue of the response of basophils from IgE⁺ donors to stimuli, such as HrHRF, D₂O, and IL-3, these donors would be considered hyperresponsive. In view of differential expression of Lyn and Syk in nonreleaser basophils compared with normal basophils, one could consider that either these kinases or another signaling molecule might be differentially expressed in hyperresponsive IgE⁺ donors.

A clue to increased hyperreleasability was demonstrated in the mouse knockout of the gene encoding Src homology 2 domain–containing inositol 5′ phosphatase (SHIP). SHIP was cloned in 1996 by 3 independent groups.^{15–17} It has 1188 amino acids, is present on chromosome 2, and becomes tyrosine phosphorylated and associated with the adapter protein Shc in hematopoietic cell lines after stimulation with cytokines.¹⁸ The full-length protein is 145 kd, but other molecular forms are known to exist, such as those at 135, 125, and 110 kd.¹⁸ It has been demonstrated to be a negative regulator of B-cell antigen receptor signaling, as well as rodent mast-

cell signaling, through recruitment of SHIP to the tyrosine phosphorylated inhibitory motif of FcγRIIB.^{19,20} Very recently, negative regulation of FcεRI signaling by cocrosslinking through FcγRIIB in human basophils implicates the phosphatase SHP-1 rather than SHIP.²¹ What is very intriguing is that SHIP knockout mice generated by means of homologous recombination in embryonic stem cells showed hydrolysis of phosphatidylinositol (3,4,5) triphosphate (PIP₃) and degranulation of mouse bone marrow–derived mast cells (BMMCs) sensitized with IgE alone in the absence of antigen.²² More recently, monomeric IgE was shown to stimulate cytokine production and cell survival in mouse BMMCs.²³ This suggests that lower levels of SHIP would correspond to hyperreleasability, at least in the murine system. The current studies were undertaken to address the presence and levels of SHIP in human basophils and to compare it with the levels of Lyn and Syk, 2 tyrosine kinases known to be important in human basophil signaling, in basophils from 2 populations of patients, both IgE⁺ and IgE[−] donors.

METHODS

Recombinant HrHRF production

The cDNA encoding glutathione-S-transferase (GST)-HrHRF fusion protein was excision cloned by means of digestion from the pGEX-2T vector into the baculovirus vector pVL1393. Plasmid DNA was cotransfected into Sf9 cells for viral isolation and amplified according to the company's specifications (Invitrogen, Carlsbad, Calif). Subsequently, the Sf9 insect cells were infected with recombinant baculovirus and grown commercially on a large scale in serum-free media (Paragon Biotech, Baltimore, Md). The cell pellets from 8 L of Sf9 cells were dissolved in 800 mL of NBB buffer (20 mmol/L sodium phosphate and 500 mmol/L sodium chloride, pH 7.8) to which 800 μL of BD Pharmingen (San Diego, Calif) inhibitor cocktail was added. The suspension was freeze-thawed 3 times and centrifuged at 9000 rpm for 20 minutes. Purification of the supernatant, which contained the GST-HrHRF fusion protein, was accomplished by means of affinity chromatography on immobilized glutathione and confirmed with SDS-PAGE and Western blotting by using a polyclonal anti-HrHRF antibody generated against the recombinant material produced in *Escherichia coli*. Protein concentration was determined by using the BioRad protein assay and was found to be 160 μg/mL HrHRF. HrHRF was dialyzed against physiologic 1× PIPES (25 mmol/L Pipes, 110 mmol/L NaCl, and 5 mmol/L KCl, pH 7.4) for use in all assays. HrHRF was found to contain a single band on SDS-PAGE and was judged to be greater than 95% pure.

Basophil, monocyte, and lymphocyte isolation

By definition, IgE⁺ donor basophils demonstrate 10% or greater histamine release to HrHRF. For histamine-release studies, basophils were isolated by means of our standard dextran sedimentation method²⁴ and stimulated with polyclonal goat anti-human IgE, recombinant HrHRF, or the phosphatidylinositol 3 kinase (PI3-K) inhibitor LY294002 (Sigma, St Louis, Mo) for 45 minutes, and histamine was measured by using our automated fluorometric method.²⁵ For studies investigating levels of Lyn, Syk, and SHIP, basophils were purified from 150 mL of peripheral blood drawn from volunteers after obtaining informed consent. All blood drawing was conducted under a protocol approved by the Johns Hopkins University

Institutional Review Board. Blood was layered onto a discontinuous double Percoll (Amersham-Pharmacia, Piscataway, NJ) gradient consisting of 53.2% and 62% Percoll, respectively, in Accuspin tubes (Sigma) and centrifuged at 130g for 20 minutes at room temperature. After centrifugation, 2 bands of white cells were visible above the filter, and the majority of red blood cells (RBCs) sedimented below the filter. The cell suspension from the lower white cell band (consisting primarily of basophils and lymphocytes, trace numbers of RBCs, and, infrequently, eosinophils and neutrophils) was then further purified by means of negative selection on a Midi-MAC LS+ gravity column (Miltenyi Biotech, Auburn, Calif) with the Miltenyi basophil isolation kit. Routinely, basophil preparations were 87% to 99% pure. For real-time RT-PCR, which required basophils of 98% to 100% purity, the StemCell (Vancouver, British Columbia, Canada) basophil isolation kit and pump feed column were used to further purify basophils isolated from a double Percoll gradient.

Ten milliliters of blood was collected in 0.1 mol/L EDTA to prepare enriched monocyte and lymphocyte suspensions, and then both monocytes and lymphocytes were prepared by using the Rosette-SEP (StemCell) cell-enrichment cocktail for monocytes or lymphocytes. Cells were enriched according to the manufacturer's protocol. Of note, Ficoll-Paque (Pharmacia Biotech AB, Uppsala Sweden) was used as the density gradient, and residual RBCs were removed by using RBC lysis buffer (150 mmol/L NH_4Cl , 10 mmol/L KHCO_3 , and 0.1 mol/L EDTA). Monocytes and T cells were counted with erythrosin B stain (Sigma). Forty million cells per milliliter of either monocytes or lymphocytes were lysed in lysis buffer and Western blotted for SHIP, as noted below. Flow cytometry was used to determine the purity of these 2 cell populations. Mononuclear lymphocyte specimens were labeled for direct immunofluorescence and flow cytometry in the presence of 4 mg/mL human IgG to minimize nonspecific binding to $\text{Fc}\gamma\text{R}$, as previously described.²⁶ For each leukocyte subset, both light-scatter characteristics and specific surface markers were used for analysis (CD14 for monocytes and CD3 for T cells). Antibodies used for these studies included an FITC-conjugated mouse anti-human CD14 (Pharmingen, San Diego, Calif), phycoerythrin (PE)-conjugated mouse anti-human CD3 (Becton Dickinson, San Jose, Calif), FITC-conjugated irrelevant mouse IgG₁ (Becton Dickinson), or PE-conjugated irrelevant mouse IgG₁ (Biosource, Camarillo, Calif).

Cells were incubated with either the FITC IgG control or FITC CD14 for monocytes or PE IgG control or PE CD3 for T cells for 30 minutes on ice. After washing, the cells were resuspended in 1% paraformaldehyde (Sigma). Samples were analyzed by using a Coulter EPICS Profile II flow cytometer with appropriate compensation. Identical photomultiplier tube settings were used for each sample, and the flow cytometer was fully aligned and calibrated with fluorospheres daily. At least 5000 events per experimental condition were counted. Data were expressed as net mean fluorescence intensity (MFI; sample MFI minus MFI of the irrelevant IgG control).

Cell lysis and Western blotting

Cells were washed twice with 1× PIPES buffer and then lysed at 40×10^6 cell equivalents/mL by means of resuspending the cells in 1× PIPES buffer containing 1× PharMingen Protease Inhibitor Cocktail, 5 mmol/L EDTA, 1 mmol/L Na_3VO_4 , and 5 mmol/L $\text{Na}_4\text{P}_2\text{O}_7$ as inhibitors and adding an equal volume of 2× TRIS glycine SDS sample buffer (Invitrogen) to determine relative Lyn, Syk, and SHIP levels in purified human basophils. To measure SHIP levels in human lymphocytes and monocytes, the cells were washed twice in 1× PIPES buffer and then lysed as above for basophils. Two hundred thousand cell equivalents were loaded per lane and run on SDS-PAGE under nonreducing conditions on a 10% TRIS-glycine gel (Invitrogen) to detect Lyn and Syk. A SHIP Western blot standard was prepared from primary T lymphocytes purified by means of

countercurrent elutriation and lysed in TRIS glycine SDS sample buffer. For Lyn and Syk Western blot standards, a similar whole-cell lysate was prepared from the monocytes of a single donor. The standard lysates were placed in aliquots for single use before being frozen at -80°C . Western blots were developed with rabbit anti-human Lyn antibody (UBI, Lake Placid, NY) at a dilution of 1:5000 or mouse anti-human Syk (Santa Cruz Biotechnology, Santa Cruz, Calif) at a dilution of 1:500, followed by anti-rabbit IgG-HRP or anti-mouse IgG-HRP (Amersham-Pharmacia) at a dilution of 1:10,000 and developed with enhanced chemiluminescence (Pierce Supersignal West Pico, Rockford, Ill). To detect SHIP in human basophils, 2×10^6 cell equivalents were loaded per lane and run on an 8% TRIS-glycine gel under nonreducing conditions. Rabbit anti-human SHIP (Santa Cruz) was used at 1:500 dilution, and Western blots were developed as described above.

The method of loading the same amount of cell equivalents and comparing levels of intracellular signaling molecules with the same standard lysate loaded on every blot is a standard method when using human basophils from different donors.^{13,27,28} Additionally, SHIP samples from the same donor were run on different blots to document reproducibility, and compared with the internal T-cell lysate standard and the interblot, variability was less than 10%, and the intrablot variability was less than 6% when SHIP samples from the same donor were run on the same blot. Furthermore, the linearity of staining with the antibodies used for Western blotting was confirmed by scanning blots loaded with increasing amounts of lysate containing Lyn, Syk, or SHIP to verify that doubling the specific protein concentration doubled the blot intensity.

SHIP mRNA quantitation

Total RNA was isolated from highly purified human basophils (98%-100%) by using RNeasy B, according to the manufacturer's instructions (Tel-Test, Friendswood, Tex). All samples were stored at -20°C until mRNA was quantitated with real-time RT-PCR.²⁹ RNA concentration was determined by using UV absorbance. Forward and reverse oligonucleotide primers for SHIP were constructed with Primer Express software (Perkin-Elmer, Foster City, Calif) from a previously published SHIP cDNA sequence (accession No. U84400). The forward primer was 5'-CAAGAAGCTGAGTCCCTTTAACATC-3' (nucleotide position 1782), and the reverse primer was 5'-GTAGGCAGATCCACACGGTAGTT-3' (nucleotide position 1846). The probe for use in real-time RT-PCR was also designed with Primer Express and the previously reported cDNA sequence for human SHIP. The probe sequence for SHIP was 5'-ACCGCTTCACGCACCTCTTCTGGTT-3'. The 5'-reporter dye was 6-carboxyfluorescein, and the 3'-quencher dye was 6-carboxy-N,N,N',N'-tetramethylrhodamine. Primers and probe were prepared and purified by BioSource International (Camarillo, Calif). The real-time RT-PCR method is based on the cleavage of fluorescent dye-labeled probes by the exonuclease activity of Taq-DNA polymerase during PCR and measurement of fluorescence intensity with a Sequence Detection System (Perkin-Elmer Model 7700). With this instrumentation, 7 measurements of fluorescence per well per PCR cycle were made during a 40-cycle amplification, and the point at which fluorescence exceeds a predetermined threshold (occurring within the linear region of the amplification curve) determines the cycle number (so-called C_T) for the sample. Because multiple measurements are made during each cycle, the analytic software produces fractional cycle numbers for each sample. Using this system, we have determined mRNA levels for SHIP in human basophils from a selection of IgE⁺ and IgE⁻ donors using a 1-step RT-PCR protocol. The final PCR products were run on a 2.5% agarose gel to confirm that a single band of the correct size (87 bp) was amplified. The mRNA levels of SHIP from the donor samples were quantified as the fold increase above a positive control RNA

prepared from a single donor's monocytes and stored in aliquots at -20°C . Each basophil RNA sample was amplified at 5 serially diluted amounts of template (10-160 ng) in parallel with the similarly diluted positive control RNA. Reactions were prepared by mixing 22 μL of a master mix consisting of 5.5 mmol/L MgCl_2 ; 300 $\mu\text{mol/L}$ each of deoxyadenosine triphosphate, deoxycytidine triphosphate, and deoxyguanosine triphosphate; 600 $\mu\text{mol/L}$ deoxyuridine triphosphate; 50 nmol/L each of forward primer, reverse primer, and probe; 0.4 U/ μL RNase inhibitor; 0.25 U/ μL reverse transcriptase; and 0.025 U/ μL AmpliTaq Gold polymerase (all from Perkin-Elmer) with 3 μL of template RNA diluted in diethylpyrocarbonate- H_2O in 96-well plates. The amplification program consisted of an RT step (48°C for 30 minutes) and an AmpliTaq Gold activation step (95°C for 10 minutes), followed by a PCR step (40 cycles each of 95°C for 15 seconds [denature] and 60°C for 1 minute [anneal-extend]). The difference in C_T value between basophil RNA and positive control RNA (ΔC_T) at each dilution of template was averaged to give the average difference in cycle number ($\Delta C_{T\text{ave}}$). The fold increase in SHIP message above the positive control RNA was calculated with the following formula: fold increase = $2^{\Delta C_{T\text{ave}}}$.

RESULTS

Correlation between the ability to respond to HrHRF and cellular amount of SHIP, but not Lyn or Syk protein

The current study was designed to elucidate a molecular basis for the ability of basophils from IgE^+ donors to respond directly to HrHRF (ie, the hyperresponsiveness of these basophils). An increase in a positive regulatory molecule, such as Lyn or Syk, or a decrease in a negative regulatory molecule, such as SHIP, would be expected to increase releasability. Highly purified human basophils (87%-99%) were isolated from peripheral blood (where they constitute only 1% of the total cell population) by means of double Percoll gradients and negative selection with magnetic beads. Whole-cell lysates were then Western blotted with antibodies to 3 different signal-transduction proteins (Lyn, Syk, and SHIP). Simultaneous with basophil purification, histamine release to a dose response of HrHRF or 100 ng/mL anti-IgE was performed on dextran-sedimented basophils from the same donor. As before, all donors were degranulated on Fc ϵ RI aggregation ($54.4\% \pm 5.3\%$ [mean $\pm$ SEM], $n = 19$), but only a subset were degranulated to HrHRF (IgE^+ donor basophils, $32.6\% \pm 3.1\%$; IgE^- donor basophils, $2.1\% \pm 0.9\%$; $n = 9$ or 10, respectively).

As shown in Fig 1, A, the 145-kd SHIP isoform was detected in human basophils on an immunoblot from both types of donors. Three different blots are shown demonstrating SHIP levels from 2 IgE^+ and 2 IgE^- donors. For comparison, the T-cell lysate standard is shown. In each case the IgE^- donors had more SHIP protein than the IgE^+ donors when compared with the standard. The presence of SHIP in human basophils was recently described, but its function has not been explored.²¹ We then isolated and blotted basophils from additional IgE^+ and IgE^- donors. The cellular amount of SHIP varied 10-fold among the donors tested. As shown in Fig 1, B, there was a striking negative correlation

between the amount of SHIP per cell equivalent and the maximum histamine release to HrHRF ($n = 11$ preparations from 8 donors, $R = 0.84$). Western blotting of whole-cell basophil lysates for Lyn from 2 IgE^+ and 2 IgE^- donors, as shown in Fig 2, A, indicated that both IgE^+ and IgE^- donor basophils contained high and low levels of Lyn tyrosine kinase. Fig 2, B, demonstrates that the levels of Lyn varied greater than 40-fold among the 9 donors tested, and the levels of Lyn did not correlate with histamine release to HrHRF. Similar experiments were conducted, and basophils from both types of donors were Western blotted for Syk tyrosine kinase (Fig 2, C). The Syk levels between these 2 donors were similar. Fig 2, D, indicates that the amounts of Syk varied greater than 10-fold ($n = 8$) and also did not correlate with HrHRF-induced histamine release. As mentioned in the "Methods" section, the linearity of staining with the antibodies used for Western blotting was confirmed by means of scanning blots loaded with increasing amounts of lysate containing Lyn, Syk, or SHIP to verify that doubling the antigen concentration doubled the blot intensity (data not shown). For each type of blot, the scanned value was normalized to the same amount of standard loaded onto the gel (primary T-cell lysate for SHIP and monocyte lysate for Lyn and Syk). To correct for differences in basophil purity (87%-99%) between preparations, we Western blotted the corresponding amount of column contaminants. To be more specific, if the basophil preparation purity was 87%, then 13% would constitute the contaminants. In this instance an equivalent number corresponding to 13% of the column contaminants were loaded onto the gel. This number of contaminating cells was sufficiently low that no detectable signal could be seen, and therefore the signal that was detected came from the basophils alone. In most instances (9/11 SHIP samples, 9/9 Lyn samples, and 5/8 Syk samples) the basophil purity was sufficiently high that no signal was detected in the column contaminants. In the few instances in which a signal was detected, the basophil value for SHIP, Lyn, or Syk was corrected accordingly (average = 12% for the correction factor). These results suggest that the hyperreleasability of IgE^+ HrHRF-responsive donors is associated with low levels of SHIP and implicate SHIP as a crucial negative regulator of secretion in human basophils.

SHIP protein levels in human lymphocytes and monocytes

A disparity in SHIP levels between our 2 donor basophil populations raised the issue of whether SHIP levels in IgE^+ donors are reduced in additional cell types.

The purity of monocytes using the RosetteSEP reagent (see "Methods" section) from 5 IgE^+ and 5 IgE^- donors was $83\% \pm 2\%$ and $82\% \pm 2\%$, respectively. Likewise, the purity of T cells from 5 IgE^+ and 5 IgE^- donors was $98\% \pm 0.5\%$ and $97\% \pm 0.6\%$, respectively. Lysates of human monocytes and lymphocytes were prepared from both IgE^+ and IgE^- donors and were analyzed by means of anti-SHIP Western blotting in comparison with the same p145 SHIP standard lysate. SHIP levels in mono-

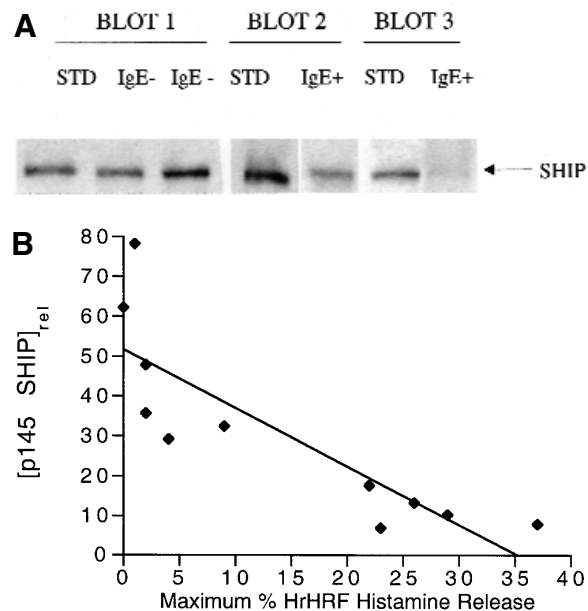


FIG 1. p145 SHIP protein is reduced in human basophils from IgE⁺ donors who respond directly to HrHRF. **A**, Anti-SHIP Western blot. Human basophils were isolated from peripheral blood to a final purity of 87% to 99%. Whole-cell lysates (2×10^6 cell equivalents) of 2 IgE⁺ and 2 IgE⁻ donor basophils were Western blotted with a polyclonal anti-SHIP antibody. A whole-cell lysate prepared from primary T cells is shown for comparison on each blot. **B**, Negative correlation between p145 SHIP protein expression and histamine release to HrHRF. Quantitation of SHIP protein was performed by scanning blots with Kodak Image software and normalizing to a fixed amount of primary T-cell lysate run on each gel. The value on the ordinate was calculated by dividing the basophil SHIP value by the T-cell standard value to give [p145 SHIP]_{rel}. Histamine release was performed on the same day on dextran-sedimented basophils. Stimulation was with 2 to 64 μ g/mL GST-HrHRF fusion protein or 100 ng/mL polyclonal anti-IgE for 45 minutes. All donors in Figs 1 and 2 released histamine to anti-IgE ($54.4\% \pm 5.3\%$ [mean $\pm$ SEM], $n = 19$; $R = 0.84$).

cytes and lymphocytes from 2 IgE⁺ and 2 IgE⁻ donors are shown in Fig 3, A. Quantitation of blots from 5 IgE⁺ and 5 IgE⁻ donors showed that the levels of SHIP (per cell equivalent) were not statistically different in monocytes and lymphocytes from the 2 donor populations. Although there was a tendency toward lower levels of SHIP in the IgE⁺ donors, the error bars overlapped compared with those of the IgE⁻ donors ($P > .29$ for monocytes and $P > .53$ for T cells, paired t test). Therefore SHIP levels appear to be regulated in basophils, but not in monocytes or lymphocytes, from our 2 donor populations.

Histamine release to HrHRF by IgE⁺ basophils requires PIP₃

To further investigate the role of SHIP in HrHRF-induced histamine release in IgE⁺ basophils, we used the PI3-K inhibitor LY294002, which has been used to prevent PIP₃ formation in a variety of cell types.³⁰ LY294002 was recently shown to prevent Fc ϵ RI-induced signaling and histamine release in human basophils.^{27,31} Dextran-sedimented IgE⁺ basophils were incubated simultaneously

with stimulus (32 μ g/mL GST-HrHRF or 100 ng/mL anti-IgE) and various doses of LY294002 for 45 minutes. Histamine release was quantitated by means of automated fluorometry from duplicate samples. Values obtained from stimulated samples were corrected for spontaneous release and expressed relative to basophils stimulated in the absence of drug ($29.9\% \pm 6\%$ for GST-HrHRF and $52.5\% \pm 6\%$ for anti-IgE [mean $\pm$ SEM], $n = 4$ donors). As shown in Fig 4, treatment of dextran-sedimented IgE⁺ basophils with the LY294002 compound prevented histamine release induced by both HrHRF and anti-IgE. These data are consistent with a SHIP deficiency in IgE⁺ basophils causing an excess PIP₃ accumulation, which is reversed by treating the cells with LY294002. As expected, histamine release to anti-IgE from both types of donor basophils was inhibited by incubation with LY294002 (50% inhibition at 2 μ mol/L for IgE⁺ [Fig 4] or IgE⁻ (data not shown) basophils, respectively).

Posttranscriptional regulation of SHIP protein levels in human basophils

To begin to understand the molecular mechanisms regulating the steady-state levels of SHIP protein in human basophils, we used real-time PCR to quantitate the message for SHIP from both IgE⁺ and IgE⁻ basophils (Fig 5). Real-time PCR was performed on total RNA prepared with RNazol B from 98% to 100% pure basophils. One-step RT-PCR analysis was conducted by using primers and a probe directed to the SHIP catalytic domain (87-bp amplicon). Results are expressed relative to a total RNA standard prepared from human monocytes and analyzed in each run. The value for each sample was calculated from the average ΔC_T value between monocyte standard RNA and basophil RNA over 5 serial dilutions of template. Each basophil sample ($n = 8$ donors) was tested in duplicate in 2 separate runs. Electrophoresis of amplified samples on a 2.5% agarose gel stained with ethidium bromide detected a single band of approximately 90 bp, confirming the predicted amplicon (results not shown). Surprisingly, the quantitation of SHIP message levels by real-time PCR indicates no correlation between the amount of SHIP mRNA and the amount of SHIP protein expressed in human basophils. These data suggest that SHIP protein levels are controlled by a posttranscriptional mechanism in human basophils.

DISCUSSION

In the current study we chose HrHRF-induced histamine release from human basophils as a model of basophil hyperreleasability in allergic patients. Previous studies had established that IgE⁺ donor basophils from highly allergic patients were distinguished by their ability to respond to HrHRF ($\geq 10\%$ histamine release at ≤ 64 μ g/mL GST-HrHRF)¹ and pharmacologic doses of IL-3 (46% histamine release at 300 ng/mL IL-3) and D₂O ($> 50\%$ histamine release to 44% D₂O).^{8,9}

The concept of basophil releasability was proposed to explain the inability of serum IgE concentrations and

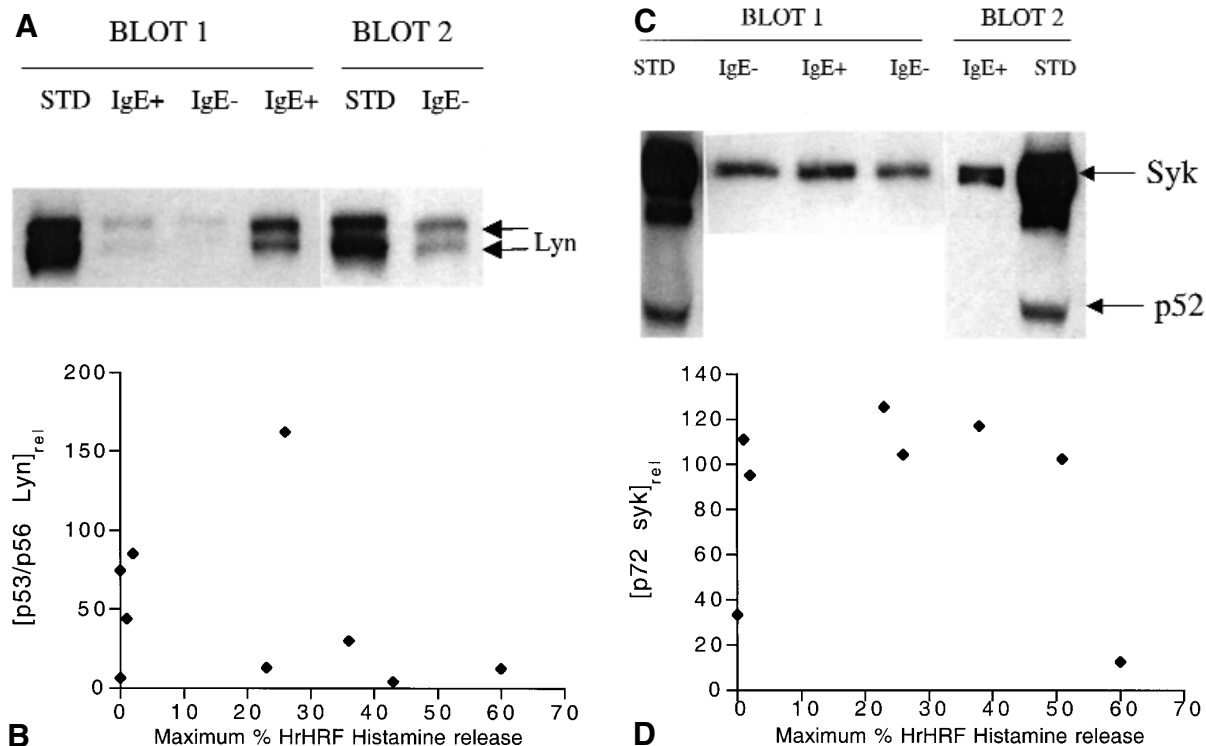


FIG 2. Lack of correlation between cellular levels of p53/p56 Lyn or p72 Syk and histamine release to HrHRF. **A**, Western blot of human basophil tyrosine kinase Lyn from 2 IgE⁺ and 2 IgE⁻ donors. Western blots were performed on whole-cell lysates (200,000 cell equivalents) prepared from purified human basophils by using a polyclonal anti-Lyn. **B**, Blots were scanned with Kodak Image software, and values were normalized to a monocyte lysate run on each gel. The value on the ordinate was calculated by dividing the basophil Lyn value by the monocyte standard value to give [p53/56 Lyn]_{rel}. HrHRF-induced histamine release was determined as described in the legend to Fig 1. **C**, Western blots were performed as in **A** with a monoclonal anti-Syk antibody. The value on the ordinate was calculated by dividing the basophil Syk value by the monocyte p52 standard value to give [p72 Syk]_{rel}. **D**, Again, HrHRF-induced histamine release was determined, as described in **B**.

skin test reactivity to various types of allergens to predict the extent of a patient's *in vivo* allergic response.^{32,33} For example, in one study of *in vitro* releasability, human basophils isolated from 3 different donors, all containing 100,000 sensitized FcεRI molecules, released 10% to 95% of total cellular histamine to polyclonal anti-IgE as the stimulus. It has been previously determined that donor basophils containing as few as 5000 IgE molecules or as many as 500,000 IgE molecules release similar amounts of histamine to polyclonal anti-IgE. They proposed that additional nonimmunoglobulin biochemical mediators must contribute to the amount of inflammatory mediators released by a particular donor and that these differences could contribute to the complexity of symptoms exhibited by various allergic patients. Additional studies conducted by the Marone laboratory found a positive correlation between donor age and histamine release to anti-IgE, as well as histamine release to the same stimulus, between monozygotic, but not dizygotic, twins.^{34,35}

The elucidation of the secretory pathway in rodent BMMCs, as well as in rodent cell lines (eg, RBL), has defined several key regulatory sites that may be applicable to secretion in human basophils and mast cells as

well. Potentially, an increase in the specific activity or amount of a positive regulatory molecule or a decrease in a negative regulatory molecule could contribute to basophil hyperreleasability. We chose to quantitate the cellular amounts of the tyrosine kinases Lyn and Syk and the 5'-inositol phosphatase SHIP in human basophils. The tyrosine kinase Lyn, which initiates signaling through FcεRI, was proposed to be limiting in RBL cells on the basis of studies noting a plateau in receptor phosphorylation with time while new aggregates continued to form.³⁶ In fact, in FcεRI-transfected Chinese hamster ovary fibroblasts further transfected with increasing amounts of Lyn, aggregation of the receptors by covalent IgE dimers led to a linear increase in receptor tyrosine phosphorylation.³⁷ Furthermore, Lyn is associated with the common β chain of the IL-3 receptor.³⁸ Syk-deficient RBL cells generated by means of chemical mutagenesis failed to degranulate in response to antigenic challenge; transfection of Syk restored phospholipase C-γ phosphorylation and sustained calcium mobilization and histamine release, indicating the essential role of Syk kinase in RBL secretion.³⁹ Human basophils that fail to degranulate to FcεRI-mediated stimulation (nonreleaser

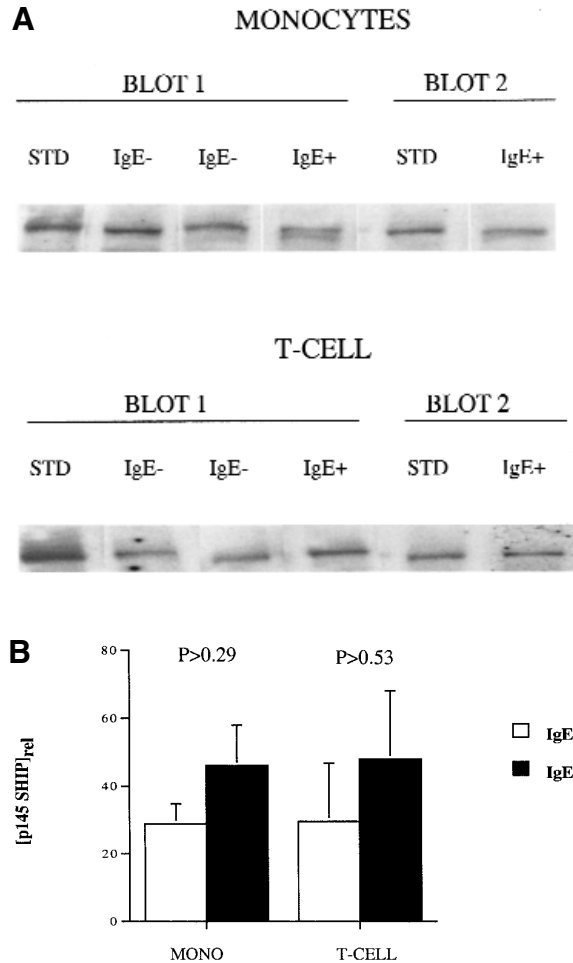


FIG 3. SHIP deficiency in IgE⁺ donors is not found in their monocytes or lymphocytes. **A**, Human lymphocytes and monocytes were purified, and whole-cell lysates were prepared, as described in the "Methods" section. Anti-SHIP Western blots for each cell type were quantitated relative to a primary T-cell standard lysate, as described in the legend of Fig 1. Data from 2 blots demonstrating SHIP levels in purified monocytes and T cells from 2 IgE⁺ and 2 IgE⁻ donors are shown. **B**, As described in the legend to Fig 1, quantitation of SHIP protein is demonstrated. The data depict the average $\pm$ SEM for 5 donors of each type (IgE⁺ and IgE⁻ donor cells).

basophils) were recently shown to be deficient in Syk alone or Lyn and Syk, depending on the donor population.^{12,13} As shown in Fig 2, degranulation of IgE⁺ basophils to HrHRF did not correlate with increased amounts of these 2 kinase proteins. Although possible, it is not likely that differences in the specific activity of the 2 kinases are responsible for increased releasability because a study by Lavens-Phillips and MacGlashan¹³ found maximum histamine release to anti-IgE correlated with protein levels of Lyn and Syk in human basophils.

A negative regulatory role of SHIP in limiting murine mast cell degranulation was proposed on the basis of the hyperresponsiveness of BMMCs from SHIP knockout mice.^{22,40} SHIP-deficient mice have a myeloproliferative-like syndrome, with lung infiltration and increased num-

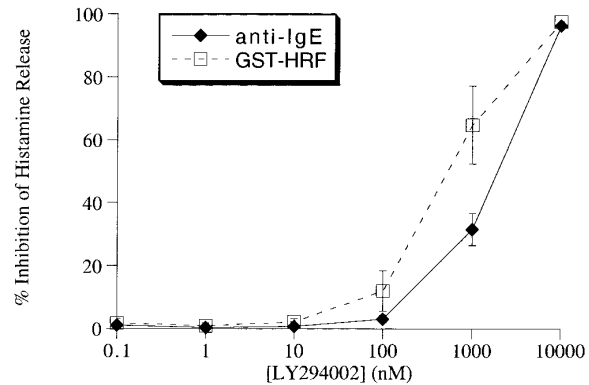


FIG 4. PIP₃ is required for histamine release to HrHRF. Dextran-sedimented IgE⁺ basophils were incubated simultaneously with stimulus (*open squares*, 32 mg/mL GST-HRF; *filled diamonds*, 100 ng/mL anti-IgE) and various doses of LY294002 for 45 minutes. Histamine release was quantitated by means of automated fluorimetry from duplicate samples. Values obtained from stimulated samples were corrected for spontaneous release and expressed relative to basophils stimulated in the absence of drug (10%-42% for GST-HRF and 34%-64% for anti-IgE). The results shown are the average $\pm$ SEM for 4 IgE⁺ donors.

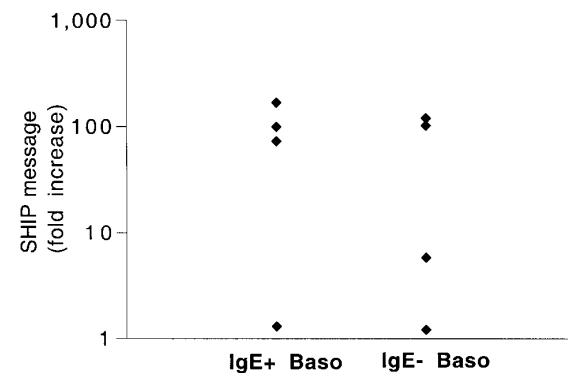


FIG 5. SHIP protein levels do not correlate with message levels. Real-time PCR was performed on total RNA prepared by using RNAzol B from 98% to 100% pure basophils. One-step RT-PCR analysis was conducted by using primers and a probe directed to the SHIP catalytic domain. Results are expressed relative to a total RNA standard prepared from human monocytes and analyzed in each run. The value for each sample was calculated from the average ΔC_T value between monocyte standard RNA and basophil RNA over 5 serial dilutions of template. Each basophil RNA sample was tested in duplicate in 2 separate runs. The results shown depict SHIP mRNA levels for 4 IgE⁺ and 4 IgE⁻ donors.

bers of granulocyte-macrophage progenitor cells in bone marrow and the spleen, leading to a reduced life span.⁴¹ When BMMCs from wild-type mice are incubated with IgE or Steel factor, no degranulation results. However, IgE or Steel factor stimulation alone resulted in up to 80% degranulation in SHIP^{-/-} BMMCs. As shown in Fig 1, a striking negative correlation was seen between the amount of SHIP protein per cell and the degree of degranulation to HrHRF in human basophils, implicating SHIP in limiting degranulation to incomplete stimuli in IgE⁻ basophils.

More recently, a related 5' inositol phosphatase, SHIP-2, has been cloned, and its expression has been reported in a variety of hematopoietic and nonhematopoietic tissues and cell lines.¹⁸ Like SHIP, it is tyrosine phosphorylated on growth factor stimulation and associates with Shc and Fc γ RIIB. However, the failure of SHIP-2 to substitute for SHIP in Fc ϵ RI-mediated signaling in SHIP knockout BMMCs would indicate that the 2 have nonredundant roles, at least in rodent mast cells. We are currently examining human basophils for the presence of SHIP-2.

The 2 functions that have been proposed for SHIP in immune-system cells are regulation of plasma membrane-bound PIP₃ levels and competition for binding to the adaptor Shc with Grb-2.¹⁸ In vitro phosphatase assays indicate that SHIP has the ability to hydrolyze PIP₃ and inositol 1,3,4,5 tetraphosphate to phosphatidylinositol 3,4 bisphosphate (PIP₂) or inositol 1,3,4 trisphosphate, respectively.¹⁵ Membrane-bound PIP₃ can bind to Pleckstrin homology domains found in a variety of proteins, including members of the Tec family of protein kinases, such as Btk.⁴² Activation of Btk through aggregation of the B-cell antigen receptor or Fc ϵ RI acts on phospholipase C- γ , which generates inositol 1,3,4 trisphosphate, which is responsible for transient calcium release from the endoplasmic reticulum.^{43,44} Sensing of endoplasmic reticulum calcium depletion then results in the opening of a store-operated calcium channel and then leads to the sustained increase in cytosolic calcium required for secretion in mast cells or proliferation in B cells.⁴⁵ In B cells transfection studies and coimmunoprecipitation studies indicate that Shc forms mutually exclusive complexes with either Grb-2 leading to ras activation or SHIP, which thereby acts as a sink for Shc.⁴⁶ In human basophils SHIP may function either way because Miura and MacGlashan²⁷ and Lavens-Phillips and MacGlashan¹³ have demonstrated the presence of Shc, ras, Grb-2, and Btk by means of Western blotting.

Furthermore, the apparent requirement for PIP₃ for HrHRF-mediated secretion in responsive IgE⁺ basophils demonstrated by our experiments with the PI3-K inhibitor LY294002 may dovetail with the ability of D₂O to activate these same cells. Previously, Maeyama et al⁴⁷ reported that D₂O stimulates phosphatidylinositol turnover in RBL cells.

Our results (Fig 3) indicate the expression of SHIP is not differentially regulated in the lymphocytes or monocytes from our 2 donor populations. As previously mentioned, 2 groups have recently reported either a deficiency of Syk or both Syk and Lyn in human basophils that do not degranulate in response to Fc ϵ RI crosslinking (ie, so-called nonreleaser basophils).^{12,13} The B cells, eosinophils, and neutrophils from one nonreleaser donor group contained normal levels of Syk, indicating that a basophil-specific regulatory mechanism was operating in nonreleaser basophils.¹³ Murine SHIP protein expression is developmentally regulated during hematopoiesis and spermatogenesis.⁴⁸ The recent cloning of the murine SHIP promoter revealed several putative hematopoietic transcription factor-binding sites that may be candidates for regulation of human SHIP.⁴⁹

SHIP is constitutively phosphorylated during the chronic phase of chronic myelogenous leukemia, and its expression appears to be regulated by the oncogene Bcr/Abl generated during the Philadelphia chromosome translocation, which is characteristic of the disease.⁵⁰ In transfected myeloid cells, induction of Bcr/Abl reduces the half-life of SHIP protein from 18 to 3 hours, as well as decreasing SHIP mRNA levels. Therefore both transcriptional and posttranscriptional mechanisms are responsible for the Bcr/Abl-mediated SHIP downregulation. In human basophils we have found that SHIP message levels, as monitored with real-time PCR, are not correlated with the amount of SHIP protein expressed. A similar posttranscriptional mechanism for Lyn and Syk protein expression was suggested by Lavens-Phillips and MacGlashan¹² to explain their observation that releasing and nonreleasing human basophils have similar message levels but differ on the protein level by 10-fold. Likewise, Kepley et al⁵¹ postulated a posttranslational mechanism for Syk levels in nonreleaser basophils.

A variety of mechanisms have been identified that regulate the efficiency of translation for a given mRNA. RNA transcription begins in the nucleus with the assembly of a protein-DNA complex at a sequence-specific initiation site. Both the 5' untranslated region (UTR) and the 3' UTR contain information that will regulate the half-life, spatial localization, and translation efficiency of a given mRNA. For the mRNA to be exported to the cytoplasm, where it is translated on ribosomes, the initial immature message must be spliced and polyadenylated, and any nuclear retention signal must be masked. Also, once the mature message reaches the ribosomes, the rate at which the message will be translated to protein, as well as the lifetime of the message, can be regulated. A variety of physiologic and pathologic changes in gene expression have been reported to be controlled by posttranscriptional mechanisms, including the cellular iron-mediated regulation of ferritin mRNA and its dysregulation, leading to hereditary hyperferritinemia-cataract syndrome (HHCS).⁵²⁻⁵⁴ The production of ferritin protein, which binds iron itself and stores it in the cell until needed, allows cells to regulate cellular iron availability. In HHCS a mutation in the stem-loop structure in the 5' UTR of the ferritin mRNA prevents regulation of ferritin protein production. The resulting excessive ferritin accumulation leads to cataract formation in the affected patient with HHCS. The cloning and sequencing of SHIP cDNAs from both hyperresponsive IgE⁺ human basophils and their IgE⁻ counterparts may identify a posttranscriptional regulatory mechanism that affects the severity of an individual's allergic response.

In conclusion, we have demonstrated a deficiency of the negative regulatory molecule SHIP in the basophils of allergic donors whose hyperresponsiveness is characterized by degranulation to 3 stimuli (ie, HrHRF, IL-3, and D₂O) that are incomplete stimuli for IgE⁻ basophils. Inactivation of PI3-K with the specific inhibitor LY294002 prevented HrHRF-induced degranulation in IgE⁺ basophils, which is consistent with a requirement for PIP₃. The extent of SHIP expression in human

basophils appears to be regulated at both the cellular and posttranscriptional levels.

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REFERENCES

- MacDonald SM, Rafnar T, Langdon J, Lichtenstein LM. Molecular identification of an IgE-dependent histamine releasing factor. *Science* 1995;269:688-90.
- Schroeder JT, Lichtenstein LM, MacDonald SM. An immunoglobulin E-dependent recombinant histamine releasing factor induces IL-4 secretion from human basophils. *J Exp Med* 1996;183:1265-70.
- MacDonald SM, Lichtenstein LM, Proud D, et al. Studies of IgE-dependent histamine releasing factors: heterogeneity of IgE. *J Immunol* 1987;139:506-12.
- Schroeder JT, Lichtenstein LM, MacDonald SM. Recombinant histamine-releasing factor enhances IgE-dependent IL-4 and IL-13 secretion by human basophils. *J Immunol* 1997;159:447-52.
- Bheekha-Escura R, Chance SR, Langdon JM, MacGlashan DW Jr, MacDonald SM. Pharmacologic regulation of histamine release by the human recombinant histamine-releasing factor. *J Allergy Clin Immunol* 1999;103:937-43.
- Wantke F, MacGlashan DW Jr, Langdon JM, MacDonald SM. The human recombinant histamine releasing factor: Functional evidence that it does not bind to the IgE molecule. *J Allergy Clin Immunol* 1999;103:642-8.
- Bheekha-Escura R, MacGlashan DW Jr, Langdon JM, MacDonald SM. Human recombinant histamine-releasing factor activates human eosinophils and the eosinophilic cell line, AML 14-3D10. *Blood* 2000;96:2191-8.
- MacDonald SM, Schleimer RP, Kagey-Sobotka A, Gillis S, Lichtenstein LM. Recombinant IL-3 induces histamine release from human basophils. *J Immunol* 1989;142:3527-32.
- MacDonald SM, White JM, Kagey-Sobotka A, MacGlashan DW Jr, Lichtenstein LM. The heterogeneity of human IgE exemplified by the passive transfer of D₂O sensitivity. *Clin Exp Allergy* 1991;21:133-8.
- Findlay SR, Lichtenstein LM. Basophil "releasability" in patients with asthma. *Am Rev Respir Dis* 1980;122:53-9.
- Nguyen KL, Gillis S, MacGlashan DW Jr. A comparative study of releasing and nonreleasing human basophils: nonreleasing basophils lack an early component of the signal transduction pathway that follows IgE cross-linking. *J Allergy Clin Immunol* 1990;85:1020-9.
- Kepley CL, Youssef L, Andrews RP, Wilson BS, Oliver JM. Syk deficiency in nonreleaser basophils. *J Allergy Clin Immunol* 1999;104:279-84.
- Lavens-Phillips SE, MacGlashan DW. The tyrosine kinases p53/561yn and p72syk are differentially expressed at the protein level but not at the messenger RNA level in nonreleasing human basophils. *Am J Respir Cell Mol Biol* 2000;23:566-71.
- Okazaki H, Siraganian RP. Signal transduction for secretion in basophils. *ACI Int* 1999;11:86-92.
- Damen JE, Liu L, Rosten P, et al. The 145-kDa protein induced to associate with Shc by multiple cytokines is an inositol tetraphosphate and phosphatidyl 3,4,5-trisphosphate 5-phosphatase. *Proc Natl Acad Sci U S A* 1996;93:1689-93.
- Lioubin MN, Algate PA, Tsai S, Carlberg K, Aebersold A, Rohrschneider LR. p150ship a signal transduction molecule with inositol polyphosphate-5-phosphatase activity. *Genes Dev* 1996;10:1084-95.
- Kavanaugh WM, Pot DA, Chin SM, et al. Multiple forms of an inositol polyphosphate 5-phosphatase form signaling complexes with Shc and Grb2. *Curr Biol* 1996;6:438-45.
- Krystal G. Lipid phosphatases in the immune system. *Semin Immunol* 2000;12:397-403.
- Ono M, Bolland S, Tempst P, Ravetch JV. Role of the inositol phosphatase SHIP in negative regulation of the immune system by the receptor Fc-gamma-RIIB. *Nature* 1996;383:263-6.
- Kimura T, Sakamoto H, Apella E, Siraganian R. The negative signaling molecule SH2-domain containing inositol-polyphosphate 5-phosphatase (SHIP) binds to the tyrosine-phosphorylated beta subunit of the high affinity IgE receptor. *J Biol Chem* 1997;272:13991-6.
- Kepley CL, Cambier JC, Morel PA, et al. Negative regulation of FcεRI signaling by FcγRII costimulation in human blood basophils. *J Allergy Clin Immunol* 2000;106:337-48.
- Huber M, Helgason CD, Damen JE, Liu L, Humphries RK, Krystal G. The src homology 2-containing inositol phosphatase (SHIP) is the gatekeeper of mast cell degranulation. *Proc Natl Acad Sci U S A* 1998;95:11330-5.
- Kalesnikoff J, Huber M, Lam V, Damen JE, Zhang J, Siraganian RP, Krystal G. Monomeric IgE stimulates signaling pathways in mast cells that lead to cytokine production and cell survival. *Immunity* 2001;14:801-11.
- Schleimer RP, MacGlashan Jr DW, Gillespie E, Lichtenstein LM. Inhibition of basophil histamine release by antiinflammatory steroids. II. Studies on the mechanism of action. *J Immunol* 1982;128:1632-6.
- Siraganian RP. Refinements in the automated fluorometric histamine analysis system. *J Immunol Methods* 1975;7:283-90.
- MacGlashan DW, Bochner BS, Adelman DC, et al. Downregulation of FcεRI expression on human basophils during in vivo treatment of atopic patients with anti-IgE antibody. *J Immunol* 1997;158:1438-45.
- Miura K, MacGlashan Jr DW. Phosphatidylinositol-3 kinase regulates p21ras activation during IgE-mediated stimulation of human basophils. *Blood* 2001;96:2199-205.
- Miura K, MacGlashan DW. Dual phase priming by IL-3 for leukotriene C4 generation in human basophils: difference in characteristics between acute and late priming effects. *J Immunol* 2000;164:3026-34.
- Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. *Genome Res* 1996;6:986-94.
- Vlahos CJ, Matter WF, Hui KY, Brown RF. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J Biol Chem* 1994;269:5241-8.
- Gibbs BF, Grabbe J. Inhibitors of PI 3-kinase and MEK kinase differentially affect mediator secretion from immunologically activated human basophils. *J Leukoc Biol* 1999;65:883-90.
- Marone G, Spadaro G, Patella V, Genovese A. The clinical relevance of basophil releasability. *J Allergy Clin Immunol* 1994;94:1293-303.
- Lichtenstein LM, MacGlashan DW Jr. The concept of basophil releasability. *J Allergy Clin Immunol* 1986;77:291-4.
- Marone G, Poto S, di Martino L, Condorelli M. Human basophil releasability. I. Age-related changes in basophil releasability. *J Allergy Clin Immunol* 1986;77:377-83.
- Marone G, Poto S, Celestino D, Bonini S. Human basophil releasability. III. Genetic control of human basophil releasability. *J Immunol* 1986;137:3588-92.
- Wofsy C, Torigoe C, Kent UM, Metzger H, Goldstein B. Exploiting the difference between intrinsic and extrinsic kinases: implications for regulation of signaling by immunoreceptors. *J Immunol* 1997;159:5984-92.
- Wofsy C, Vonakis BM, Metzger H, Goldstein B. One lyn molecule is sufficient to initiate phosphorylation of aggregated high-affinity IgE receptors. *Proc Natl Acad Sci U S A* 1999;96:8615-20.
- Adachi T, Pazdrak K, Stafford S, Alam R. The mapping of the Lyn kinase binding site of the common β subunit of IL-3/granulocyte-macrophage colony-stimulating factor/IL-5 receptor. *J Immunol* 1999;162:1496-501.
- Zhang J, Berenstein EH, Evans RL, Siraganian RP. 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* 1996;184:71-9.
- Huber M, Helgason CD, Scheid MP, Duronio V, Humphries RK, Krystal G. Targeted disruption of SHIP leads to Steel factor-induced degranulation of mast cells. *EMBO J* 1998;17:7311-9.
- Helgason CD, Damen JE, Rosten P, et al. Targeted disruption of SHIP leads to hematopoietic perturbations, lung pathology, and a shortened life span. *Genes Dev* 1998;12:1610-20.
- Mano H. Tec family of protein-tyrosine kinases: an overview of their structure and function. *Cytokine Growth Factor Rev* 1999;10:267-80.
- Scharenberg AM, El-Hillal O, Fruman DA, et al. 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* 1998;17:1961-72.
- Turner H. Kinet J-P. Signalling through the high-affinity IgE receptor FcεRI. *Nature* 1999;402:B24-30.
- Putney JW Jr, McKay RR. Capacitative calcium entry channels. *Bioessays* 1999;21:38-46.
- Coggeshall KM. Inhibitory signaling by the B cell FcγRIIB. *Curr Opin Immunol* 1998;10:306-12.

47. Maeyama R, Hohman JJ, Metzger H, Beaven MA. Quantitative relationships between aggregation of IgE receptors, generation of intracellular signals and histamine secretion in rat basophilic leukemia (2H3) cells. *J Biol Chem* 1986;261:2583-92.
48. Liu QF, Shalaby F, Jones J, Bouchard D, Dumont DJ. The SH2-containing inositol polyphosphate 5-phosphatase, SHIP, is expressed during hematopoiesis and spermatogenesis. *Blood* 1998;91:2753-9.
49. Wolf I, Lucas DM, Algate PA, Rohrschneider LR. Cloning of the genomic locus of mouse SH2 containing inositol 5-phosphatase (SHIP) and a novel 110-kDa splice isoform, SHIP δ . *Genomics* 2000;69:104-12.
50. Sattler M, Verma S, Byrne CH, et al. BCR/ABL directly inhibits expression of SHIP, an SH2-containing polyinositol-5-phosphatase involved in the regulation of hematopoiesis. *Mol Cell Biol* 1999;19:7473-80.
51. Kopley CL, Youssef L, Andrews RP, Wilson BS, Oliver JM. Multiple defects in Fc ϵ RI signaling in syk-deficient nonreleaser basophils and IL-3-induced recovery of syk expression and secretion. *J Immunol* 2000;165:5913-20.
52. Allerson CR, Cazzola M, Rouault TA. Clinical severity and thermodynamic effects of iron-responsive element mutations in hereditary hyperferritinemia-cataract syndrome. *J Biol Chem* 1999;271:26439-47.
53. Cazzola M, Bergamaschi G, Tonon L, et al. Hereditary hyperferritinemia-cataract syndrome: relationship between phenotypes and specific mutations in the iron-response element of ferritin light-chain mRNA. *Blood* 1997;90:814-21.
54. Thomson AM, Rodgers JT, Leedman PJ. Iron-regulatory proteins, iron-response elements and ferritin mRNA translation. *Int J Biochem Cell Biol* 1999;31:1139-52.

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