

Early signal protein expression profiles in basophils: a population study

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ABSTRACT

IgE-mediated histamine release from peripheral blood basophils is highly variable within the general population. Recent studies have shown that the ability of anti-IgE antibody to induce release can be predicted reasonably well by knowing the level of syk expression in the cells. The current study expands a previous survey to include 14 additional early elements known to be involved in activation and deactivation of basophils and showed that with the exception of syk, the variance of expression of 19 other elements (lyn, fyn, csk, cbp/PAG, CIN85, Bob1, c-cbl, SHIP1, SHIP2, p85 α , p110 δ , btk, PLC γ 1, PLC γ 2, SHP-1, PTEN, SOS2, CRACM1, and IL-3R α) was narrow despite a broad range of functional capability in the basophils under study. With syk as the only element with high variance and well-correlated to maximum histamine release and cellular sensitivity, this survey examined the expression levels of two proteins thought to regulate syk expression: Bob1/OCA-B and CIN85. Expression of CIN85 was not correlated to syk expression, but Bob1 expression was negatively correlated to expression of syk and maximum histamine release. However, the expected behavior for this protein should have been as a protector of post-translational syk loss and therefore, positively correlated. Previous studies suggested that post-translational control mechanisms regulated syk expression. However, in this study, steady-state mRNA levels for syk in resting basophils showed a correlation with syk protein expression levels ($r=0.593$). It is concluded that with the exception of syk expression, the expression of 19 early signaling elements is tightly regulated and that a component of the regulation of syk may be related to control of transcription or processing of syk mRNA. *J. Leukoc. Biol.* 86: 313–325; 2009.

Abbreviations: CIN85=Cbl-interacting 85-kDa protein, CRACM1=calcium release-activated calcium modulator 1, CV=coefficient of variation, ED₅₀=effective density for 50% of maximal histamine release expressed in molecules of antigen-specific IgE/basophil required for 50% of the maximum response, Grb2=growth factor receptor-bound protein 2, HSA=human serum albumin, Km=Michaelis-Menton-like constant, OCT-1=octamer transcription factor-1, PAG=PIPEs-albumin-glucose, PIP3=phosphatidylinositol-3,4,5-trisphosphate, PLC=phospholipase C, PTEN=phosphatase and tensin homolog, RIN=RNA integrity number, SHIP=SH2-containing 5'-inositol phosphatase, SHP-1=Src homology 2-containing tyrosine phosphatase 1, SOS2=son of sevenless 2, Vmax=maximum initial velocity

Introduction

In atopic diseases, the secretion of mediators from mast cells and basophils is an important component of the disease process. Studies of basophils in the general population show that there is a wide range in the intrinsic ability to secrete mediators in response to IgE-mediated stimulation. Studies over several decades have found that the relative ability to respond to IgE-mediated stimulation is enhanced in atopic diseases or asthma [1–3]. In a recent pilot survey of the relationship between signal element expression and IgE-mediated histamine release from human basophils from the general population, an excellent correlation was found between syk expression and maximum histamine release induced by antigen/anti-IgE antibody [4]. There was a modest inverse correlation between the expression of SHIP1 and histamine release but no relationship between maximum histamine release or the ED₅₀ and the expression of SHIP2, lyn, c-cbl, or the p85 α subunit of PI3K. The relationship between syk expression and histamine release is likely the result of syk being a critical, early, nonredundant signaling kinase in the signaling pathway and that syk is present in limiting amounts (the syk:Fc ϵ RI ratio is 1:7). Various studies in human basophils support the criticality of syk in IgE-mediated signaling [5, 6] and in a small study of syk substrate phosphorylation, the level of syk expression if basophils of different subjects were mirrored by the extent of phosphorylation of shc or c-cbl [4]. The pilot survey examined only six elements thought to be involved in IgE-mediated signaling, but the survey provided material for a broader study. The current study represents an extension of this pilot survey, adding 14 new signaling elements to the analysis. The results are informative and serve to further highlight the relevance of the observations about syk.

In addition, some of the elements examined may have relevance to understanding the source of variance in syk. Prior to the current study, the evidence supported the possibility that the level of syk expression is under post-translational control. Recently, two proteins have been identified that appear to have some role in regulating post-translational loss of syk. One of these proteins, OCA-B/Bob1, is a surprising participant, as

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it also functions as a transcription factor. Preliminary studies indicate that OCA-B/Bob1 interacts directly with syk kinase and while doing so, protects syk from engagement with cbl (c-cbl or cbl-b) [7]. In this model, the cell must experience some level of prior stimulation. A second protein, CIN85, has also been implicated in the regulation of syk [8]. This protein is thought to interact directly with cbl, decreasing the interaction of cbl with sts1, which is a negative regulator of cbl, thereby increasing the activity of cbl. As c-cbl or cbl-b is thought to mediate the loss of syk through the proteasome pathway, CIN85 acts to enhance the loss of syk, an effect that is opposite the apparent role of OCA-B/Bob1. Once again, however, some degree of stimulation must occur for this mechanism to alter the expression of syk. Although the details of syk regulation by these proteins are not fully understood yet, a simple question to ask is whether the expression of syk is regulated by the expression of OCA-B/Bob1 or CIN85. Samples from our previous population survey were available to examine whether there was a correlation between syk expression and OCA-B/Bob1 or CIN85. Neither of these molecules has enzymatic activity that is relevant to their protective roles, so expression of protein levels might be expected to be relevant to their ability to protect syk. For OCA-B/Bob1, one might expect to observe a positive correlation, and for CIN85, the correlation might be expected to be negative.

MATERIALS AND METHODS

Materials and buffers

Materials, buffers, and antibodies

The following were purchased: PIPES, BSA, EGTA, EDTA, D-glucose, NaF, Na₂VO₄, 2-ME, Nonidet P-40, and PP1 (Calbiochem, San Diego, CA, USA); crystallized HSA (Miles Laboratories, Elkhart, IN, USA); FCS and RPMI 1640 containing 25 mM HEPES and L-glutamine (BioWhittaker, Walkersville, MD, USA); Percoll (Pharmacia, Piscataway, NJ, USA); Tris(hydroxymethyl)-aminomethane and Tween-20 (Bio-Rad, Hercules, CA, USA); 4-(2-aminoethyl) benzene sulfonyl fluoride (Sigma-Aldrich, St. Louis, MO, USA); anti-syk mAb (4D10), anti-SHIP1 (P1C1), anti-SHIP2 (I20,G20), anti-c-Cbl (sc15), anti-lyn (44), anti-p110δ (H219), anti-fyn (fyn3), anti-PLCγ1 (530), anti-PLCγ2 (Q-20), anti-Src homology 2 domain containing leukocyte protein of 76 kDa (F-7), anti-Grb2 (C-23), anti-SOS2 (C-19), and anti-VAV (C-14; Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-p85 recombinant lyn and syk, anti-PLD1/2, and anti-Grb2-associated binder 2 (Upstate Biotechnology, Charlottesville, VA, USA); anti-btk, anti-shc, and anti-SHP-1 (BD Transduction Labs, Lexington, KY, USA); anti-PTEN, anti-Akt, anti-Erk, and anti-linker of activated T cells (Cell Signaling Technology, Beverly, MA, USA); anti-p56dok (Calbiochem); 22E7 (anti-FcεRIα, gift from Hoffman LaRoche, Nutley, NJ, USA); and HRP-conjugated donkey anti-rabbit Ig antibody, HRP-conjugated sheep anti-mouse Ig antibody, and protein G sepharose beads (Amersham Life Science, Arlington Heights, IL, USA). Goat anti-human IgE antibody was prepared as described [9]. PAG buffer consisted of 25 mM PIPES, 110 mM NaCl, 5 mM KCl, 0.1% glucose, and 0.003% HSA. Countercurrent elutriation and labeling with antibodies for flow cytometry were conducted in PAG containing 0.25% BSA in place of 0.003% HSA. SDS stripping buffer for Western blots was 65 mM Tris (pH 6.7), 100 mM 2-ME, and 2% SDS.

Basophil purification

Two sources of basophils were used for these experiments. To develop the population profile of signal element expression, basophils were obtained by venipuncture for functional analysis and signal element expression. For this

study, blood was first enriched in basophils by a two-step Percoll gradient (53%/62% Percoll) [10]. Approximately one-third of the enriched cells was set aside for functional analysis (below), and the remaining cells were purified to near homogeneity by negative selection using the basophil purification kit (Stem Cell Technologies, Vancouver, BC, Canada) and columns from Miltenyi Biotec (Auburn, CA, USA) [11]. Following the two-step enrichment step, the basophils were maintained at 4°C throughout the steps necessary for final purification. The average purity of these basophils by alcian blue staining [12] was 99%. For many of the remaining studies, residual cells of normal donors undergoing leukapheresis were enriched in basophils using Percoll density gradients and countercurrent flow elutriation, as described previously [10]. Basophils were purified further by negative selection as described above. Basophil purity exceeded 99%.

Subjects

Recruitment was quasi-random, and there were no specific exclusion criteria other than those specified by good practice for blood drawing. The mean age was 35 (22–58), and 61% were female. With respect to atopy, subjects were simply asked if they had any known allergies after the study was complete; 21% reported having allergies to common antigens (animal danders, aeroallergens, insects). A medication history was not obtained.

Functional analysis

A portion of the two-step Percoll-enriched cells was divided for sensitization under five conditions: no IgE antibody and 5, 2.5, 1.25, and 0.625 μg/ml anti-gp120 peptide-specific, biotinylated IgE (SE44B, a gift from Tanox Corp., Houston, TX, USA). Sensitization was done at 4°C for 1 h in PAG buffer containing 10 μg/ml heparin. Following washing, an aliquot of cells from each sensitization condition was set aside for flow cytometry (see below), and the remaining cells were stimulated with 0.2 μg/ml goat polyclonal anti-IgE antibody or 0.6 μg/ml gp120-peptide-OVA conjugate (both concentrations were determined previously to be optimal for histamine release at all IgE densities [13, 14]). For the cells not sensitized with SE44B, the antigen-challenge condition was replaced with a mouse IgM anti-IgE mAb, 6061 (Hybridoma Reagent Laboratory, Baltimore, MD, USA), so that for comparative purposes, nonsensitized cells were tested for response to goat anti-IgE antibody or IgM anti-IgE antibody. Supernatants were harvested after 45 min of stimulation in PAG supplemented with 1 mM CaCl₂ and 1 mM MgCl₂ and analyzed for histamine. The maximum histamine value was obtained from the best response to antigen or goat anti-IgE antibody from any condition. The ED₅₀ for the response was extrapolated from the antigen responses plotted against the SE44B density obtained from flow cytometry, as described previously [14].

Flow cytometry and its calibration

The cells set aside for flow cytometry were incubated on ice for 30 min with anti-CD123 PE and streptavidin-Alexa647. The nonsensitized cells acted as the control for the Alexa647 signal, and median fluorescence for the control was subtracted from the values obtained for the other conditions. Basophils were gated first on forward/side-scatter and PE-positive cells (IL-3Rα-positive).

To calibrate the flow cytometric results with absolute gp120-specific IgE density, RBL-SX38 cells (transfected with human FcεRI, gift of Dr. Jean-Pierre Kinet, Harvard University, Cambridge, MA, USA) were loaded with SE44B. A portion was analyzed by flow cytometry, as above, and a portion was treated with acetate buffer to dissociate bound IgE, which was measured in a total IgE assay (ImmunoCap®), as described previously [13]. The density of cell-surface IgE was calculated from the total IgE measurement and a count of the cells used for dissociation. The flow cytometric results were then equated with this measured density of IgE/basophil.

Calibrated Western blots

These methods and a presentation of example blots from these studies have been published previously [4]. All sampling was based on lysing equiv-

alent cell numbers, as this approach removed the assumption that cells from different subjects would contain equivalent amounts of total protein or equivalent amounts of a reference protein (e.g., actin; normalizing to protein is traditional but not appropriate when there is no *a priori* knowledge about the assumption of equivalence). The purified basophils were counted, and 300,000 pelleted cells were lysed in 20 μ l hot Novex electrophoresis sample buffer containing 5% 2-ME, the tube placed in boiling water for 5 min, and the samples stored at -80°C until electrophoresis. When six to eight samples were accumulated, they were run in a 15-well 8% tris-glycine gel with molecular weight markers in the first and last lanes. After blocking, the nitrocellulose was cut horizontally at the 98-kD marker level. The $>98\text{-kD}$ and $<98\text{-kD}$ portions were blotted on 2 or 3 successive days with a choice of antibodies that did not interfere with each other. No stripping step occurred between blotting. Many of these antibodies produced single bands at the loading levels in this study so that they do not interfere with each other. In addition, in the successive blotting, the antibodies were chosen to be progressively more sensitive detectors, so there is little evident crossover of information between measurements. Each Western blot included a three-point standard curve of a basophil lysate prepared prior to the survey (aliquoted and stored at -80°C) to bracket the range of expected expression for each of the elements tested. Multiple film exposures were made to optimize linearity, the films were digitized, and the band intensities calculated against the standard curve (see the online repository of ref. [4]).

Quantitative real-time PCR for syk mRNA expression

Total RNA was extracted from 0.25×10^6 intact cells using a HighPure RNA isolation kit (Roche, Indianapolis, IN, USA), according to the manufacturer's instructions. Samples were kept at -135°C until several samples were collected and then analyzed by quantitative real-time PCR using a TaqMan primer/probe set (Applied Biosystems, Foster City, CA, USA) with primers to the human syk cDNA sequence. Integrity of the RNA at the time of the analysis was measured on an Agilent bioanalyzer and was expressed as a RIN, which ranged from 6.6 to 8.3. A pilot survey established that a RIN number >4 did not alter the measurement of syk mRNA. The forward PCR primer for syk was 5'-AGCAGAAGCAAATGTCATGCA-3', and the reverse PCR primer was 5'-CCTCGCATATCCCGATCATC-3'. The probe sequence was 5'-CAGCTG-GACAACCCGTACATCGTGC-3', and FAM was the 5'-reporter dye, and TAMRA was the 3'-quencher dye. A two-step PCR protocol was run using murine leukemia virus RT and the TaqMan PCR reagent kit (Applied Biosystems). RNase inhibitor (Applied Biosystems) was also included in the reactions to prevent RNA degradation. Samples were run in duplicate (5 μ l sample/reaction=1/14th of the total sample) and analyzed on an Applied Biosystems 7300 real-time PCR system with the following program: 50°C for 30 min RT, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. Comparative threshold values were determined using the auto-calculation feature. A 50-ng sample of Jurkat total RNA (Stratagene, La Jolla, CA, USA) was included on each PCR plate to normalize samples run on different days and different plates. Expression of syk RNA in the sample relative to the Jurkat RNA was reported. The average efficiency per cycle of replication for this reagent set was equal to 1.95 ± 0.04 , based on twofold serial dilutions of Jurkat RNA.

Analysis of syk protein expression by intracellular flow cytometry

The measurement of syk protein expression by flow cytometry using the anti-syk antibody 4D10 has been described previously and validated with respect to standard Western blotting [15]. This method has been used on impure as well as purified cell fractions with equivalent results. Briefly, cells (100,000–200,000/labeling condition) were resuspended in PBS containing 0.04% BSA and blocked with 0.2 mg/ml nonspecific human IgG. After labeling with an appropriate gating antibody, samples were fixed with Medium A (Fix/Perm kit, Caltag Laboratories, S. San Francisco, CA, USA). Fixed samples were permeabilized in Medium B (Fix/Perm kit) in the presence of the anti-syk antibody 4D10 (2 $\mu\text{g}/\text{ml}$) or a mouse IgG_{2a} isotype control (2 $\mu\text{g}/\text{ml}$). An Alexa647-conjugated anti-mouse IgG_{2a} secondary

antibody was used for detection. syk protein expression is reported as net mean fluorescence intensity, the difference between 4D10 and isotype-labeled cells. Flow data were normalized for variability between samples using CaliBRITE allophycocyanin calibration beads (BD Biosciences, San Jose, CA, USA).

Statistical modeling and correction factors

Two aspects of the dataset were manipulated to generate information about the significance of the various statistical outcomes. It was suspected (see Results) that there was a systematic procedural error in the generation of the signaling element expression profiles. The Western blot method is dependent on the accuracy of cell counting and repeatable aspiration of cell pellet supernatants, and both of these procedures introduce variance that results in a systematic bias to measurements of signaling elements and to the statistical correlations between signaling element expression levels. For example, if a sample were assumed to have 0.3×10^6 basophils, but as a result of a combination of the procedural errors, there were actually only 265,000 basophils, then all signaling elements for this one sample would decrease by 15% in concert.

To examine how this procedural error would be reflected in the correlations, several artificial datasets (with different initial correlation coefficients) were used as a starting point in a model that mimicked the precise methods used to determine signal element expression. The model injected noise into each procedural step, the counting of basophils, the resuspension of cells in electrophoresis buffer, and the running of a Western. In each case, the amount of random noise that was injected into a data point was based on known levels of noise in the various techniques. Different amounts of noise injection were explored, but we calculated from known methodological errors that the loading variance based on cell counting, volume estimates after pelleting, and pipetting error would be $\sim 14\%$ (CV).

To explore the use of a correction factor, three of the signaling elements, lyn, p85, and c-cbl, with low levels of CV, no known expression linkage, but correlated in their expression, were used as an indicator to the procedural noise noted above. For example, if lyn, p85, and c-cbl looked low in a sample, then we assumed this indicated that the combination of cell count and sample preparation conspired to produce a sample that was short of cellular proteins. Therefore, the average deviance of a particular sample's lyn, p85, and c-cbl from the mean of the population was used to calculate a correction factor. For example, the average expression of lyn, p85, and c-cbl in the entire dataset was 0.345 ± 0.087 , 0.293 ± 0.090 , and 0.322 ± 0.081 standard units. If a given sample showed values of lyn, p85, and c-cbl that were 0.2, 0.3, and 0.25 sds higher than the mean, the average of 0.25 sds was assumed to represent how far off all other signaling elements from the same sample would also be from their measured value. The expression level of each of these other signaling elements could then be adjusted accordingly for this discrepancy. After presentation of the raw data, all data were corrected this way, and this approach necessarily excluded from further analysis lyn, p85, and c-cbl.

Parenthetically, data (see Fig. 5B) are a combination of data from the histogram of correlations between function and signaling element expression and the correlations between ED₅₀ (cellular sensitivity) and element expression. However, the correlation between ED₅₀ (cellular sensitivity) and element expression would be expected to be negative if more of an element led to a more-sensitive cell (fewer IgE molecules needed to reach 50% of the maximum response). To combine the correlations for sensitivity versus element expression and maximum histamine release versus element expression, the correlation values for "sensitivity" versus element were multiplied by -1 .

Heuristic statistical modeling

One of the earliest heuristic models of basophil histamine release was explored extensively 30 years ago [16]. At the time, the intention of the modeling was to simply generate a function that described the nature of the relationship between the strength of a signal (which was left relatively undefined) and the outcome, histamine release. Using various approaches to

vary signaling, the heuristic equation (see Fig. 6A, right side) was derived. The signal was somewhat ambiguously defined but was considered to be related to receptor activation. Recently, we generated a control systems model that described a few early steps in IgE-mediated signaling [17], which incorporated several known aspects of early IgE-mediated signaling. The goal in this case was not to explicitly describe the mechanics and enzymology of the early reaction but to derive qualitative characteristics that held true throughout a large range of possible parameters. The initial parameters were tuned to mimic what was observed experimentally, i.e., to reproduce what occurred in cells stimulated without pharmacologic modifiers. As the activity of PI3K has been shown to lead critically to histamine release in human basophils [18], the output of the control systems model was linked to the heuristic description of histamine release. The signal for the heuristic equation of histamine release was the output of the control systems model, which was used to explore how noise in measurements and variation in activators (syk) and deactivators (SHIP) influenced the ability to detect correlations. In effect, the model became a statistical model in that the changes in correlations were explored for different noise injection into an otherwise deterministic outcome.

The scheme (see Fig. 6A) roughly represents the signaling cascade that proceeds from FcεRI receptor aggregation to PI3K activity (with PIP3 as the marker of this activity). In this scheme, activation of P2 and N2 (the later down-regulatory step (see Fig. 6A) requires activation of an earlier activating step, represented by P1³ (see Fig. 6A). The reactions in this signaling model have kinetics with enzymatic characteristics (a V_{max} and Km). The distinction in steps is motivated by the knowledge that there is a step that precedes activation of syk, thought to be a src-family kinase member, which also appears to regulate the activation of SHIP, independent of syk activity [5]. syk is known to lead to activation of PI3K [19], and SHIP is known to regulate PIP3 levels [20], one possible output of the activity of PI3K. Therefore, P1, P2, and P3 might represent lyn, syk, and PI3K, respectively. N2 is being represented by SHIP, but we do not know what N1 represents. We had once assigned a role for actin polymerization in this step [17], but recent data suggest that this is not the case. As it is modeled, it is now simply a process that can inactivate P1. Each equation has a parameter that could be equated to V_{max} and a parameter related to Km. The substrate for each step is the next step in the cascade, mimicking the fact that these are kinase enzymes that phosphorylate their downstream substrate. The final step is the generation of a signal level that is used in the histamine release heuristic. For the purposes of this modeling, it is known that PI3K activity is required for histamine release [18, 21, 22], so the signal is being represented by the presence of PIP3. Clearly, this is not the signal that couples directly to the granule extrusion apparatus, but the feedback loop involving PI3K and SHIP is the most downstream step in signaling, relevant to degranulation and for which there is an activator/deactivator pair, which has been examined in basophils. As we were interested in the effects of SHIP and syk more or less, the model captures only those elements we have studied. Therefore, PIP3 is the surrogate metric for whatever is generated by PI3K activity that regulates histamine release.

For the purposes of modeling, the parameters of the coupled equations representing the various enzymatic reactions are expressed in arbitrary units. The parameters were obtained from previous studies [17]. The heuristic equation for histamine release was tuned to replicate the kinetics and magnitude of histamine release in human basophils. As we were only interested in predictions about correlations between expression and function, thousands of model datasets for different values of P2 ("syk") and N2 ("SHIP") were generated with noise injected according to known limitations in their measurement.

RESULTS

For the most part, these results are dependent on a semiquantitative Western blot. The expression of a given element is expressed in terms relative to the standard, the highest concen-

tration of which is assigned the value 1.0. **Figure 1** shows the flow diagram for generating expression results. **Figure 2** shows the frequency distributions of 13 signaling elements (for similar distributions of six additional elements; see ref. [4]).

Table 1 shows the correlations among the various elements as well as the their relationship to the functional endpoints, maximum histamine release, and ED₅₀ (a measure of basophil sensitivity that is expressed as the density of cell-surface antigen-specific IgE for one-half maximal response) and to the expression of previously measured signaling elements (notably syk). One "element", the p85/PI3Kδ ratio, is synthesized from the two independent measurements of p85 and PI3Kδ, and one element, IL-3Rα, is a surface measurement by flow cytometry.

syk mRNA levels and syk expression

Previous studies of nonreleasers and releasers (a grouping of basophils based on their responsiveness to stimulation with anti-IgE antibody) suggested that there was no relationship between the cellular level of syk mRNA and expression of syk protein [11, 23]. This conclusion was re-examined in a subset of 16 individuals using a more stringent methodology (see Discussion and ref. [15] for examples of the flow cytometric method for determining syk expression). We found that the CV for syk mRNA and protein expression was 0.47 and 0.63, respectively (**Fig. 3, A and B**). There was a statistically significant correlation ($r = 0.593$; $P = 0.019$) between the levels of mRNA and syk protein (**Fig. 3C**). A similar analysis for syk mRNA expression in the contaminating mononuclear cells showed no correlation with syk mRNA in basophils ($r = 0.187$). We also noted that syk protein expression (by flow cytometry) and maximum histamine release from basophils of these subjects were highly correlated ($r = 0.789$), as found in

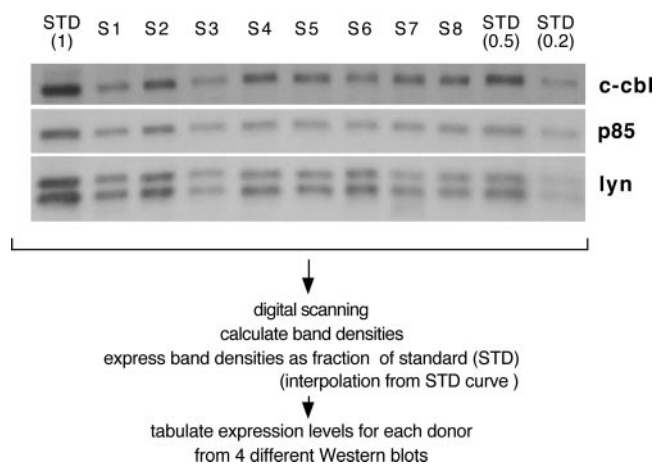


Figure 1. An example of the semiquantitative Western blotting protocol and the process for expressing results as a fraction of a basophil "standard" (STD). Each gel included a three-point standard curve to bracket eight samples. The standard was a lysate generated from a large number of purified basophils at an approximate concentration of 0.7 million cell/20 μ l lysate with aliquots stored at -80°C . Using molecular weight standards on the leftmost and rightmost lanes, the nitrocellulose was cut to generate two to three strips for antibody blotting (depending on the antibodies to be used).

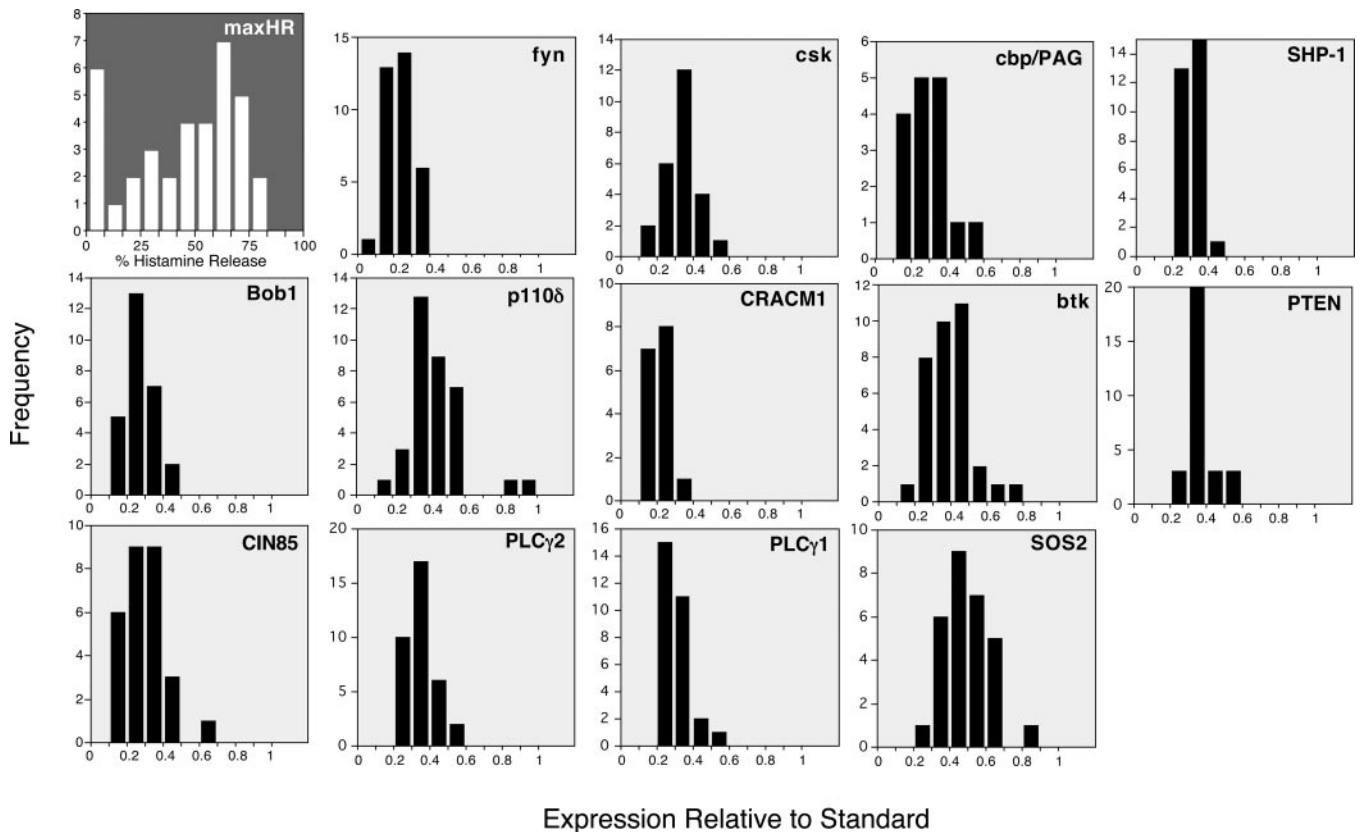


Figure 2. Population distributions of signal element expression. The abscissa is the expression level of a signaling element relative to the basophil standard. To generate the distribution, expression levels for each subject's basophils were placed in one of 13 "bins", each bin, 0.1 unit wide, ranging from 0 to 1.3 standard "units" and the number in each bin plotted. maxHR, Maximum histamine release.

the survey study of 36 subjects using Western blotting methods.

Statistical and heuristic modeling

With the exception of cbp/PAG, the distributions of the 13 additional signaling elements are quite narrow. This behavior is similar to the results for lyn, c-cbl, SHIP2, and p85 α in our previous study; i.e., despite the large range of functional abilities in these cells, most of the signaling species that we have studied are expressed at a similar level among basophils from different subjects. The greatest population variance is found in syk expression, as reported previously in the original pilot study [4]. **Figure 4** shows the CV for 20 elements, including surface IL-3R α . Previous studies determined that the Western blot procedure generates a CV of $\sim 16\%$, and the average CV for all of the signaling elements was $24 \pm 8\%$ (the dotted line on the plot with the SD displayed as a gray region around the dotted line). With the exception of syk and IL-3R α surface expression, none of the other signaling elements showed a variance that statistically exceeded this range, which itself was only marginally above expectation for the measurement technique itself.

Notably, OCA-B/Bob1 and CIN85 expression distributions were also quite narrow. However, Bob1 and CIN85 showed a negative correlation with maximum histamine release (Table

1). Their relationship to syk expression is less clear, but this issue will be explored in greater detail below.

When considering the normal expectations for correlation coefficients and confidence intervals for $\alpha < 0.05$ from standard statistical tables, there were several other signaling elements with statistically significant correlation values to histamine release, in order of statistical significance: PLC γ 2, Bob1, btk, p110 δ , CIN85, and SHIP1. Notably, each of these correlations was negative. The negative correlation for a signaling element such as PLC γ 2 was surprising. There was no correlation between PLC γ 1 and function nor did the sum of PLC γ 1 and PLC γ 2 expression change the inverse correlation observed for PLC γ 2 alone (see Discussion). In a similar context, a negative correlation between expression of p110 δ and maximum histamine release would be surprising. Parenthetically, in recent studies, it has been proposed that too much p85 α , relative to the presence of the p110 subunits of PI3K, inhibits the activity of PI3K [24, 25]. In effect, the regulatory subunit of PI3K acts as a competitive inhibitor of the combined p85/p110 enzyme. In human basophils, only p110 δ is expressed [18]. Therefore, the ratio of p85/p110 δ was calculated and correlated to maximum histamine release or ED $_{50}$; no correlation was found.

The negative correlation between PLC γ 2 and function is one unexpected observation arising from this survey. The narrowness of the PLC γ 2 population distribution makes the pres-

TABLE 1. Correlation Matrix for Function and Expression Levels

n	maxHR 36	ED50 30	svk 26	Bob1 26	c-cbl 26	CIN85 26	SHIP1 26	SHIP2 26	p85a 26	p110d 35	85a/011C 35
maxHR											
ED50	−0.486										
svk	0.766	−0.367									
Bob1	− 0.461	0.115	−0.341								
c-cbl	0.101	−0.090	0.398	0.340							
CIN85	−0.429	0.058	0.145	0.718	0.243						
SHIP1	−0.343	0.201	0.027	0.416	0.560	0.237					
SHIP2	−0.049	0.028	−0.127	0.424	0.480	0.250	0.546				
p85a	0.052	−0.098	0.322	0.519	0.530	0.401	0.542	0.348			
p110d	−0.391	0.153	−0.326	0.501	0.224	0.281	0.190	0.292	0.109		
p85/p110c	0.200	−0.105	0.389	−0.028	0.002	0.059	0.211	−0.054	n/a	n/a	
lyn	−0.014	−0.054	0.299	0.403	0.531	0.371	0.610	0.427	0.604	0.126	0.259
fyn	−0.094	0.137	0.149	0.395	0.460	0.438	0.494	0.377	0.573	0.059	0.361
csk	−0.195	0.085	−0.155	0.780	0.409	0.554	0.402	0.434	0.288	0.314	−0.103
cbp/PAG	−0.114	−0.277	−0.220	0.524	−0.032	0.458	0.354	0.592	0.416	0.228	0.092
btK	−0.394	0.026	−0.233	0.736	0.464	0.706	0.249	0.319	0.270	0.463	−0.198
PLCq1	−0.012	−0.015	0.343	0.249	0.311	0.540	0.015	−0.047	0.281	−0.105	0.254
PLCq2	−0.447	0.199	−0.236	0.673	0.432	0.549	0.345	0.504	0.322	0.605	−0.301
SHP-1	−0.078	0.092	0.206	0.628	0.307	0.644	0.153	0.005	0.258	0.282	−0.037
PTEN	−0.221	0.155	−0.160	0.188	0.101	0.280	−0.089	0.113	−0.177	0.054	−0.275
SOS2	−0.275	0.103	−0.063	−0.007	0.173	0.332	−0.134	−0.309	0.028	−0.054	0.022
IL3Ra	0.002	0.084	0.130	0.487	0.074	0.315	0.059	−0.032	0.192	−0.109	0.186
CRACM1	−0.136	0.313	0.033	0.341	0.328	0.249	0.315	−0.020	0.355	0.678	−0.277

Statistically significant, positive correlations are noted in bold; statistically significant, negative correlations are in a table cell with a gray background. Correlations to ED₅₀ necessarily excluded subjects whose basophils did not release histamine, as the ED₅₀ cannot be determined under such conditions. Therefore, the number of subjects decreases by six. Not all measurements could be made on the entire population; therefore, some correlations were run on a subset of the entire population (those for whom enough whole cell lysate sample remained). The number of subjects is noted in the second row of the table.

ence of a negative correlation more surprising. There is no mechanistic basis for a negative correlation, and this suggests that the correlation is spurious. In addition, the five statistically significant correlations for signaling elements generally considered activating elements appeared skewed. Indeed, there also appeared to be a bias to the correlations between various signaling elements. To explore this issue further, **Figure 5A** shows a “heatmap” of signaling element expression for 25 subjects. As not all signaling elements could be analyzed for the full survey of 36 subjects (see sample numbers at the top row of Table 1), this subset represents a balance of the most signaling elements for the most subjects. The ordering is derived from a multidimensional hierarchical clustering algorithm. The heatmap shows that some samples cluster (to the left) as having an overall higher level expression of all elements and others, a generally low expression (to the right), suggesting there is a sampling bias in the dataset. Parenthetically, average histamine release for the two halves of the heatmap is statistically the same. To explore sample bias further, **Figure 5B** shows a distribution of all of the correlations in the correlation matrix for signaling element expression versus functions (maximum histamine release and ED₅₀) and a distribution for the correlations between signaling elements (**Fig. 5C**). For the element versus function distribution, there is a slight negative skewing (-0.078 ± 0.229). More notably, there is a net-positive bias to the element versus element distribution,

where the average is 0.259 ± 0.268 . The tendency in the heatmap for common high or low expression, the bias in the correlations, the finding of several highly correlated species, and the narrow distributions in expression for most signaling elements suggest that the expression of many of these elements is under common control or that there is a procedural or methodological error that creeps into the generation of the data.

Although both of these possibilities are good candidate explanations, we were intrigued by the latter possibility, as it offered a way to correct for the bias. In Materials and Methods, there is a description of the issues that arise when assessing signal element expression by Western blotting.

Systematic shifts as a result of methodology would be manifested as a positive bias in the population correlations. Indeed, repetitive simulations showed that injecting the 14% procedural error into a dataset with an initial correlation of $r = 0$ would produce a mean for the population correlation distribution of 0.239, a value very close to the value 0.259 (**Fig. 5C**). It should also be noted that there was a modest inverse correlation between the size of a signaling element's CV and whether it generates a correlation with another signaling element (data not shown), again suggesting that some of the noise is correlated. Therefore, it is reasonable to conclude that the bias we observed is a result of this known procedural error. This conclusion has two implications. The first is that the distributions in **Figure 5, B and C**, should be used as the guide for whether

TABLE 1. (Continued)

lyn 36	fyn 35	csk 26	cbp/PAG 16	btb 35	PLC γ 1 29	PLC γ 2 35	SHIP-1 29	PTEN 29	SOS2 29	IL3Ra 36
0.615										
0.469	0.443									
0.181	0.576	0.668								
0.293	0.613	0.776	0.778							
0.070	0.513	0.317	0.540	0.617						
0.456	0.281	0.546	0.280	0.598	0.194					
0.388	0.504	0.581	0.339	0.736	0.604	0.355				
-0.145	0.310	0.358	0.636	0.347	0.313	0.305	0.159			
-0.239	0.128	-0.185	-0.149	0.339	0.408	0.065	0.198	0.421		
0.216	0.069	0.653	0.221	0.092	-0.001	0.138	0.233	0.111	-0.022	
0.462	0.155	0.046	-0.114	0.264	-0.022	0.618	0.174	-0.252	0.134	0.032

a correlation was significant. Second, a sample of presumptively unrelated elements with small CVs could be used as a measure of the loading variance.

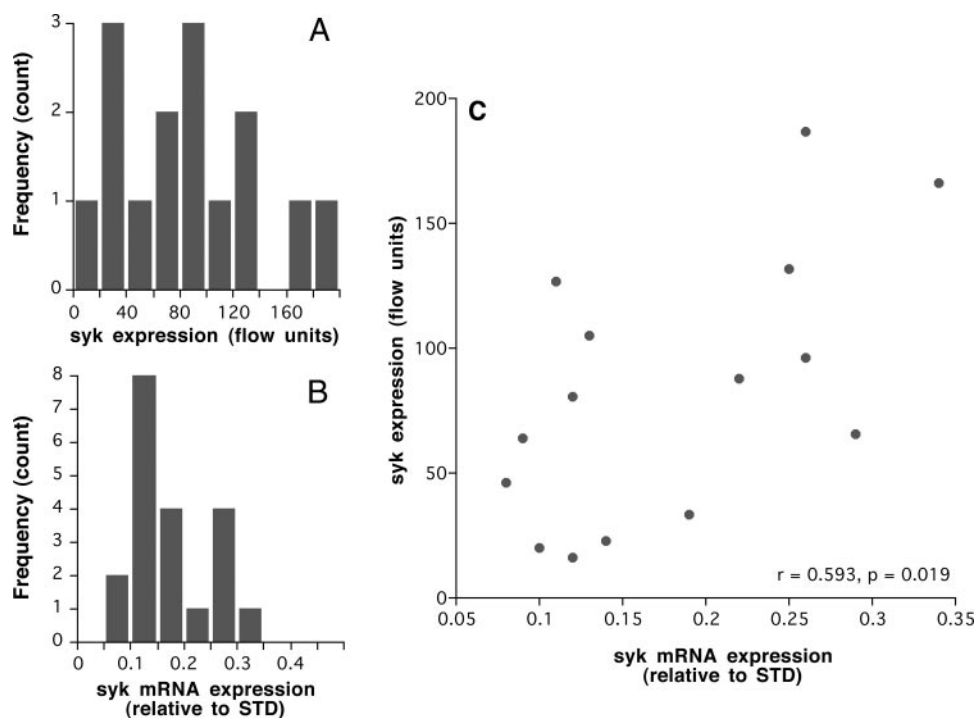
With respect to the first implication, the confidence intervals for a significant correlation coefficient change to -0.451 to 0.295 ($\alpha=0.05$) for the signaling element versus functions (note the negative bias) and -0.178 to 0.696 for signaling element versus signaling element (note the positive bias). With this interval as a guide, only two correlations remain positive for signaling element versus function: syk versus maximum histamine release (0.766) and Bob1 versus maximum histamine release (-0.461). There are 15 correlations for signaling element versus signaling element. They are noted in Table 1 by bolding or highlighting the text. All of the positive correlations were related to btk expression. Five of the negative correlations relate to syk; notably, Bob1 and syk expression were negatively correlated.

For the second implication, a group of unrelated signaling elements that showed a small population CV was used to generate a correction factor. The particular interest is whether this kind of correction factor (see Materials and Methods) would improve some of the correlations or eliminate some of the spurious correlations between element expression and function. This was indeed the case, although weakly so. The data for c-cbl, p85, and lyn, each with low CVs (Fig. 4) and reasonable cross-correlations, were used as an aggregate indi-

cator of the hidden loading variance among samples (see Materials and Methods). When this correction was applied to syk, SHIP1, and PLC γ 2, and the correlation with histamine release was re-evaluated, the syk correlation increased to 0.823 (from 0.776), the SHIP1 correlation improved to -0.41 (from -0.343), and the PLC γ 2 and Bob1/OCA-B correlations changed to -0.490 (from -0.447 and -0.461 , respectively). Other signaling element correlations did not shift significantly.

As noted in a previous publication [4], we explored how the statistics would appear for a completely deterministic model of histamine release. This model is diagrammed in **Figure 6A** (see Materials and Methods). Figure 6B shows the purely deterministic relationships that this model produces when varying syk and SHIP and calculating the outcome measures of maximum histamine release and ED₅₀ (cellular sensitivity). Most notable is that variation in SHIP has little influence on the ED₅₀, and it does control maximum histamine release. Figure 6B also shows that for the same region (gray shading) of syk expression, a linear relationship between expression and maximum histamine release would be expected to provide an excellent correlation, and the nonlinear relationship between expression and ED₅₀ would be expected to generate a poorer correlation. Figure 6C shows an example of introducing the known measurement noise of Western blotting, histamine release determinations, and ED₅₀ calculations on the relationship between syk and histamine release/ED₅₀ and SHIP and

Figure 3. Expression distribution of syk mRNA and protein and correlation between the two parameters. (A) "Binned" frequency distribution of syk mRNA expression in 16 subjects' basophils. (B) Binned frequency distribution of syk protein expression in the same 16 samples. (C) Correlation between syk protein and syk mRNA.



histamine release/ ED_{50} . This one example (left panels of Fig. 6C) is but one of 100,000 such simulations. The distributions of possible correlations produced from these simulations are shown in the right panels of Figure 6C. It is notable that even with the deterministic model, the mode for the SHIP correla-

tion is approximately -0.5 . The mode for the syk versus histamine release correlation is ~ 0.82 , which is similar to the experimental value. If the counting/loading correction is applied to the actual data for syk and SHIP, then the correlations match model expectations more closely.

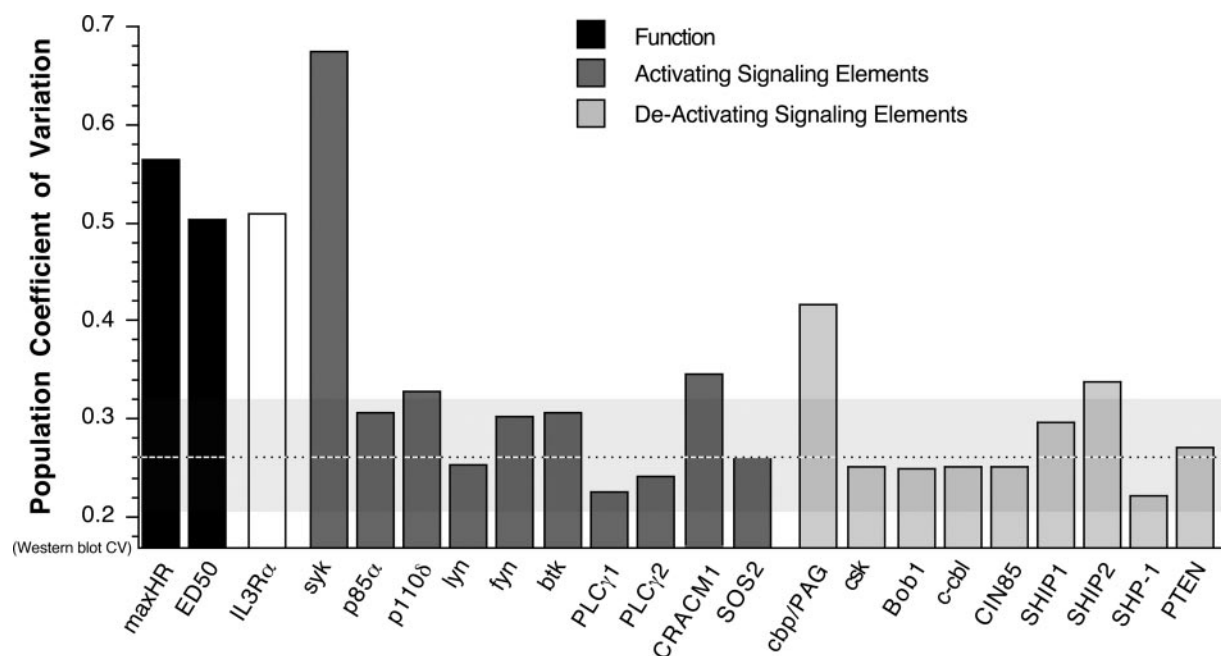


Figure 4. CV for each of the measured signaling elements and two basophil functional parameters. The plot shows three characteristics: the baseline represents the expected CV for the population based on previous analysis of the noise intrinsic to Western blotting; the dotted horizontal line represents the average CV value for all of the signaling elements; and the gray band is the ± 1 SD "region" of the various CV assessments for each element.

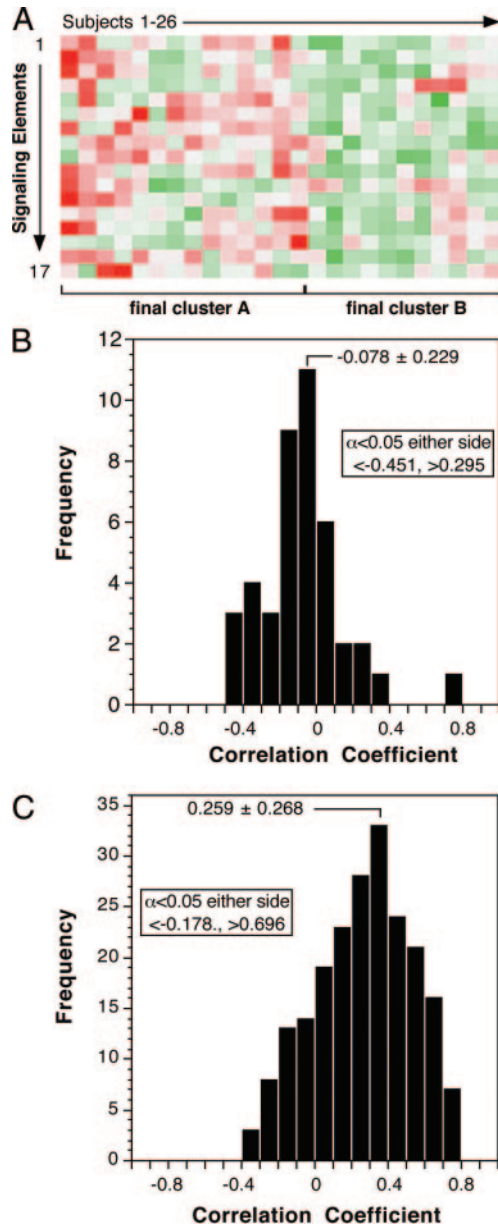


Figure 5. Heatmap and distributions of correlation coefficients from the correlation matrix shown in Table 1. (A) Heatmap of signaling element expression for 26 subjects. The columns represent subject number, the rows represent a particular signaling element, the color of a block is red for strong expression and green for low expression, and white is the average expression. The ordering of subjects is derived from a hierarchical clustering algorithm, and the final two largest clusters are shown as “final cluster A” and “final cluster B.” The signaling element order, from 1 to 17, is: csk, CIN85, SHP-1, PTEN, SHIP2, SHIP1, c-cbl, p85, lyn, Bob1, SOS2, PLCγ1, PLCγ2, btk, p100δ, fyn, and syk. (B) Correlations between signaling element expression and the two measured basophil functions—maximum histamine release and ED₅₀ (the sensitivity measure; see Materials and Methods). (C) Correlations between signaling elements.

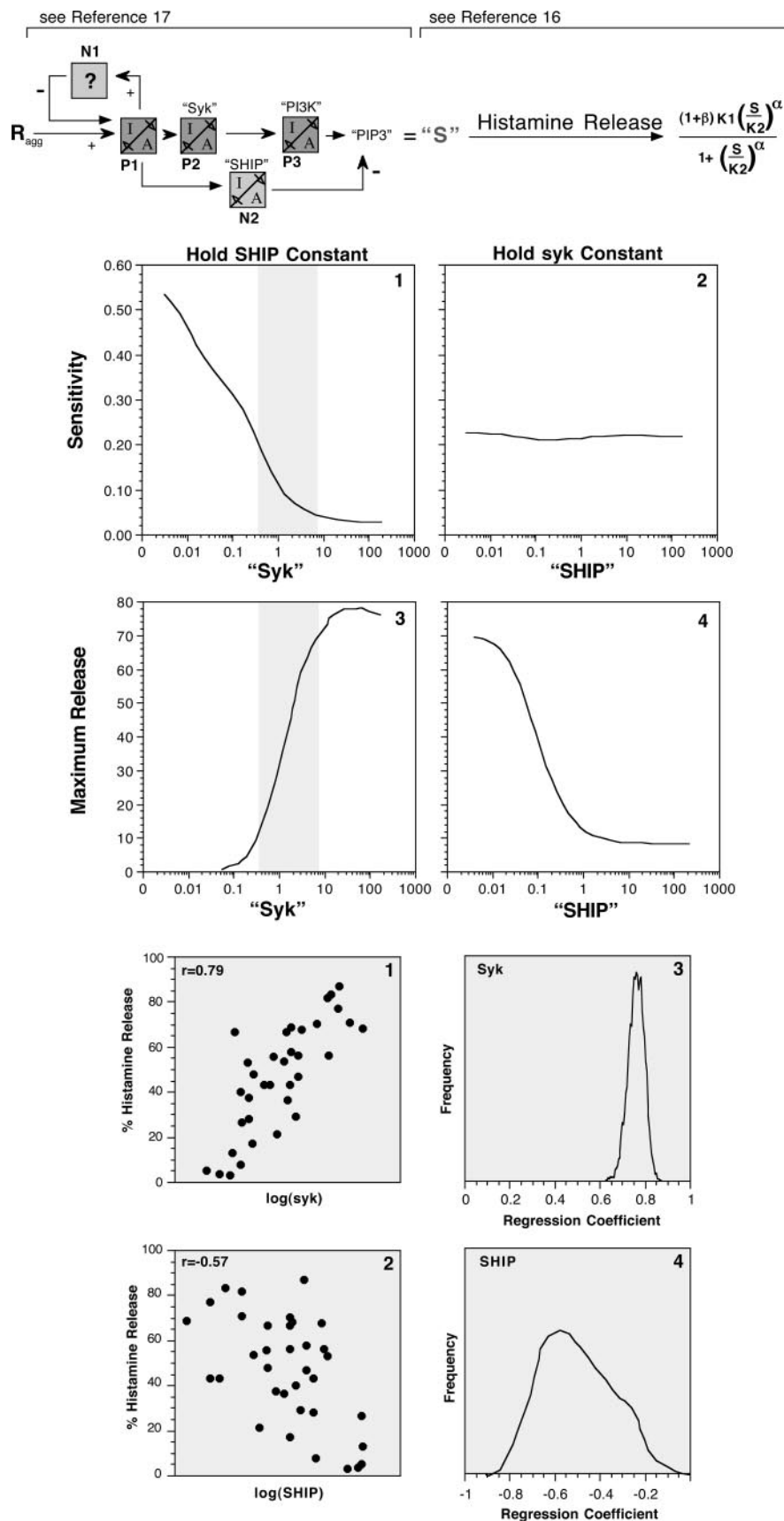
DISCUSSION

We have now examined the relative expression of 20 signaling elements and one synthesized element (p85/p110 ratio) in the

general population of human basophils. The original study focused on syk and SHIP1, as these enzymes had been speculated to be gatekeepers of function [26] and because of two identified basophil phenotypes [11, 23, 27] and a phenotype of mast cells derived from SHIP^{−/−} mice. However, many other signaling elements have a place in signaling, and this survey has examined groups based on function. Elements were chosen on the basis of their demonstrated roles in IgE-mediated signaling in rodent studies, cell lines, or human basophil studies. They were also grouped into positive and negative regulators. In the category of negative regulation, several phosphatases, SHIP1, SHIP2, SHP-1, and PTEN, which are thought to possibly regulate secretion negatively, were examined. In the context of a negative signal, the expression of c-cbl was also studied [28, 29]. With the exception of SHIP1 (see below as well), there was no correlation with basophil function. The family of src-family kinases that is found expressed in basophils, lyn, fyn, and csk was found to be well-correlated with each other. However, it should be noted that csk acts as a negative regulator of lyn function. In this context, we also examined the expression of cbp/PAG, an adaptor molecule that recruits csk to the cell membrane, resulting in phosphorylation of lyn on its negative regulatory tyrosine [30, 31]. Current evidence suggests that lyn itself has positive and negative regulatory effects on secretion [32]; therefore, the relationships between these various species and basophil function would be difficult to anticipate. Several enzymes known to have a positive role in secretion have now been examined; the only class 1A PI3K in basophils, p85α/p110δ, is critical for secretion [18], PLCγ1/γ2 are thought to mediate the generation of IP3 [33–35] and are therefore important in generating the cytosolic calcium response, and btk is thought to participate in the phosphorylation of several signaling proteins, such as PLCγ. In basophils, one linkage between the activation of the ras-dependent pathways (such as MAPK) includes the Grb2-SOS2 adaptor molecules. It appears that p21ras activation is important in the basophil response [18], and as basophils express only SOS2, this adaptor was also examined. Finally, there is a strong correlation between the rise in free cytosolic calcium during stimulation and subsequent histamine release among the general population of basophil donors [36, 37], and CRACM1/Orai-1 has been identified tentatively as a critical component on the influx phase of the cytosolic calcium response [38, 39].

The large number of possible correlations between signaling element expression and function or between the expression of different signaling elements raises questions about how these data should be analyzed. In addition, the narrowness of the CVs for many elements and the bias in the correlation coefficients suggest there are some procedural issues to address. It would also be useful to adopt a variant of Koch's postulates when considering the results, notably, whether a given correlation makes sense with respect to the mechanisms of secretion that are currently understood. Factoring in the statistical analysis presented in Results, there are only several significant correlations and only two between expression and function. The syk correlation is the stand-out correlation, and at the other end of the distribution, there is a negative correlation between Bob1 and maximum histamine release. However, even this

Figure 6. Heuristic model of histamine release. (A) Schematic version of the model. The boxes on the left side labeled P1, P2, P3, N1, and N2 represent a kinase with an on-and-off state determined by the activity of the preceding kinase. The exception is P1, which is turned "on" by the presence of aggregated receptor (R_{agg}). The substrate for one kinase is the next kinase or phosphatase, as in the case of P1 driving the activation of N1 or N2. The amount of "S," a product of P3, is used in the heuristic equation on the right that determines the rate of histamine release (see Materials and Methods). (B) The four subpanels show the relationship between syk (P2) expression and ED_{50} /sensitivity (1), SHIP (N2) expression and ED_{50} (2), syk and maximum histamine release (3), and SHIP and maximum histamine release (4). (B1 and B3) The gray shading is present to show the same range of syk expression to compare the relative linearity of the responses in this range. (C) Injection of methodological noise into the deterministic relationships shown in B. (C1 and C2) A single example of a sample of randomly chosen syk and SHIP expression levels for a population size similar to our experimental study and the effect of methodological noise injection on the scatter in the relationships. (C3 and C4) The distribution of correlation coefficients for thousands of simulations such as those shown in C1 and C2.



negative correlation was marginal, and the expected correlation is not negative but positive. This transcriptional element, which also acts as a protector of syk loss in B cells [7], would be expected to be correlated positively with function and syk expression. It is inverted in both cases. The next-closest negative correlations are PLC γ 2 and CIN85. There is also no reason to expect a negative correlation between PLC γ 2 and histamine release. A negative correlation might be expected for CIN85, but these unexpected correlations suggest that they are indeed spurious, and their marginality (the PLC γ 2 correlation doesn't make the statistical cut based on the distribution in Fig. 5B) leads us to conclude that there are no reasonable correlations between function and element expression except for syk.

This conclusion, however, raises a question about the negative correlation between SHIP1 and histamine release. This correlation, -0.343 (uncorrected), does not make the cut. There may be some factors mitigating against the possibly spurious nature of the SHIP1 correlation. First, there are studies of human basophils that appear to indicate that significant changes in SHIP1 or SHIP2 are associated with the IgE-mediated ability of the cells to release. This implicates SHIP in function. Our own modeling raises the possibility that even when SHIP has an explicitly determined function, its correlation with function may not be very strong. Finally, entering SHIP1 into a multiple regression does improve the relationship between expression of syk and maximum histamine release. These are all relatively weak arguments, and the strongest conclusion we can draw at this point is that in the general population, SHIP expression variability is not marked and accounts for, at best, a modest amount of variation in function. There remains the possibility that in certain disease states, SHIP can become an important regulator if its expression varies significantly from the norm, although such a statement would probably be true of many signaling elements.

Two target elements for the current study were OCA-B/Bob1 and CIN85, each of which appears to influence how effectively the cbl ubiquitin ligases mediate the loss of syk by the proteasome pathway. As noted above, expression of Bob1 was negatively correlated to release and syk expression, which makes no sense if the studies in B cells are an accurate guide to what is happening in basophils. OCA-B/Bob1 is also a cofactor for the transcription factor OCT-1/2. In B cells, regulation of κ -light chain synthesis is diminished severely in OCA-B $^{-/-}$ mice [40]. The 4000-bp upstream promoter of syk contains at least 15 possible OCT-1-binding sequences (15 core match of which three are high matrix match as well), although there are no reports about the requirements for OCT-1/OCA-B in syk gene transcription. However, if the B cell studies are used again as a guide, the correlation with syk expression, via a gene transcription effect, should have been positive, not negative. CIN85 showed no statistically meaningful correlation with function or syk expression but oddly, was highly correlated with expression of Bob1/OCA-B. If the mechanisms for these two proteins were taken at face value, Bob1 protecting syk during stimulation and CIN85 accelerating syk loss during stimulation, their covariance would appear to counterbalance each other. The mechanisms for how these proteins regulate syk expression are not fully known, and the point in basophil maturation at

which they may exert an influence on syk expression is not clear. Each protein may also require prior stimulation of IgE-mediated pathways to exert their influence on syk expression. Furthermore, although OCA-B/Bob1 has two forms, both of which can mediate protection of syk, the details of the relative efficacy of the two forms are not fully appreciated. As the relative presence of the two forms could not be assessed, it also remains possible that there is a subtlety to OCA-B/Bob1 expression levels that isn't appreciated in these studies.

The remaining correlations between signaling element expression are also difficult to understand. Five of the 10 negative correlations are relationships between syk expression and other elements considered involved in activation arms of the signaling cascades. The remaining negative correlations are between elements that would not be expected to be correlated inversely. There is no basis for drawing strong conclusions about these negative correlations. With the exception of the positive correlation between CIN85 and Bob1/OCA-B, the remaining four positive correlations are between btk and another element. btk and cbp/PAG show modest CVs for this population, so these highly significant correlations may be biologically meaningful, but the mechanistic reason for linkage is not clear.

Interesting aspects of these results are that for an intracellular element, only syk showed a CV that was as broad as histamine release, and the distributions of the other elements were tightly regulated among very different types of subjects (atopic, nonatopic, and nonreleasers). Indeed, this tight regulation of most elements (in the face of significant functional variation) is a remarkable result from this expanded study of signal element expression. How expression is controlled this tightly even when some elements, e.g., syk, are undergoing significant variation remains an interesting question.

Besides syk, only cell-surface expression of IL-3R α appears to show a CV that is similar to the CV for maximum histamine release. As IL-3 is a strong modulator of secretion [41–43], this variance may be relevant. However, one characteristic of exposure of peripheral blood basophils to IL-3 is the broad up-regulation of a variety of signaling elements. In addition, all functions of the cell are enhanced, but expression of IL-3R α showed no correlations with function or signaling element expression. Therefore, the broad CV is present without an obvious association with other characteristics of the basophil.

syk mRNA levels were examined in two previous studies of nonreleasing basophils. Although the definition of a nonreleasing basophil is somewhat flexible, the term generally refers to basophils that are completely unresponsive to stimulation with anti-IgE antibody or respond with less than 5–10% histamine release. The stringency of the definition may define the frequency of this phenotype in the population. For example, basophils from four subjects in the current survey fit the more stringent definition, and six fit the 5% cutoff rule. In the previous studies, the steady-state level of syk mRNA did not differ significantly between nonreleasers and the rest of the population (i.e., releasers) [11, 23, 44]. However, in our own nonreleaser study, the variation in syk mRNA expression appeared broad in nonreleasers, and we surmised that a better measurement technique might allow a more refined study of the question. The methodology used was refined from our previous

methods to include quality of mRNA assessment, measurements against a standard for all real-time PCR measurements, and analysis of syk protein with flow cytometry [15]. On the latter point, syk mRNA is measured with an extrinsic method (i.e., dependent on cell number) and syk protein with an intrinsic method (flow cytometry), meaning that the two assessments will be independent (unlike the cross-correlations manifested in the data shown in Fig. 5B). With the more stringent method, a modest correlation was found between Syk mRNA and syk protein. The syk mRNA levels were not distributed as broadly as syk protein, but the theoretical relationship between mRNA levels and protein levels follows an unknown function, so it is difficult to interpret the meaning of the difference in CV for mRNA and protein. Based on the earlier studies, we had concluded that control of syk expression was something that occurred post-translationally. syk ubiquitination would be a mechanism consistent with such an interpretation. The current evidence suggests that at least some of the variability (36%) in syk protein expression might derive from differences in syk transcription or processing of syk mRNA.

In summary, an analysis of the expression of 20 signaling elements in basophils from a general population survey demonstrates a remarkably narrow population distribution of all signaling elements except syk. With some caveats, the only element that clearly correlated with function was also syk. The expression of Bob1/OCA-B, a putative post-translational syk expression regulator, was only marginally correlated to syk expression, and the correlation was opposite of expectation. CIN85, a second post-translational syk expression regulator, was not correlated with syk expression. Some variance in the level of syk protein expression correlated with syk mRNA levels.

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