

Induced Loss of Syk in Human Basophils by Non-IgE-Dependent Stimuli

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In the general population, Syk expression in human basophils is highly variable and correlates well with the IgE-mediated responsiveness of these cells. Previous studies established that IgE-mediated stimulation results in loss of Syk expression. The current studies investigated whether stimulation through other receptors results in loss of Syk. Two classes of stimulation were examined, those that operate through the kinase Syk and those that operate through a GTP-binding protein. These studies demonstrated that aggregation of leukocyte Ig-like receptor LILRA-2 resulted in phosphorylation of Syk and c-Cbl, was inhibited by a third generation Syk inhibitor with an expected IC₅₀, and induced histamine release in strict proportion to release induced by anti-IgE Ab. Stimulation of LILRA-2 for 18 h resulted in modest loss of Syk that correlated with the more profound loss of Syk induced by anti-IgE Ab. Human recombinant histamine-releasing factor has also recently been shown to induce Syk phosphorylation and in the current studies has also been shown to induce loss of Syk in 18-h cultures. fMLP stimulation for 18 h was also found to induce modest loss of Syk. fMLP induced phosphorylation of c-Cbl that was sustained for at least 45 min. Phosphorylation of c-Cbl was inhibited by a Syk kinase inhibitor but with an IC₅₀ that was not consistent with Syk activity, suggesting another kinase was responsible for Cbl phosphorylation following fMLP. These studies demonstrate that it is possible to induce the loss of Syk expression in human basophils by a non-IgE-dependent mechanism and even by a mechanism that does directly involve Syk in the reaction complex. *The Journal of Immunology*, 2008, 180: 4208–4217.

The IgE-mediated signal transduction in mast cells and basophils is dependent on the tyrosine kinase Syk (1–5). This enzyme, in collaboration with one or more Src family kinases (e.g., Lyn and Fyn), provides the enzymatic activity that the high affinity IgE receptor (FcεRI) lacks. By binding to the FcεRIγ subunit, Syk becomes enzymatically more active and phosphorylates a variety of proteins generating the full signaling reaction that drives mediator secretion and a host of other cell responses. In this signaling cascade, Syk expression is an almost absolute requirement; there is no known redundancy. Recent studies have noted that Syk in human basophils is broadly expressed and correlates well with the ability of these cells to respond to IgE-mediated stimulation (6). These studies generalize observations made when comparing so-called nonreleasers and releasers (7–9). Other signaling elements display a much narrower population distribution. Indeed, for elements like Lyn and p85α (the regulatory subunit of PI3K), the population distribution is narrow enough to be considered a constant (the resulting distribution being no more than “noise” in the Western blot technique). Therefore, the question arises, what is the source of the variance in Syk expression when other signaling elements are consistently expressed in the population?

In the previous studies of nonreleasing basophils (8, 9), no statistically significant difference in mRNA levels was found between nonreleaser and releaser basophils. Therefore, it has been tentatively concluded that the differences in Syk protein levels are due to a posttranslational event and the information from these studies on nonreleasers might be extrapolated to the general population until shown otherwise. This extrapolation leads to a consideration of the posttranslational mechanisms that might regulate Syk levels. IL-3 has a wide variety of effects on basophils and operates on the basophil phenotype on several time scales. Notably, IL-3 alters the functional response of basophils to all known stimuli, IgE-dependent and non-IgE-dependent. It is also essential in the maturation of basophils from CD34⁺ progenitors (10). Previous studies have shown that IL-3 can modulate Syk levels on a time scale of days (6, 8) and it partially restores IgE-mediated histamine release in nonreleaser basophils on this time scale while not doing so on a much shorter time scale (11). These results initially suggested that the presence of IL-3 in vivo, circulation or bone marrow, might explain differences in Syk expression among donors. Although increases in Syk expression induced by IL-3 may be responsible for the increased IgE-mediated responsiveness in nonreleasers (8, 12), the survey study mentioned (6) also found that the IL-3 induced changes in a number of signaling elements and the changes were most marked in signaling elements that were otherwise nearly invariant in the general population. For example, IL-3 induced an average Syk increase of 38% and a 175% increase in c-Cbl. c-Cbl shows no correlation to function or to Syk protein levels in the population survey and shows one of the most narrow coefficients of variation in the general population. Indeed, there was reasonable covariance in the increases observed for all the signaling elements measured. Thus, the IL-3 “signature” for inducing changes in signaling elements argues against a dominant role for this cytokine in generating the observed selective variation of Syk. In contrast, aggregation of the IgE receptor induces a marked loss in Syk

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expression (6, 13, 14). The strength of stimulation does not need to be sufficient to cause mediator release in order for some Syk to be lost. Furthermore, Syk activity is not necessary for Syk to be lost by this mechanism (6). Of 25 signaling elements studied, Fc ϵ RI aggregation induced some loss of only two other elements, a small loss (~10–20%) of Lyn kinase and a somewhat greater loss (20–30%) of the receptor itself. Therefore, although not a perfect mechanism to explain the rather selective variance of Syk expression in the general population, it is possible that variable low level aggregation of the IgE receptor *in vivo* might result in variable expression of Syk in a circulating basophil.

Another mechanism that might explain variable Syk expression is activation of the basophil through non-IgE-dependent receptors that also use Syk kinase to regulate their signaling cascades. Hypothetically, any receptor that uses Syk as an early signaling element could drive a reduction in Syk expression. In recent years, numerous receptors have been identified that use Syk as an early step in signaling. The tandem Src homology 2 (SH2)² domain of Syk binds to specific ITAMs like those found in the Fc ϵ RI γ subunit, and Fc ϵ RI γ is used by several receptors to direct their early signal transduction. In addition to Fc ϵ RI γ , the small adaptor protein DAP12 binds to Syk through ITAMs, and this small membrane protein also associates with several receptor types (reviewed in Ref. 15), e.g., inhibitory receptors (16). Recent studies in human basophils have identified two possible candidate receptors. The first of these is the so-called human recombinant histamine-releasing factor (HrHRF)-dependent receptor that induces secretion of all three classes of mediators from basophils (17, 18). The profile of mediators released by this stimulus as well as their kinetic characteristics suggest that the receptor for this ligand uses a mechanism similar to that of IgE-mediated secretion. In studies being presented elsewhere, HrHRF was observed to induce Syk phosphorylation and secretion induced by HrHRF was found to be very sensitive to the presence of a third generation Syk selective inhibitor, strongly suggesting a role for Syk in early signaling induced by HrHRF (29).

A second candidate receptor that generates a secretory response with the characteristics of IgE-mediated secretion is the leukocyte Ig-like receptor (LILR) A-2 (formerly LIR7) (19). Recent studies have demonstrated that LILRA-2-mediated signaling (initiated by cross-linking Abs to LILRA-2) also induces a profile of mediators with kinetic characteristics similar to an IgE-mediated process. However, the signaling mechanisms of LILRA-2 in the human basophil have not, until the current study, been explored.

There is evidence that the Cbl family of proteins, notably, c-Cbl and Cbl-b, mediate the ubiquitination of Syk and therefore its directed loss through a 26 S proteasome mechanism (reviewed in Ref. 20). Therefore, a related but more general hypothesis is that any receptor that generates an activated Cbl might also lead to loss of Syk protein. This possibility is more difficult to visualize because current models suggest that there must be interaction between Cbl and Syk for ubiquitination to occur and for the IgE-mediated response in basophils, Cbl phosphorylation is a Syk-dependent event (21) and so is the autophosphorylation of Syk tyrosines (22). But, there is at least one example of a receptor that induces phosphorylation of c-Cbl without apparent phosphorylation of Syk. In human basophils, the bacterial tripeptide fMLP, which operates through a heterotrimeric GTPase, has been noted to induce phosphorylation of c-Cbl (13) and not induce phosphorylation of Syk during stimulation (23). There is a characteristic of

the IgE-mediated phosphorylation of c-Cbl that suggests how fMLP-induced c-Cbl phosphorylation could nevertheless alter Syk expression. We have noted an interesting persistence to Cbl phosphorylation following anti-IgE Ab that is not seen with other signaling elements, suggesting that Cbl may act over a relatively long time period to reduce Syk expression (13). This persistent phosphorylation might explain the ability of very weak stimuli to induce Syk loss even when there is no mediator secretion. This process has characteristics of an integrating signal that requires many hours to be manifested. With this in mind, it is possible that a receptor that induces Cbl activity, without apparent Syk involvement, might nevertheless induce a slow loss of Syk expression by an association that could not be easily measured.

The current study tests the two suggested mechanisms noted earlier: 1) that non-IgE receptors that use Syk for signaling may also induce loss of Syk expression and 2) receptors that induce Cbl activity/participation, but not Syk, may also induce loss of Syk expression. Although there may be numerous receptors that fit these criteria, only three have been studied in sufficient detail that they could be tested in the current study. For LILRA-2 activation, this study also explores the signaling characteristics of activation through this receptor. For HrHRF and fMLP, we ask the simpler question of whether loss of Syk follows activation of these two receptors.

Materials and Methods

Materials

The following were purchased from the manufacturer indicated: PIPES, BSA, EGTA, EDTA, D-glucose, sodium fluoride, tetrasodium pyrophosphate, sodium vanadate, 2-ME, Nonidet P-40, fMLP (Sigma-Aldrich); crystallized human serum albumin (HSA; Miles Laboratories); FCS and RPMI 1640 containing 25 mM HEPES and L-glutamine (BioWhittaker); Percoll, (Amersham Biosciences); Tris and Tween 20 (Bio-Rad); leupeptin, DTT, PMSF (Boehringer Mannheim); biotinylated m.w. markers (New England Biolabs); anti-Syk mAb, 4D10; mouse anti-Fc ϵ RI α mAb, 22E7 (a gift from Hoffmann-LaRoche); HRP-conjugated donkey anti-rabbit Ig Ab, HRP-conjugated sheep anti-mouse Ig Ab, and protein G-Sepharose beads (Amersham). Goat anti-human IgE Ab was prepared as previously described (24). Anti-LILRA-2 Ab was provided by Amgen and anti-mouse IgG (mIgG) Ab (F(ab')₂) from The Jackson Laboratory. Anti-phospho-c-Cbl both pY731 and pY774 and anti-phospho-Erk1/2 were from Cell Signaling Technology, anti-c-Cbl and anti-Cbl-b from Santa Cruz Biotechnology and anti-phosphotyrosine (4G10; Upstate Biotechnology).

Buffers

PIPES-albumin-glucose (PAG) buffer consisted of 25 mM PIPES, 110 mM NaCl, 5 mM KCl, 0.1% glucose, and 0.003% HSA. PAG-CM was PAG supplemented with 1 mM CaCl₂ and 1 mM MgCl₂. PAG-EDTA consisted of PAG supplemented with 4 mM EDTA. Counter-current elutriation was conducted in PAG containing 0.25% BSA in place of 0.003% HSA. ESB is Novex electrophoresis sample buffer containing 5% 2-ME. Complete lysis buffer is 20 mM Tris-HCl (pH 7.5), 100 μ g/ml aprotinin, 10 mM benzamidine, 1 mM AEBSF or 1 mM PMSF, 100 μ g/ml leupeptin, 50 mM NaF, 1 mM Na₂VO₄, 1% Nonidet P-40, and 10% glycerol. Incomplete lysis buffer is complete lysis buffer without the protease inhibitors, Nonidet P-40, glycerol, or vanadate. Nitrocellulose stripping buffer was 65 mM Tris-HCl (pH 6.7), 100 mM 2-ME, and 2% SDS.

Basophil purification

For most of these experiments, residual cells of normal donors undergoing leukapheresis were enriched in basophils using a combination of Percoll density gradients and counter-current flow elutriation, as previously described (25). The cells were further purified by negative selection using MACS basophil isolation kit (Miltenyi Biotec). More recently, we have used a mix of Abs for negative selection from StemCell Technologies (basophil purification kit) and columns from Miltenyi Biotec. The purity of basophils was determined by Alcian blue staining (26) and basophils purified from leukapheresis packs generally exceeded 99% purity.

² Abbreviations used in this paper: SH2, Src homology 2; HrHRF, human recombinant histamine-releasing factor; LILR, leukocyte Ig-like receptor; PAG, PIPES-albumin-glucose; HSA, human serum albumin; mIgG, mouse IgG.

Basophil cultures

Purified basophils were cultured at 2 million/ml in RPMI 1640 medium supplemented with 10 μ g/ml gentamicin and 300 μ g/ml HSA. In experiments in which anti-IgE Ab, fMLP, anti-LILRA-2/anti-mIgG, or HrHRF was used to down-regulate receptor expression, the medium included enough additional calcium to equal 1 mM and usually excluded IL-3. Cultures were performed in tilted polypropylene tubes so that after overnight incubation, the tubes were centrifuged at high-speed and 1 $\times$ hot electrophoresis sample buffer added to the tubes for lysis (this procedure mitigates against cell loss that might occur with further handling). To insure that equivalent cell loading occurred, results were normalized to the presence of the p85 subunit of PI3K, which provides a stable indicator of cell number during stimulation (6).

Stimulation with anti-LILRA-2

Based on a earlier protocol, basophils were stimulated with anti-LILRA-2 in a staged process that included IL-3. The cells were incubated in PAG with IL-3 (10 ng/ml) containing 1 μ g/ml anti-LILRA-2 Ab for 30 min at 4°C. After one wash with ice-cold PAG plus IL-3, the reaction was started when cells were resuspended in PAG-CM containing 10 ng/ml IL-3 and 10 μ g/ml anti-mIgG (F(ab')₂) Ab and incubated at 37°C. This protocol was only used in initial experiments with the Syk inhibitor NVP-QAB205. For all remaining experiments, basophils were incubated with 1 μ g/ml anti-LILRA-2 Ab for 2 min with cells prewarmed to 37°C, and the reaction was initiated with the addition of 10 μ g/ml anti-mIgG. No IL-3 was used.

HrHRF production and donor selection

HrHRF was generated as previously described (17) and was judged to be >95% by Coomassie blue staining of an SDS-PAGE gel. HrHRF protein was dialyzed into RPMI 1640 medium. Only a small percentage of the general population has circulating basophils able to respond to HrHRF at any given time. In general, the threshold for defining a basophil response as positive to stimulation with HrHRF is 10% histamine release to an optimal or maximum concentration. Our initial attempts to use leukocyte pack-derived cells were not productive but it was also discovered that purification and culturing reduced the response of otherwise positively responding basophils to levels that might be considered, using fresher cells, positive. The solution was to do the experiments with basophils isolated on a single-step Percoll gradient and analyze Syk expression by flow cytometry.

Flow cytometry for the determination of Syk expression

The use of the Santa Cruz Biotechnology anti-Syk Ab, 4D10, for Western blot analysis demonstrated that there were very few bands observed except for the p72 band associated with Syk. All other bands, together, represent $\leq$ 10% of the signal observed for Syk obtained from a lysate of human basophils. Therefore, this Ab appears to have sufficient selectivity in the basophil context to assess Syk expression by flow cytometry. Initial testing used purified basophils but the procedure for measuring Syk in impure basophils required identifying basophils with anti-IL-3R Ab. Basophils prepared by single-step Percoll gradients (0.5–2% purity) were resuspended in PBS buffer containing 0.04% BSA and 0.2 mg/ml nonspecific human IgG; $\sim$ 100,000 total cells per labeling condition. After incubation at 4°C for 5 min, anti-IL-3R Ab (PE-labeled mIgG1, anti-CD123; BD Pharmingen) at 2 μ g/ml was added and the cells incubated at 4°C for 15 min. The tubes were warmed in 37°C water for 2 min and transferred back to the bench top for a 20-min incubation with Medium A (Fix/Perm kit; Caltag Laboratories). Cells were washed once with PBS plus 5% FCS, resuspended in Medium B, and split and incubated with either control IgG2a Ab (Caltag Laboratories) at 2 μ g/ml or 4D10 (an IgG2a Ab) at 2 μ g/ml for 20 min. After one additional wash with PBS plus FCS, the cells were resuspended in PBS plus 4% BSA for an overnight labeling with anti-mIgG2a Alexa Fluor 647 Ab at 4°C. After washing, the cells were analyzed by flow cytometry for Alexa Fluor 647 positivity after gating on IL-3R positivity and a forward or side light scatter gate appropriate for basophils. Syk expression is quantified by calculating the net median fluorescence intensity of cells labeled with nonspecific IgG2a vs 4D10 Abs. In a population experiment, the net value is also expressed as a fraction of a standard (CaliBRITE APC beads; BD Biosciences) to control for day-to-day variation in the flow cytometer performance. As normalized, net median fluorescence, the low end of Syk expression, can be zero. For experiments comparing control vs stimulated loss of Syk, a standard is not necessary. Preliminary studies established that plasmacytoid dendritic cells, which express levels of IL-3R that are similar to basophils, can be distinguished from basophils by their forward or side scatter characteristics and by the fact that they express considerably higher levels of Syk expres-

sion. In these preliminary studies, the use of blood DC Ag-2 (CD303) as a marker of plasmacytoid dendritic cells verified their scatter characteristics and Syk expression differences from basophils so that they could be excluded from subsequent analysis without the use of blood DC Ag-2 (although routine use of blood DC Ag-2 labeling is a possible refinement of the method).

Statistics

In general, data shown are mean $\pm$ SEM, both in text and figures. In some instances, the experiments show mean $\pm$ SEM, although the analysis is done with paired statistics.

Results

Histamine release induced by anti-LILRA-2 is sensitive to a Syk inhibitor

There has been some controversy over the selectivity of early generations of Syk inhibitors (27) but there are now third generation inhibitors that show some of the expected characteristics for relatively selective inhibition of Syk. Previous studies of the Novartis inhibitor NVP-QAB205 show that it displays the expected characteristics of Syk inhibitor with an IC₅₀ for inhibition of early signaling steps that lies somewhere between 0.01 and 0.1 μ M (typically, $\sim$ 0.04 μ M) (21). Human basophils were stimulated with either anti-IgE Ab or anti-LILRA-2 Ab in the presence of NVP-QAB205 or equivalent concentrations of its DMSO carrier solvent. In this protocol, the basophils were stimulated with anti-LILRA-2 Ab following a previously established protocol that included a 30' preincubation with the primary anti-LILRA-2 Ab (19), centrifugation, and stimulation with a secondary anti-mIgG F(ab')₂ Ab, all in the presence of IL-3, to initiate strong aggregation of the LILRA-2 receptor and to augment mediator release (see *Materials and Methods*). Fig. 1A shows the concentration-dependence of the response on the presence of NVP-QAB205 for anti-LILRA-2 Ab. The IC₅₀ for this curve, $\sim$ 0.2 μ M, was somewhat higher ($\sim$ 4-fold) than expected for an IgE-mediated stimulus. In Fig. 1A, *inset*, three experiments are shown in which the concentration-dependence of the Syk inhibitor for both anti-IgE Ab and the stimulation pair of anti-LILRA-2/anti-mIgG is assessed. In these experiments, the protocol for stimulation with anti-LILRA-2 is as described for the signaling experiments below, i.e., no IL-3 present and anti-LILRA-2 added 2 min before the direct addition (without washing) of anti-mIgG. In these experiments, there remained a slight rightward shift for the potency with anti-LILRA-2 as the stimulus, although the difference was not statistically significant. Although this rightward shift in potency was very modest, we felt it useful to explore how the conditions used to stimulate basophils with anti-LILRA-2 Ab might have modified the relative potency. Experiments in Fig. 1, B and C, show two manipulations that might influence the potency of the drug, but using anti-IgE Ab or anti-receptor to stimulate the cells. Previous studies of this drug did not include IL-3, a cytokine that can dramatically enhance the response of basophils. Fig. 1B demonstrates that inclusion of IL-3 did not influence the potency of NVP-QAB205. The study of anti-LILRA-2 also required the use of a secondary Ab. To test whether a secondary Ab effects the potency of NVP-QAB205, basophils were stimulated with a mouse anti-Fc ϵ RI α Ab, $\pm$ the same anti-mIgG F(ab')₂ Ab used for the LILRA-2 experiments. The inclusion of the secondary Ab also shifted the IC₅₀ rightward, 3- to 3.5-fold. These experiments showed that the relative potency of the Syk inhibitor could be shifted somewhat by altering the conditions of stimulation.

Recent studies have established that the ability of a basophil to respond to anti-IgE Ab is tightly associated with the relative expression of Syk (6). If LILRA-2 receptor also used Syk to signal

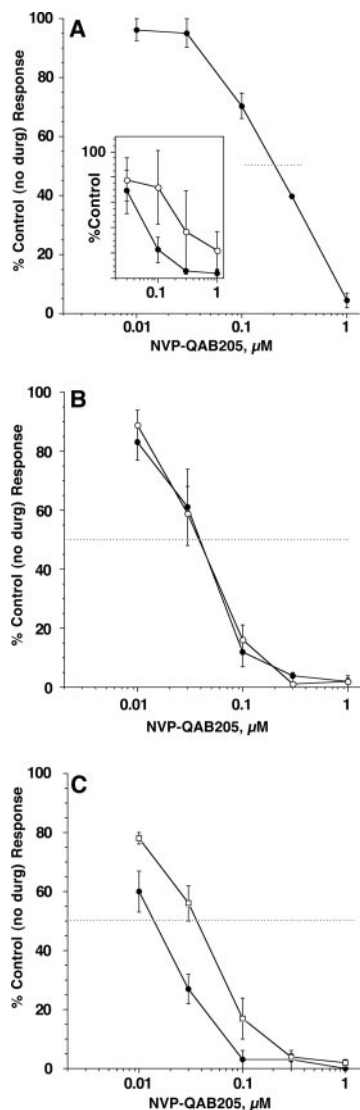


FIGURE 1. Inhibition of histamine release induced by anti-LILRA-2 Ab or anti-IgE Ab. **A**, Basophils were “loaded” with anti-LILRA-2 Ab (1 $\mu\text{g/ml}$) for 30 min at 4°C in the presence of IL-3 at 10 ng/ml. After centrifugation, the cells were resuspended in PAG-CM buffer plus IL-3 and bound anti-LILRA-2 was cross-linked with anti-mIgG (F(ab')_2) for 45 min in the presence or absence (vehicle control) of NVP-QAB205 (Syk inhibitor) at the concentrations shown ($n = 3$). Data are plotted as the fraction of the release in the presence of vehicle control (DMSO). Average histamine release to anti-LILRA-2 was $46 \pm 5\%$. A similar experiment is shown (*inset*) except that the cells were stimulated with either anti-IgE Ab (0.2 $\mu\text{g/ml}$) (●) or anti-LILRA-2 Ab (1 $\mu\text{g/ml}$) (○) ($n = 3$). In this alternate protocol, the cells were incubated at 37°C with anti-LILRA-2 in PAG-CM without IL-3 for 2 min followed by the addition (without washing) of anti-mIgG Ab (10 $\mu\text{g/ml}$). Average vehicle control histamine release to anti-IgE Ab was $48 \pm 4\%$ and to anti-LILRA-2 Ab, $10 \pm 5\%$. **B**, Basophils were first incubated with (●) or without (○) 10 ng/ml IL-3 for 10 min before addition of anti-IgE Ab at 0.2 $\mu\text{g/ml}$ for 45 min with NVP-QAB205 at the concentrations shown. Average vehicle control histamine release without prior IL-3 was $52 \pm 3\%$ and with prior IL-3, $86 \pm 4\%$. **C**, Basophils were stimulated with anti-Fc $\epsilon\text{RI}\alpha$ Ab (22E7) with (○) and without (●) anti-mIgG (F(ab')_2) added 2 min after the addition of 22E7 with the concentrations of NVP-QAB205 shown. Average vehicle control histamine release was $19 \pm 5\%$ without the anti-mIgG and $58 \pm 6\%$ with anti-mIgG.

secretion, then it might be expected that a reasonably good correlation would exist between IgE- and LILRA-2-mediated histamine release. Fig. 2 demonstrates this relationship was indeed very

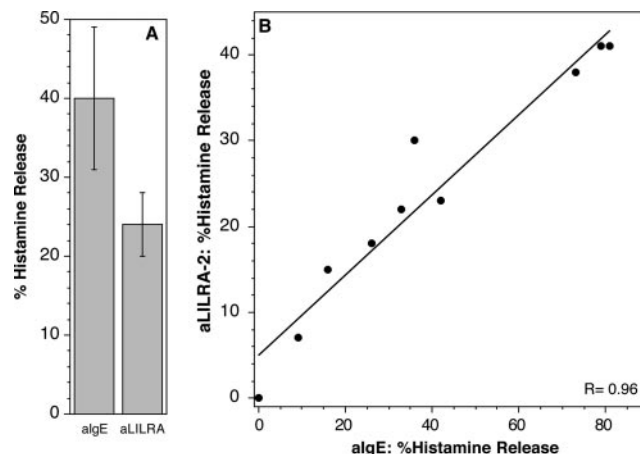


FIGURE 2. Relationship between magnitude of histamine release induced by anti-LILRA-2 (aLILRA) or anti-IgE Ab (aIgE). **A**, Average for subjects ($n = 11$). **B**, The correlation for the same subjects. For these experiments, the stimulation with anti-LILRA-2 Ab was performed by adding anti-LILRA-2 Ab (1 $\mu\text{g/ml}$) for 2 min followed by the addition of anti-mIgG (10 $\mu\text{g/ml}$). Anti-IgE was added at 0.2 $\mu\text{g/ml}$.

good. Note that the protocol for stimulating the cells used for the data points in the experiments shown in Fig. 2 followed the method used below for the signaling studies, notably there was no IL-3 present. These results suggest that LILRA-2 signaling is also dependent on the relative expression of Syk.

Signaling induced by anti-LILRA-2 Abs

Previous studies established a protocol for stimulating human basophils through the LILRA-2 receptor but this protocol was not very amenable to these initial studies of signaling through LILRA-2 because they required a long sequential incubation with anti-LILRA-2 Ab and a somewhat ambiguous start time (resuspension of centrifuged cells with warm buffer plus anti-mIgG). Therefore, based on previous Ag binding studies, the protocol was modified to omit IL-3 and to preincubate the cells with anti-LILRA-2 Ab for 2' followed by the addition (without washing) of anti-mIgG Ab. In some experiments, the signal element phosphorylation was examined without and with the secondary anti-mIgG Ab. The primary interest was to establish whether anti-LILRA-2 induced phosphorylation of Syk, but two potential Syk substrates were also examined for phosphorylation, Shc and c-Cbl. The phosphorylation of c-Cbl was of interest because of its role in mediating Syk ubiquitination and Syk loss. Basophils were stimulated and Syk isolated by immunoadsorption and under these conditions, Syk phosphorylation could be observed. The amount of Syk phosphorylation was weak, although in these experiments using L-pack basophils, the anti-LILRA-2-induced histamine was proportionally weak. Fig. 3A shows one of three of these experiments, with the response to anti-IgE Ab included for comparison. This experiment also shows that inclusion of NVP-QAB205 at 0.3 μM reduced the weak Syk phosphorylation following anti-LILRA-2 to resting levels ($4 \pm 3\%$ of the no drug response to anti-LILRA-2 ($n = 2$)) (Fig. 3A, compare lanes 2, 4, and 5). With the existing anti-phospho-Shc Abs, we could not demonstrate phosphorylation of Shc in whole cell lysates following anti-LILRA-2 (data not shown), but could observe phosphorylation of c-Cbl ($n = 3$) (Fig. 3B). Erk phosphorylation was $\sim 50\%$ of the anti-IgE signal ($n = 2$) (Fig. 3C). These experiments demonstrated that anti-LILRA-2 induced some of the same early signaling steps observed for IgE-mediated secretion, notably phosphorylation of Syk and c-Cbl.

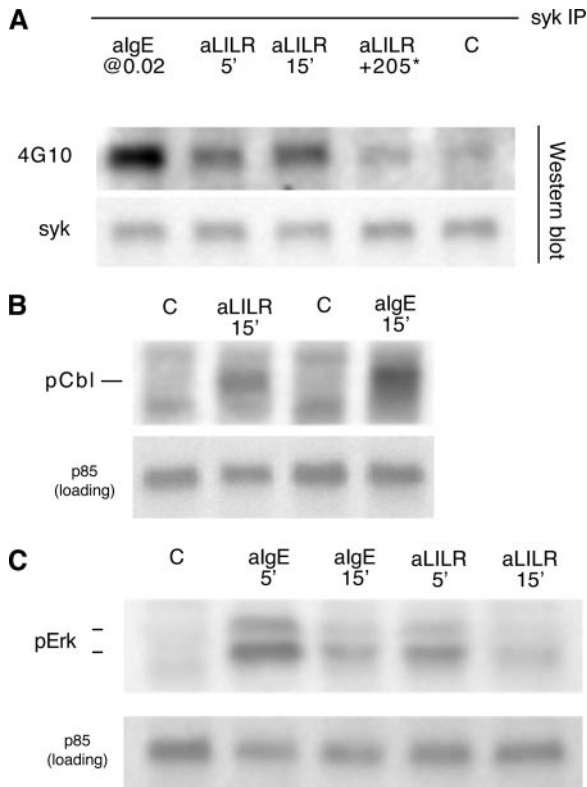


FIGURE 3. Signal element phosphorylation induced by anti-LILRA-2 (labeled aLILR) vs anti-IgE Ab (labeled algE). Anti-LILRA-2 Ab (1 $\mu\text{g}/\text{ml}$) was added for 2 min followed by the addition of anti-mIgG (10 $\mu\text{g}/\text{ml}$). The addition of anti-mIgG marked time zero. **A**, Syk phosphorylation was measured by immunoadsorption and blotting with anti-phosphotyrosine 4G10. The blots were stripped and reblotted with anti-Syk. **B**, Whole cell lysates were Western blotted with anti-phospho-c-Cbl (p-Cbl) and the blots were then further blotted for p85 levels to act as the lane loading control. **C**, Whole cell lysates were Western blotted with anti-phospho-Erk1/2 and further blotted with p85 to act as the lane loading control.

Loss of Syk expression following LILRA-2 stimulation

Having established that stimulation through LILRA-2 could induce Syk and c-Cbl phosphorylation, the basophils were also ex-

amined for down-regulation of Syk expression following an overnight incubation with the stimulus. The protocol for these experiments followed those used in previous studies, cells were stimulated in RPMI 1640 culture medium, supplemented to 1 mM Ca^{2+} but containing no IL-3. Whole cell lysates were obtained and to control for lane loading, Western blots included a test for the p85 regulatory subunit of PI3K, which we have previously shown not to change during overnight stimulation (6). Fig. 4 summarizes these results. Syk expression following anti-LILRA-2 was statistically different from the control expression level, although the change was modest. However, there was a good correlation ($R = 0.63$, $p = 0.013$) between the down-regulation of Syk expression induced by anti-IgE Ab and the loss following anti-LILRA-2. These results revealed an aspect of the Syk loss phenomenon not previously appreciated. Fig. 4C shows our accumulated experience (all experiments during the last several years that have used anti-IgE Ab for overnight stimulation) for loss of Syk when induced by stimulation with an optimal concentration of anti-IgE Ab. It is notable that there is a primary peak in the frequency distribution that lies between 80 and 90% reduction, but the distribution is otherwise broad and there are individuals who lose very little Syk when stimulated overnight. There is no correlation between Syk loss and the maximum histamine release induced by an optimal concentration of anti-IgE Ab nor is there a correlation between the initial level of Syk and its subsequent loss (data not shown). The mechanism underlying Syk loss is not completely understood and neither are the factors that modulate its loss. However, if two stimuli share similar signaling mechanisms, as do Fc ϵ RI and LILRA-2, then whatever the mechanism of loss, one might expect similar influences to operate on Syk loss due to aggregation of Fc ϵ RI or LILRA-2. Therefore, the correlation between loss of Syk following anti-IgE Ab vs anti-LILRA-2 Ab might be expected. Fig. 4C also shows the cumulative experience for loss of Syk when stimulating with anti-LILRA-2 Ab (data represent results with anti-IgE Ab).

The use of an intact mouse anti-LILRA-2 Ab raises the possibility of the Ab co-cross-linking Fc γ RIIb and Fc ϵ RI. We have previously shown that mIgG anti-IgE Abs probably engage both receptors because inclusion of a high concentration of human IgG (25 μM) enhances the response to these Abs (this experimental

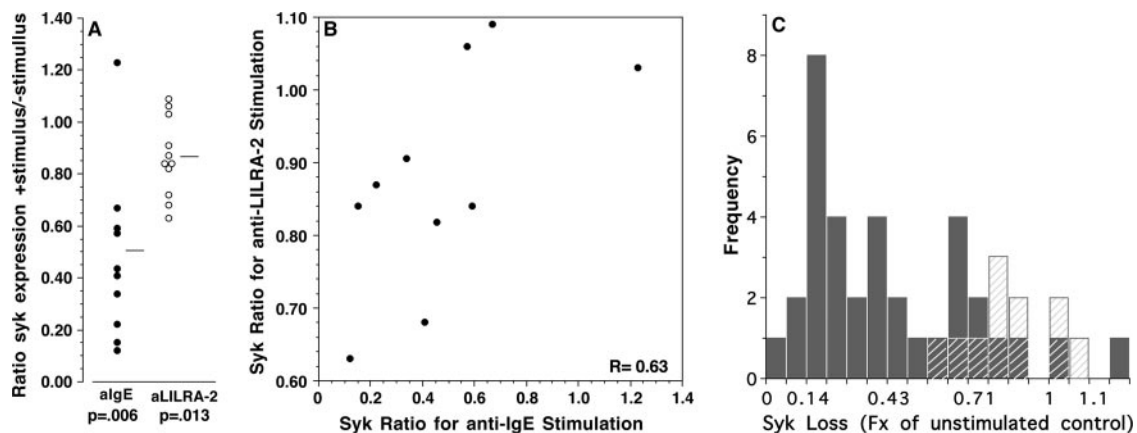


FIGURE 4. Loss of Syk kinase following overnight incubation with either anti-IgE or anti-LILRA-2 Ab. Basophils were stimulated for 18 h with optimal anti-IgE Ab (0.2 $\mu\text{g}/\text{ml}$) or anti-LILRA-2 Ab (1 $\mu\text{g}/\text{ml}$) in medium. Two minutes after the addition of anti-LILRA-2 Ab, anti-mIgG Ab (10 $\mu\text{g}/\text{ml}$) was added. **A**, distribution of loss induced by anti-IgE Ab (aIgE) or anti-LILRA-2 (aLILRA-2). Syk expression in whole cell lysates for cells incubated with no stimulus was compared with those incubated with stimulus to calculate the ratio of loss. **B**, Correlation between loss induced by anti-IgE Ab vs anti-LILRA-2 Ab. **C**, Frequency distribution of Syk loss induced by anti-IgE Ab (dark gray) or anti-LILRA-2 Ab (light gray) in the general population of basophil donors. Each distribution represents our cumulative experience for the Syk loss obtained from a variety of experiments that included incubation with an optimal concentration of anti-IgE Ab (dark gray). The anti-LILRA-2 experiments from **A** are overlaid for comparative purposes. Incubation time was 18 h with optimal concentrations of stimulus.

maneuver does not alter the response to goat polyclonal anti-IgE Ab) (28) presumably by blocking interaction with Fc γ RIIb. At this time, a direct test of the possibility that anti-LILRA-2 was susceptible to such an effect was not possible. However, it was possible to use the experimental maneuver noted to test whether Fc ϵ RI-mediated down-regulation of Syk was influenced by Fc γ RIIb co-cross-linking. We have tested the effects of high concentrations of human nonspecific IgG on the ability of 22E7 to induce loss of Syk and not found an effect. With the addition of 25 μ M HSA or 25 μ M human IgG in the culture with 22E7, Syk expression was 0.43 ± 0.15 and 0.40 ± 0.13 of nonstimulated control, respectively, or a ratio difference of -0.03 ± 0.07 ($n = 3$; p was not significant). However, we have noted that use of anti-mIgG F(ab')₂ to further cross-link 22E7 reduces the loss of Syk; for stimulation with 22E7, with and without anti-mIgG F(ab')₂, Syk expression was 0.81 ± 0.09 vs 0.38 ± 0.15 of nonstimulated control, respectively ($n = 4$; $p = 0.022$). This result is surprising, but suggests that the reduced ability of anti-LILRA-2 to stimulate Syk loss may be partially related to a blunting effect of the secondary anti-mIgG that is necessary for these studies of LILRA-2.

HrHRF-induced loss of Syk

In a companion study, it has been shown that HrHRF induces Syk phosphorylation (29). Therefore, we examined the ability of HrHRF to induce loss of Syk in overnight cultures. These experiments were complicated by the fact that the responsiveness to HrHRF, even in subjects whose basophils were known to respond well to this stimulus, is very sensitive to handling. The long periods of purification progressively reduce the response to HrHRF, making this stimulus very weak by the time purified cells could be tested for overnight down-regulation of Syk. Furthermore, only HrHRF-sensitive donors could be examined, generally excluding the use of leukocyte packs. A new method of measuring Syk expression was determined to work well on impure cells as part of a pilot study (Fig. 5). In an initial test of two purified preparations using Western blotting to detect Syk, there was an indication of some Syk loss following HrHRF (Fig. 6A), but the histamine release response, due to the longer preparation time, was quite poor. We therefore used the new method of measuring Syk expression on impure cells. Some background on this flow cytometric method is needed. First, of several anti-Syk Abs explored, the 4D10 Ab used in this method was determined to be optimal. (It is worth noting that we had some difficulty using a commercially available FITC-labeled 4D10 in these experiments and therefore used unlabeled 4D10. See *Materials and Methods*.) To provide confidence that the flow cytometric method was replicating the Western blot data, purified basophils from different donors were examined by both Western blotting and flow cytometry for the expression of Syk. A good correlation was found between the two methodologies ($R = 0.74$, $n = 9$, $p = 0.023$). Fig. 5A shows three examples of this pilot study. In a separate survey, down-regulation with overnight anti-IgE Ab stimulation was compared using Western blotting and flow cytometry. This methodology, too, showed a good correlation ($R = 0.78$, $n = 7$, $p = 0.034$). Fig. 5B shows two examples of these experiments. We then further developed the flow cytometric method to be useful with impure basophil preparations (see *Materials and Methods*), Fig. 5C shows one example of the comparison. In a more recent survey, to be published as part of another study, Syk expression for 15 donors was determined by flow cytometry and when compared with these donors' histamine-mediated histamine release, the correlation was excellent and consistent with Syk expression determined by the Western blot method (6).

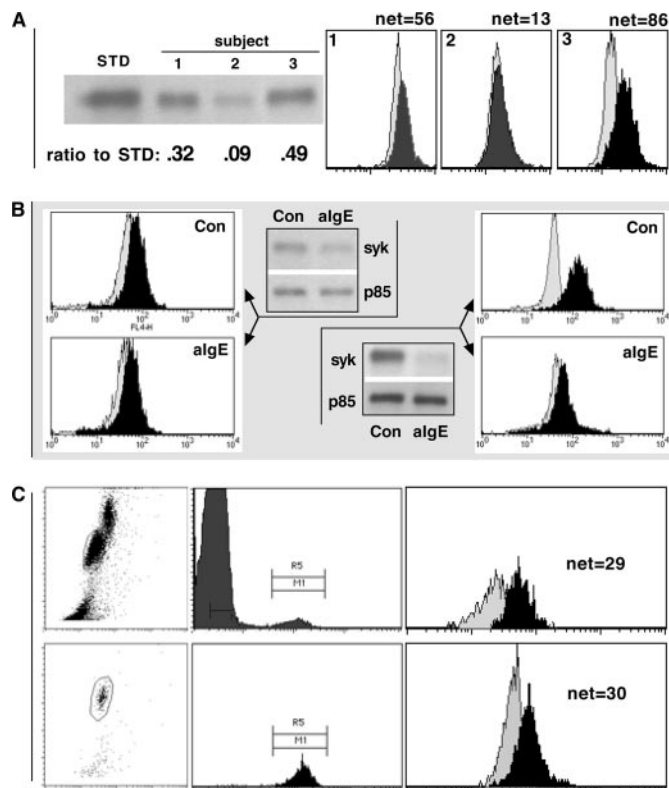


FIGURE 5. Flow cytometric method of measuring Syk expression in basophils. **A**, Three examples of a comparison of Syk measurement by Western blotting vs flow cytometry. The Western blot includes a standard (Std) against which the Syk levels obtained from 300,000 purified basophils were compared (the level of expression relative to this standard is noted underneath the Western blot). From the same basophil preparations, flow cytometry was also performed. The net values (4D10 - IgG2a control) are shown. In the flow cytogram for panels 1, 2, and 3, the IgG2a labeling (gray filled histogram) and the 4D10 labeling (filled histogram) are represented. **B**, Two examples of measuring the down-regulation of Syk expression induced by overnight culture of purified basophils without (Con) or with anti-IgE Ab (algE). After culture basophils were analyzed by lysis and Western blotting or by flow cytometry. The Western blot also shows expression of p85, which has been previously shown not to change during stimulation, to demonstrate equal loading of the gel. For the donor on the left side of the panel, Western blotting showed a Syk decrease of 25% and flow cytometry showed a decrease of 24%. For the donor on the right side, Western blotting showed a decrease of 77% and flow cytometry showed a decrease of 78%. **C**, One example of a comparison of Syk expression measured in an impure preparation of basophils (obtained by a single-step Percoll gradient) vs basophils purified from the same preparation.

Using the flow cytometric method of detecting Syk expression, basophils sensitive to HrHRF were examined for down-regulation induced by both HrHRF and anti-IgE Ab (for comparative purposes). Fig. 6 shows that HrHRF did induce loss of Syk in these overnight cultures (Fig. 6B). Using basophils that were unresponsive to HrHRF, no loss of Syk was observed in the HrHRF-challenged condition, whereas there was loss in response to anti-IgE Ab.

Syk down-regulation following fMLP

Our second hypothesis that c-Cbl phosphorylation, in the absence of measurable Syk participation, might nevertheless lead to some loss of Syk over a long time interval (by an association that would

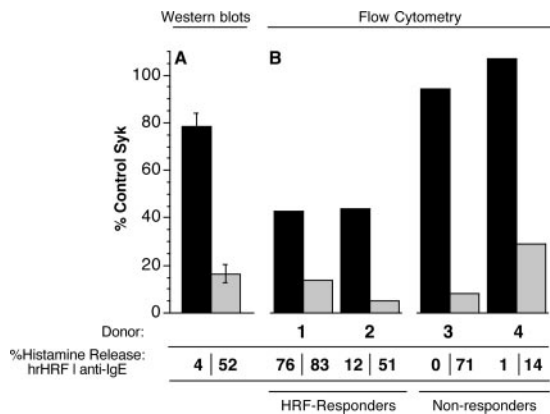


FIGURE 6. Loss of Syk induced by either HrHRF or anti-IgE Ab. *A*, Overnight incubation with medium in the absence or presence of HrHRF (1 μ M) (■) or anti-IgE Ab (0.2 μ g/ml) (□) followed by sampling for histamine release and lysis of the cell pellets for Western blots of Syk expression. Data below graph show histamine from purified basophils of these two donors and represent the relative loss of Syk (compared with medium alone controls). *B*, Similar design as in *A*, except that basophils were impure (single-step Percoll preparations) analyzed by flow cytometry, HrHRF (5 μ M) (■) or anti-IgE Ab (0.2 μ g/ml) (□). Data below graph show the amount of histamine release to either HrHRF or anti-IgE Ab and represent the amount of remaining Syk expression (relative to unstimulated controls) for the same four donors.

otherwise be difficult to detect) was tested using fMLP as a stimulus. First, previous studies have shown that fMLP does not induce measurable Syk phosphorylation (27) and a partial causal test was presented in a study in which basophils were transfected with the tandem SH2 domains of Syk (linked to GFP). This construct effectively inhibited the IgE-mediated cytosolic calcium response but not the fMLP-induced response (5). But the tandem SH2 construct acts by competing with endogenous Syk for its ITAM binding site and this may not adequately test for its participation in an fMLP-induced response. Therefore, we also tested the ability of NVP-QAB205 to inhibit fMLP-induced histamine release and the fMLP-induced cytosolic calcium response and found no inhibition of either endpoint (data not shown). Fig. 7 shows that stimulation with 100 nM fMLP induced a modest loss (13 $\pm$ 4%) of Syk expression that was statistically significant, although widely distributed (including examples of no loss) (n = 18; p = 0.006). The average loss was similar to that found for anti-LILRA-2. However, in this instance, there was no correlation between the ability of the cell down-regulate Syk expression following stimulation with anti-IgE vs the loss following fMLP (R = 0.42, p = 0.12). Two other seven-transmembrane receptors that induce strong histamine responses in basophils, C5a and SDF-1a, were examined. However, in a short series of three experiments each, no changes in Syk expression occurred. In two experiments, it was found that inclusion of fMLP would enhance the ability of anti-IgE Ab to induce loss of Syk. Although there was increased loss of Syk when both anti-IgE and fMLP were present (relative to either stimulus alone), the effect was not quite additive. At optimal and suboptimal anti-IgE Ab, there was 45% and 20% loss of Syk, respectively, whereas inclusion of 100 nM fMLP resulted in 57% and 43% loss of Syk. fMLP alone resulted in 31% loss of Syk.

An interesting characteristic of c-Cbl phosphorylation following IgE-mediated stimulation is its persistence but previous studies have not examined the kinetics of c-Cbl phosphorylation following fMLP. With the historical background that most signaling elements that are activated/phosphorylated following fMLP stimulation have been transient on a time scale of seconds to minutes,

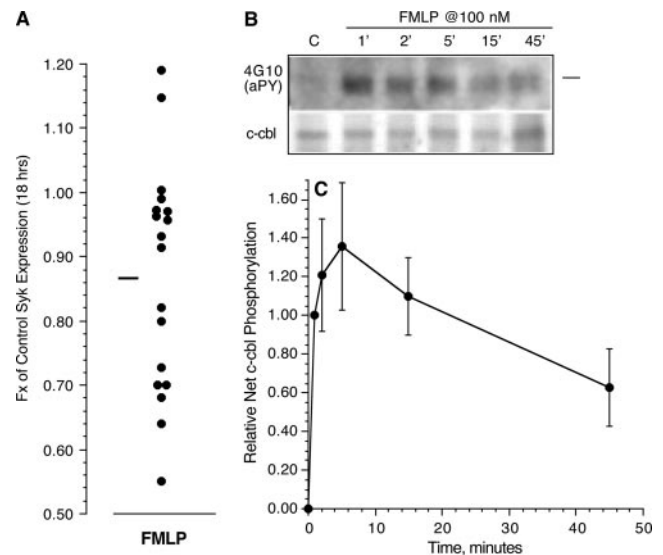


FIGURE 7. Stimulation with fMLP in Syk loss and Cbl phosphorylation. *A*, Loss of Syk induced by 18-h incubation with 100 nM fMLP cultured as described for anti-IgE Ab. Each point represents a different purified basophil preparation and the horizontal bar shows average for the group (n = 18). p = 0.006, tested against a population mean of 1.0. *B* and *C*, c-Cbl phosphorylation in basophils stimulated with 100 nM fMLP. *B*, One experiment in which the c-Cbl was immunoadsorbed, and Western blots developed with anti-phosphotyrosine, 4G10. *C*, Average kinetics of phospho-c-Cbl, band intensity normalized to the 1-min time point (n = 4).

c-Cbl phosphorylation following fMLP is surprisingly long-lived, showing only modest abatement at 45 min (Fig. 7, *B* and *C*).

It was also noted that the migration of phosphorylated c-Cbl in these SDS-PAGE gels is somewhat faster than the migration of phosphorylated c-Cbl following anti-IgE Ab, which migrates with the bulk of c-Cbl detected with c-Cbl Abs. In Fig. 7*B*, there is a horizontal marker on the right side of the panel that indicates the centerpoint of the band location for both IgE-mediated phosphorylated c-Cbl and the position found for c-Cbl when blotted with anti-c-Cbl Ab (this marks an apparent molecular mass of ~113 kDa). Following fMLP, the phosphorylated band is broader than found for phospho-Cbl following anti-IgE or broader than the band detected with anti-c-Cbl Ab, with a midpoint of the band profile occurring at an apparent molecular mass of 108–109 kDa. In Fig. 8*A*, a digitized profile of both the band that appears following fMLP and anti-IgE is shown. As time progresses, the centroid of the band profile (following fMLP) drifts toward the 113–114 kDa point. This behavior is unexpected suggesting that Cbl-b might contribute to this observation. However, although there is evidence of Cbl-b phosphorylation (30), only the Y709 of Cbl-b is similar to Y700 in c-Cbl and its flanking sequence may be not sufficiently similar to bind Abs that bind to Y700 of c-Cbl. In Cbl-b there is no equivalent of the Y774 or Y731 sites being detected in our Western blots (in addition to use of 4G10, (Fig. 8*B*)). In addition, the Ab used for immunoadsorption is considered selective for c-Cbl with no cross-reactivity with Cbl-b. We have not been able to resolve whether basophils contain Cbl-b because the commercial Abs selective for Cbl-b generate a number of possible bands that do not fall in the reported m.w. range for Cbl-b identified by others. One commercial Ab detects no bands, others detect two bands at ~114 and 124 kDa (data not shown). A test of these latter Abs using breast carcinoma cells shows a band at 109 kDa, the expected molecular mass and reported location of Cbl-b in this cell type

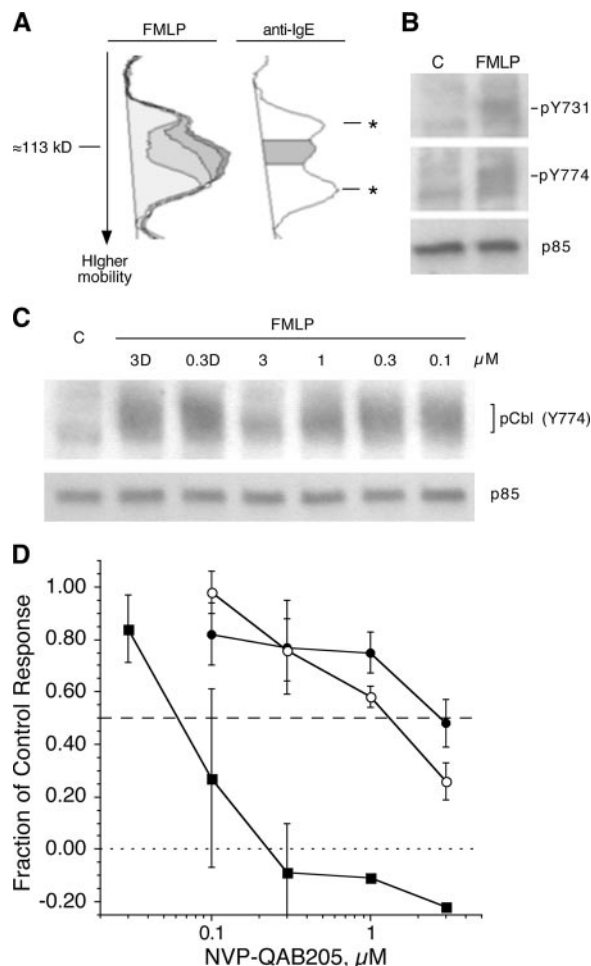


FIGURE 8. Cbl phosphorylation following stimulation with fMLP. *A*, Cells were stimulated with either fMLP (0.1 μM) or anti-IgE Ab (0.2 $\mu\text{g}/\text{ml}$), and the Western blots developed with anti-phospho-Cbl (Y774) Ab from whole cell lysates. In whole cell lysates, this Ab generates two non-specific bands (asterisks). Between these two bands, lies phosphorylated c-Cbl at a nominal 113-kDa mobility. The band that appears following stimulation with fMLP is broader as seen the digital profile. The profile shown on the *left* is a composite of four lanes: the control lane (lightest gray), 1, 5, and 15 min time points (darker gray) poststimulation with fMLP. *B*, Example Western blots using either anti-pY731 or anti-pY774 Abs to detect phospho-c-Cbl, with p85 levels used to demonstrate equal lane loading. *C* and *D*, Basophils were stimulated with 0.1 μM fMLP in the presence of either DMSO vehicle (designated as 3D or 0.3D, indicating the DMSO concentration equivalent to the amount of DMSO drug conditions) or the presence of NVP-QAB205 at 3, 1, 0.3, or 0.1 μM . Cells were incubated for 10 min with drug before stimulation and harvested 5 min after the addition of fMLP. No stimulation for 5 min (in the presence of vehicle at 3 μM equivalents of DMSO) is shown (*lane C*). *D*, Summation of results for three experiments (● and ○) and includes three experiments in which anti-IgE Ab was the stimulus (■). The inhibition of the slower mobility region of the broader band induced by fMLP was more effective than the higher mobility region, the results for the entire band (●) and for the slower mobility region (○) are represented.

(data not shown). At this time, the presence of Cbl-b or its phosphorylation in basophils cannot be definitively determined.

Although previous studies have not noted any phosphorylation of Syk during activation with fMLP, the Syk inhibitor, NVP-QAB205 was tested for its ability to inhibit fMLP-induced Cbl phosphorylation. Surprisingly, there was some inhibition and it was partially selective for the less mobile region of the nominal p110 band, i.e., the region associated with typical Cbl mobility

(~113 kDa). However, on the whole, the inhibition by NVP-QAB205 had an IC_{50} that was not consistent with expected IC_{50} for inhibition of Syk, instead shifted rightward by ~50-fold (Fig. 8, *C* and *D*). For comparison, the IC_{50} for anti-IgE-induced Cbl phosphorylation is shown and this IC_{50} is the same as found for many other downstream targets of Syk activation (21).

Discussion

These studies establish that it is possible to down-regulate Syk expression in human basophils through a non-IgE-dependent receptor. Stimulation through the LILRA-2 is shown to induce Syk and c-Cbl phosphorylation and degranulation induced by anti-LILRA-2 Ab is sensitive to a third generation Syk selective inhibitor, NVP-QAB205. There are important caveats to these observations.

First, NVP-QAB205 was not quite as potent as would be expected for inhibition of a weak stimulus. Studies with HrHRF demonstrated the expected leftward shift of NVP-QAB205 potency when comparing HrHRF to anti-IgE Ab (29). However, we did find that one of the conditions needed to demonstrate LILRA-2 responses, the inclusion of a secondary cross-linking reagent anti-mIgG (F(ab')_2), did induce a rightward shift in NVP-QAB205 potency even for stimulation through Fc ϵ RI. Whether this approach to stimulation fully explains the modest rightward shift in NVP-QAB205 when stimulating with anti-LILRA-2 remains to be seen. Mitigating this concern is the remarkable correlation between a basophil response to anti-IgE Ab and anti-LILRA-2. Because there is strong evidence that the IgE-mediated response is dependent on Syk expression levels (6), this correlation suggests that LILRA-2 responses are also dependent on Syk expression levels.

A second caveat is that although Syk phosphorylation was observed, we did not observe phosphorylation of Shc. This adaptor molecule is thought to be a direct substrate of Syk (31) and its phosphorylation following stimulation with Ags or anti-IgE Ab in human basophils is readily observed (21). Even relatively weak stimulation is generally observable, although the anti-phospho-Shc Ab we use detects some nearby bands in the Western blots of basophils that can confound a sensitive detection of phospho-Shc. It is possible that the anti-LILRA-2 mouse Ab, by potentially co-cross-linking LILRA-2 and Fc γ RIIb might alter the signaling induced by LILRA-2 aggregation. This possibility will require a different set of reagents to test. In contrast to the apparent absence of Shc phosphorylation, anti-LILRA-2 did induce c-Cbl phosphorylation. As shown in Fig. 8, the potency of NVP-QAB205 for reducing c-Cbl phosphorylation following stimulation with anti-IgE Ab is consistent with Syk inhibition and is coincident with other signaling endpoints and inhibition of histamine release. Similar concentrations of NVP-QAB205 also inhibit anti-LILRA-2 induced c-Cbl phosphorylation. Therefore, these results suggest that c-Cbl associates with Syk following stimulation with anti-LILRA-2/anti-mIgG.

On this basis, the loss of Syk following stimulation with anti-LILRA-2 would have been predicted. However, the loss was not marked. The data did reveal the importance of the intrinsic ability of a donor's basophils to down-regulate Syk expression. It is now apparent that marked loss of Syk expression following anti-IgE is not a constant feature of the response. The basis for this heterogeneity is currently unknown but it apparently led to the correlation between loss of Syk induced by anti-IgE Ab and anti-LILRA-2 Ab. Loss of Syk expression mediated by aggregation of Fc ϵ RI is not dependent on Syk activity (6). Previous studies have also noted that the signal induced by Ag or anti-IgE Ab need not be very strong to result in loss of Syk expression. Concentrations of Ag do not need to be high enough to induce degranulation,

suggesting that there is integration of a weak signal over a relatively long period of time. Although the relative weakness of anti-LILRA-2/anti-mIgG to induce loss is not well explained yet, it may be partially related to the method used to cross-link LILRA-2; notably the use of an anti-mouse F(ab')₂ Ab. It is possible that the natural ligand for LILRA-2 or in vivo conditions may produce a different relationship between activation through LILRA-2 and loss of Syk. Whether the amount of loss induced by aggregating LILRA-2 is sufficient to modify the responsiveness of basophils to IgE-mediated stimulation remains to be determined and is the subject of a study in progress. However, we can note that the level of Syk expression in basophils appears to be tuned to the range of histamine release found in the population (thus, the excellent correlation). Therefore, any change in Syk levels is sufficient to proportionally modify downstream signaling and function. For example, as part of a future manuscript, we have noted that Syk down-regulation induced by one Ag results in a second, non-cross-reacting, Ag inducing proportionally reduced signaling of several signaling intermediates downstream of Syk.

As mentioned, the broad distribution in Syk down-regulation is a new observation that currently has no explanation. However, a recent study of Syk regulation in B cells suggests the unexpected conclusion that the transcription factor (OCA-B/Bob1) can protect Syk from ubiquitination. This result suggests a mechanism by which the variability in Syk loss is explained by a heterogeneity in the presence of OCA-B/Bob1 (32). Despite being a generally weaker stimulus, HrHRF generated a more significant loss of Syk than anti-LILRA-2. However, this may be a result of the different phenotype of the basophils needed to observe an HrHRF response. In a previous study, we examined the ability of cycloheximide to alter the amount of Syk loss. The underlying hypothesis was that if Syk synthesis was faster in some subjects' basophils, then the competition between loss and synthesis might generate diversity in the loss of Syk. However, in these previous studies, the inclusion of cycloheximide did not alter the loss of Syk (13). Based on these results, we would tentatively conclude that the heterogeneity in loss we have subsequently observed is not a consequence of different rates of Syk synthesis. Further study of Syk synthetic rates would be needed to draw a stronger conclusion about this possibility.

In addition to the loss induced by two stimuli that have been demonstrated to induce Syk phosphorylation (and c-Cbl in the case of anti-LILRA-2), stimulation with fMLP induces modest loss of Syk in ~50% of the preparations tested. Previous studies have noted that Syk phosphorylation does not occur following stimulation with fMLP (27) and we would not have examined fMLP except for a notation in a previous study that this ligand induces c-Cbl phosphorylation (13). The current studies show that despite the normally transient signaling observed following stimulation with fMLP, c-Cbl phosphorylation is long-lived. However, there is an aspect to Cbl phosphorylation that is not typical of IgE-mediated stimulation; the Western blot demonstrates an unusual broadening. The centroid of the band is centered at a nominal 109 kDa and progressively shifts closer to the expected 113 kDa over time. Because this band pattern occurs with immunoadsorption of c-Cbl with c-Cbl-specific Abs and is also apparent when the blots are probed with anti-c-Cbl phosphotyrosine Abs, Y774 and Y731, it appears that the entirety of the band represents some form of c-Cbl rather than Cbl-b, which is expected to have a band at ~109 kDa. In addition, we have had difficulty demonstrating any Cbl-b band at this location. A further subtlety is that stimulation of basophils in the presence of a Syk inhibitor, shows greater inhibition of one half of the band. The IC₅₀ for inhibition of either half (by NVPQAB205) suggests that fMLP-induced c-Cbl phosphoryla-

tion is not the result of Syk activity and suggests that another kinase is involved in this process.

The mechanism by which c-Cbl phosphorylation leads to colateral loss of Syk, presumably not in the reaction complex, is unclear. A synthesis of available observations regarding how E3 ligases of the Cbl family induce loss of Syk suggests that the tyrosine kinase binding domain of Cbl binds to a phosphotyrosine on Syk (Y323 in humans and Y317 in mice) (33–35). Although c-Cbl appears to be a target of Syk activity in the IgE-mediated reaction, the fact that Syk activity is not necessary to observe loss of Syk (6) suggests that Cbl phosphorylation is not a prerequisite for binding of c-Cbl binding to Syk. Taken together, the fMLP-induced loss of Syk therefore suggests that there may be a low level of Syk phosphorylation that permits c-Cbl binding and ubiquitination of Syk. Whether fMLP induces Syk phosphorylation that is difficult to detect by conventional immunoprecipitation methods (and the high IC₅₀ for inhibition of c-Cbl phosphorylation by the Syk inhibitor, NVP-QAB205 argues against this possibility) or whether there is a low level of undetected Syk phosphorylation that exists before stimulation with fMLP remains to be seen. The relatively long kinetics for this process and the slow integration of small amounts of loss might explain the modest down-regulation of Syk. For this to occur, one would hypothesize that activated Cbl has a certain mobility outside the fMLP-induced reaction complex. However, without agents that can test the causal linkage between fMLP-induced Cbl phosphorylation and the loss of Syk, we cannot yet know whether these two observations are linked.

In summary, these studies demonstrate that it is possible to modulate Syk expression by activation of receptors that are not dependent on aggregation of FcεRI. We could speculate that any receptor that uses Syk in a reaction complex could also induce loss of Syk and even receptors that do not directly use Syk may induce modest loss as well. Whether activation of non-FcεRI receptors explains the variable expression of Syk in human basophils in the general population remains to be determined.

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Disclosures

The authors have no financial conflict of interest.

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