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Localizing a Control Region in the Pathway to Leukotriene C_4 Secretion Following Stimulation of Human Basophils with Anti-IgE Antibody[1]

Katsushi Miura, Sandra Lavens-Phillips, and Donald W. MacGlashan, Jr.[2]

Mediator release from human basophils is a self-limited process, but down-regulation of the signaling cascades leading to secretion of leukotriene C_4 (LTC_4) is controlled independently of the pathway leading to IL-4 secretion. In the current studies, we have explored the regulation of upstream signaling events leading to activation of extracellular signal-related kinases (ERKs; previously shown to be required for LTC_4 generation) in human basophils. IgE-, but not FMLP-mediated activation, induced sustained tyrosine phosphorylation of *syk*, of *shc*, and an association of *shc* to the Grb2/son of sevenless 2 complex. In contrast, IgE-mediated activation resulted in transient activation of $p21^{ras}$ and mitogen-activated protein/ERK kinase 1, which were kinetically associated with phosphorylation of ERKs. The canonical Shc/Grb2/son of sevenless pathway to activation of $p21^{ras}$ is therefore sustained, while $p21^{ras}$ activity is not. We have previously shown that phosphatidylinositol 3 kinase activity is required for $p21^{ras}$ activity and, in the current studies, we show that of the p85-sensitive forms of p110 possible, basophils express only p110 δ and that there are no changes in association between $p21^{ras}$ and p110 δ in stimulated basophils. We used the generation of phospho-Akt as a marker of the presence of phosphatidylinositol-3,4,5-trisphosphate and found that phospho-Akt is transient on a time scale consistent with $p21^{ras}$ activity. On the basis of information obtained in these and other studies, we localize down-regulation of IgE-mediated LTC_4 secretion to a region of the signaling cascade antecedent to $p21^{ras}$ activation, downstream of phosphatidylinositol 3 kinase activity and probably involving regulation of phosphatidylinositol-3,4,5-trisphosphate levels. ***The Journal of Immunology*****, 2001, 167: 7027–7037.**

Immunoglobulin E-mediated activation of basophils and mast cells results in the secretion of at least three classes of mediators. Historically, histamine release has been the most commonly studied mediator, and it represents the class of preformed mediators associated with the granules of basophils and mast cells. A second class of mediators includes the fatty acid metabolites, generally derived from free arachidonic acid, that are generated after stimulation. The most recent class includes cytokines and possibly chemokines, which are newly synthesized proteins. When considering human basophils, one distinction between the first two classes of mediators, represented by histamine and leukotriene C_4 (LTC_4),[3] and the third class, represented by IL-4, is the very different time frame for their secretion. In human basophils, histamine and LTC_4 secretion occur in a very similar time frame, with only a slight difference in the rate that they initially appear in the extracellular medium. The secretion of these two mediators is essentially complete by 15 min, when the cells are stimulated with an optimal concentration of anti-IgE Ab. In contrast, IL-4 secretion requires 2–6 h for completion, often not being detectable in the extracellular medium during the first 30–45 min of the reaction, at a time when histamine and LTC_4 release are complete (the caveat to this statement is that there are preparations of basophils where there appears to be a preformed store of IL-4 that represents a small fraction of the final amount of synthesized IL-4, which is released early in secretion). Looking at these kinetic curves from the standpoint of the rate of release, the maximum rate of release for histamine and LTC_4 occurs at 5–8 min, while the maximum rate for newly synthesized IL-4 secretion occurs at 1–2 h, i.e., a 10-fold difference. This distinction is important because it is evident that although histamine/LTC_4 release is complete with respect to the IgE-mediated stimulus, this plateau of release for these two mediators is not all the cell is capable of secreting. It has been shown that other non-IgE-dependent stimuli often initiate significantly greater release of histamine or LTC_4 than found for optimal IgE-mediated stimulation from the same cells (1–7). Therefore, the plateau observed following anti-IgE Ab is a limit imposed by the nature of this stimulus.

This is not likely to be a subpopulation issue. From single cell studies, we know that the cytosolic calcium response that accompanies activation with any secretagogue does not occur in subpopulations of basophils (8, 9). If cells are stimulated sequentially with anti-IgE Ab and FMLP, FMLP induces further elevations in cytosolic calcium in the same cells in which an IgE-mediated elevation is observed. In other words, taken together, it appears that for the IgE-mediated signal, the basophil has down-regulated its response to this stimulus, but only with respect to histamine/LTC_4 release. Since IL-4 secretion occurs much later and requires the persistence of cell surface aggregates (or newly forming aggregates) (10), the down-regulation is focused on secretory pathways leading to either histamine or LTC_4 secretion. In this context, it is

Johns Hopkins Asthma and Allergy Center, Baltimore, MD 21224

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[2] Address correspondence and reprint requests to Dr. Donald W. MacGlashan, Jr., Johns Hopkins Asthma and Allergy Center, 5501 Hopkins Bayview Circle, Baltimore, MD 21224. E-mail address: dmacglas@jhmi.edu

[3] Abbreviations used in this paper: LTC_4, leukotriene C_4; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CLB, complete lysis buffer; $cPLA_2$, cytosolic phospholipase A_2; ERK, extracellular signal-regulated kinase; ESB, electrophoresis sample buffer; GAP, GTPase-activating protein; HSA, human serum albumin; MEK, mitogen-activated protein/ERK kinase; PI3, phosphatidylinositol 3; PAG, PIPES-albumin-glucose; PIP3, phosphatidylinositol-3,4,5-trisphosphate; RBD, Ras-binding domain; RBL, rat basophilic leukemia; SOS, son of sevenless.

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also notable that there is no loss of receptor and its IgE during the first hours of the reaction (11).

We do not have a very complete picture of the signaling pathways leading to degranulation (histamine release), but have recently identified some of the components probably relevant to LTC_4 secretion from human basophils. We have recently demonstrated that extracellular signal-related kinase 1/2 (ERK1/2) and cytosolic phospholipase A_2 ($cPLA_2$) are phosphorylated during stimulation of basophils with anti-IgE Ab (12), FMLP, C5a, and IL-3 (13, 14). For all stimuli, inhibition of mitogen-activated protein/ERK kinase (MEK) activity with PD98059 inhibits ERK1/2 and $cPLA_2$ phosphorylation, without effects on some other putative upstream signaling elements. In addition, for anti-IgE, FMLP, and C5a, PD98059 selectively inhibits LTC_4 secretion, but not histamine and IL-4 secretion. This and other related evidence strongly support the role of ERK1/2 and $cPLA_2$ in the secretory pathway leading to LTC_4 generation. Studies in a variety of cell types have linked the MEK1/2 and ERK1/2 enzymes into a pathway that includes Raf-1 and $p21^{ras}$ (15), although there are alternative pathways to ERK1/2 activation (16).

Studies in rat basophilic leukemia (RBL) cells and various murine mast cells have identified some of the early signaling components that follow IgE-mediated stimulation (15, 17–23). With respect to the activation of the pathway that includes ERK1/2, MEK1/2, Raf-1, and $p21^{ras}$, it has been demonstrated that activation of *syk* kinase, phosphorylation of *shc* (by *syk*), and the subsequent association of *shc* to Grb2 and son of sevenless (SOS) may be involved in the activation of $p21^{ras}$. A few studies in basophils have also identified the presence and probably activation of one or two of these components. Notably, the presence and activation of *syk* kinase have been shown to follow stimulation of peripheral blood basophils with anti-IgE Ab (24–30). We have also recently demonstrated activation of $p21^{ras}$ following stimulation of human basophils with anti-IgE Ab (26).

The goal of the following studies is to determine which IgE-mediated signaling steps persist for times that reflect the transient nature of LTC_4 release, i.e., show their maximum state of activation between 5–8 min and quickly return to near resting levels by 15–30 min, and which are more persistent and therefore consistent with the long secretory process needed for IL-4 secretion. For example, from our previous studies of ERK activation, we found that ERK phosphorylation was transient on a time scale consistent with the activation and cessation of LTC_4 release (12). These studies ask the question: where is control exerted that it may differentiate between the cessation of histamine/LTC_4 release vs IL-4 secretion? A directly testable hypothesis is that some early signaling steps will be sustained for periods far longer than previously observed for ERK phosphorylation. In the past, it has been useful to distinguish basophil behavior when stimulated through FcεRI vs stimulation with FMLP (operating through a heterotrimeric G protein-linked receptor) because, while sharing some functional outcomes, signaling also appears independently regulated. We, therefore, examined some early signals in the context of both anti-IgE Ab and FMLP to help localize the down-regulatory alterations induced by anti-IgE.

Materials and Methods

Materials

The following were purchased: PIPES, BSA, EGTA, EDTA, D-glucose, NaF, $Na_4P_2O_7$, Na_3VO_4, 2-ME, Nonidet P-40, and FMLP (Sigma, St. Louis, MO); crystallized human serum albumin (HSA; Miles Laboratories, Elkhart, IN); FCS and RPMI 1640 containing 25 mM HEPES and L-glutamine (BioWhittaker, Walkersville, MD); Percoll (Pharmacia, Piscataway, NJ); Tris and Tween 20 (Bio-Rad, Hercules, CA); leupeptin, DTT, and PMSF (Boehringer Mannheim, Indianapolis, IN); anti-phosphotyrosine mAb (4G10) and rabbit anti-SOS1 Ab (Upstate Biotechnology, Lake Placid, NY); rabbit anti-phospho-ERK Ab, rabbit anti-phospho-MEK Ab, rERK-2 ($p42^{MAPK}$) protein, anti-phospho Akt (Thr^{308}-specific), anti-Akt, and biotinylated molecular mass markers (New England Biolabs, Beverly, MA, now Cell Signaling); anti-*shc* pAb (for immunoprecipitation), anti-*shc* mAb (for Western blotting), anti-Grb2 mAb (for Western blotting), anti-*ras* mAb, and anti-SOS1 mAb (Transduction Laboratories, San Diego, CA); rabbit anti-Grb2 Ab, rabbit anti-SOS2 Ab, anti-*syk* mAb, and anti-phosphatidylinositol 3 (PI3) kinase p110 α, β, γ, δ (Santa Cruz Biotechnology, Santa Cruz, CA); anti-MEK1 mAb (MBL, Watertown, MA); HRP-conjugated donkey anti-rabbit Ig Ab, HRP-conjugated sheep anti-mouse Ig Ab, and protein G-Sepharose beads (Amersham Life Science, Arlington Heights, IL); PP1 (Biomol, Plymouth Meeting, PA); LY294002 (Calbiochem, San Diego, CA); goat anti-human IgE Ab was prepared as previously described (31). Stock solutions of PP1 and LY294002 were prepared in DMSO; controls were incubated with an equal concentration of DMSO.

Buffers

PIPES-albumin-glucose (PAG) buffer consisted of 25 mM PIPES, 110 mM NaCl, 5 mM KCl, 0.1% glucose, and 0.003% HSA. PAGCM was PAG supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$. PAG-EDTA consisted of PAG supplemented with 4 mM EDTA. Countercurrent elutriation was conducted in PAG containing 0.25% BSA in place of 0.003% HSA. ESB is NOVEX (San Diego, CA) electrophoresis sample buffer containing 5% 2-ME. Complete lysis buffer (CLB) is 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA, 100 μg/ml aprotinin, 10 mM benzamidine, 5 mM DTT, 1 mM PMSF, 100 μg/ml leupeptin, 50 mM NaF, 5 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 1% Nonidet P-40, and 10% glycerol. Incomplete lysis buffer is CLB without the protease inhibitors, Nonidet P-40, glycerol, or vanadate. Stripping buffers were either 7 M guanidine hydrochloride or 65 mM Tris-HCl (pH 6.7), 100 mM 2-ME, and 2% SDS. The sensitivity of the subsequent blotting to the choice of stripping agent determined which of these two were used.

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 countercurrent flow elutriation, as previously described (32, 33). The cells were further purified by negative selection using MACS basophil isolation kit (Miltenyi Biotec, Aubum, CA). More recently, we have used a mixture of Abs for negative selection from Stem Cell Technologies (Vancouver, British Columbia, Canada; basophil purification kit) and columns from Miltenyi. The purity of basophils was determined by Alcian blue staining (34) and, from these leukapheresis packs, generally exceeded 99%.

Phosphorylation of ERKs, MEK, and Akt

The phosphorylation of ERKs, MEK, and Akt was assessed using phospho-ERK Ab (12, 13, 35) phospho-MEK Ab (36), or phospho-Akt Ab, respectively. After stimulating basophils ($0.5–1 \times 10^6$ cells per sample) in PAGCM buffer, reactions were stopped by adding ice-cold PAG and microfuged for 5–10 s. After collecting the supernatant (for the measurement of histamine and LTC_4), cell pellets were immediately lysed in ESB and separated on 10% or 4–20% Tris glycine gels (Novex). Electrophoresis and transfer were performed as previously described (12, 37). The membranes were immersed in TBST containing 5% nonfat dried skim milk (Carnation, Los Angeles, CA) overnight to block nonspecific binding. Immunoreactive proteins were detected using the Abs that were diluted in TBST containing 1% skim milk for 90 min. After washing, the membranes were incubated with HRP-conjugated anti-rabbit Ab for 1 h. After washing, ECL detection was performed as described previously (12, 37). In some experiments, the same membranes were sequentially blotted with both anti-phospho-ERK Ab and anti-MEK1 mAb. Between each blotting, membranes were stripped with stripping buffer (usually 7 M guanidine) (12).

Immunoprecipitation

After stimulating basophils ($1.5–5 \times 10^6$ cells per sample) in PAGCM buffer at 37°C, the reactions were stopped by adding ice-cold PAG, and the tubes were microfuged for 5–10 s. The cell pellets were immediately lysed in CLB buffer and after a 10-min incubation on ice, centrifuged for 3 min at $16,000 \times g$ to remove nuclei or undissolved components. Lysates were precleared with protein G-Sepharose beads for 1 h at 4°C to remove any nonspecific binding to the beads. The clarified lysates were then incubated with specific Ab prebound to protein G-Sepharose beads (usually 1 μg per 20 μl beads) at 4°C. After a 1-h incubation, the beads were washed three

times with CLB buffer. The immunoprecipitated proteins were eluted by boiling in ESB. Electrophoresis, transfer, and immunoblotting with anti-phosphotyrosine Ab (4G10) were performed as described previously (12). The Ab was stripped from the membranes, and then membranes were re-probed with indicated Abs.

Activated ras *affinity precipitation assay*

Activated *ras* affinity precipitation assay was performed, as described previously, with slight modifications (38, 39). A GST fusion protein containing the RBD of *raf*-1 (aa 1–149 of *raf*-1), which binds only GTP-bound (activated) *ras*, was immobilized on glutathione-agarose beads (Upstate Biotechnology, Lake Placid, NY). After stimulating basophils ($\sim 5 \times 10^6$ cells per condition), reactions were stopped by adding ice-cold PAG and microfuged for 5–10 s. The cell pellets were immediately lysed in *ras* affinity precipitation buffer (25 mM HEPES, pH 7.5, 2 mM EGTA, 150 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, 50 μg/ml aprotinin, 5 mM benzamidine, 50 μg/ml leupeptin, 25 mM NaF, 1 mM Na_3VO_4, 1% Nonidet P-40, and 1 mM PMSF). Clarified lysates were incubated with the GST-RBD beads (5 μl per sample) for 1 h at 4 °C with rocking. The GST-RBD beads were washed three times with *ras* affinity precipitation buffer. Bound proteins were eluted by boiling in ESB for 5 min. Affinity-precipitated *ras* was detected by immunoblotting with anti-*ras* mAb.

LTC_4 and histamine measurements

An RIA was performed using 100 μl supernatant to determine LTC_4 levels, as previously described (31, 40). Histamine was measured by automated fluorometry (41). The percentage of total histamine release was calculated after subtraction of spontaneous histamine release (42). Each condition tested was performed in duplicate.

Results

Although prior studies of human basophils have noted that other non-IgE-dependent secretagogues can induce greater levels of either histamine or LTC_4 release than IgE-dependent stimuli, a study of the sequential action of anti-IgE Ab and a non-IgE-dependent secretagogue like FMLP has not been done. Fig. 1, *A* and *B*, shows that for both histamine and LTC_4 release, adding FMLP to the cells after they have reached their plateau of IgE-mediated release, but at a time that still largely preceded IL-4 secretion (as shown in other studies), resulted in significantly greater secretion of both mediators (Fig. 1*C* will be discussed in detail below). In addition, the secretion of both mediators was essentially additive.

Preliminary examination of signaling element activation

Before an assessment of activation kinetics, we first established the expression and characteristics/conditions for activating signaling elements within the canonical *syk* to ERK pathway. These results are presented in Figs. 2 through 4. Previous studies have established that *syk* is rapidly phosphorylated during stimulation with anti-IgE Ab, so these data are not shown in Fig. 2. We have examined *lyn* kinase, at least with respect to its phosphorylation state during activation[4] as detected by blotting with anti-phosphotyrosine (4G10). Given the mechanism by which this kinase is activated, it is not surprising that there is little change in the ability of 4G10 to detect a change in phosphorylation state. For 13 experiments, examining activation across a range of times, there was a nonstatistically significant increase in phosphorylation; the ratio of stimulated to control was 1.26 ± 0.17. Fig. 2*A* shows one example of this observation, and also shows that for the same cells, *syk* kinase was significantly phosphorylated. Not shown in the figure is that immunoprecipitation with anti-*lyn* also resulted in coimmunoprecipitation of *syk* kinase.

Shc may be an element immediately downstream of *syk*, and Fig. 2*B* shows that basophils express this protein and that anti-IgE

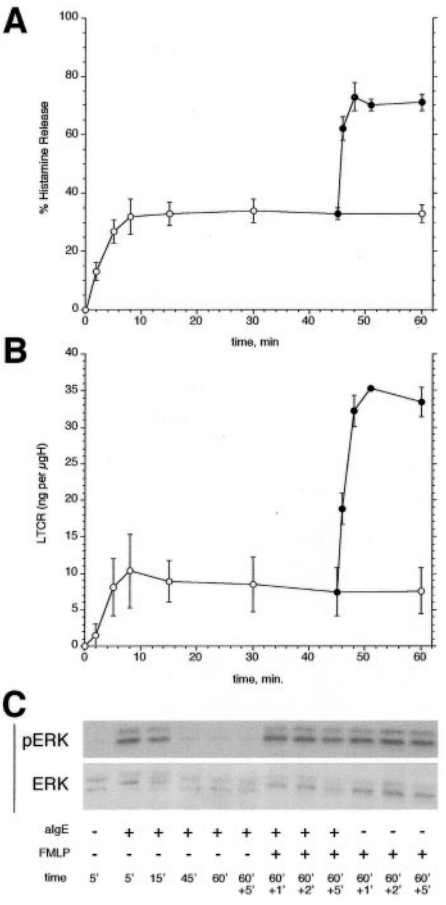

FIGURE 1. Kinetics of sequential stimulation. Basophils obtained by single Percoll gradients from selected donors were stimulated with an optimal concentration of anti-IgE Ab (0.2 μg/ml; $n = 3$). At the times shown, the reaction was stopped by the addition of ice-cold PAG buffer containing 5 mM EDTA, and the cell suspension was centrifuged immediately to recover the supernatant. At the 45-min time point, FMLP at 1 μM was added to some of the reaction tubes. Supernatants were analyzed for both histamine and LTC_4. Not shown in the figure, FMLP-induced release in the absence of prior stimulation with anti-IgE Ab was $32 \pm 6\%$ and 25 ± 5 pg LTC_4/μg histamine for histamine and LTC_4, respectively. *C*, A similar experiment, except that cell pellets were lysed in ESB and analyzed for phosphorylation of ERK1/2. As in *A* and *B*, basophils were first stimulated with an optimal concentration of anti-IgE Ab (0.2 μg/ml) and in this experiment stimulated again with 50 nM FMLP at the 60-min time point. The times indicated along the *bottom* indicate the time point for harvesting the cells, with the *rightmost* lanes indicating the presence or absence of anti-IgE Ab for 60 min and the harvesting taking place at 1, 2, or 5 min after the addition of FMLP.

Ab also induces phosphorylation of *shc*, as we previously demonstrated (26). In contrast, stimulation with FMLP does not induce *shc* phosphorylation. Within these experiments, in parallel tubes, histamine and LTC_4 release were measured. For FMLP stimulation, histamine release was $94 \pm 2\%$, and LTC_4 release was 96 ± 13 pmol/10^6 basophils, while for stimulation with anti-IgE Ab, histamine release was $45 \pm 19\%$ and LTC_4 release was 23 ± 10 pmol/10^6 basophils. Tyrosine phosphorylation of *shc* (p46) was also detected with fairly faint bands in a similar manner to phosphorylation of *shc* (p52). Provided that the anti-*shc* Ab detects the various forms equivalently, blotting showed greater expression of *shc* (p52) than *shc* (p46), and *shc* (p66) was not detected. We further confirmed the same expression pattern of *shc* (p52) and *shc* (p46) without *shc* (p66) in whole basophil lysates using different

[4] As will be noted, immunoprecipitation of *lyn* kinase during activation results in the coimmunoprecipitation of at least one other kinase, *syk*. For this reason, in vitro kinase assays of immunoprecipitated *lyn*, unless performed with well-defined substrates, would not discriminate between the activity of the various kinases that assemble in the developing aggregate that forms during stimulation.

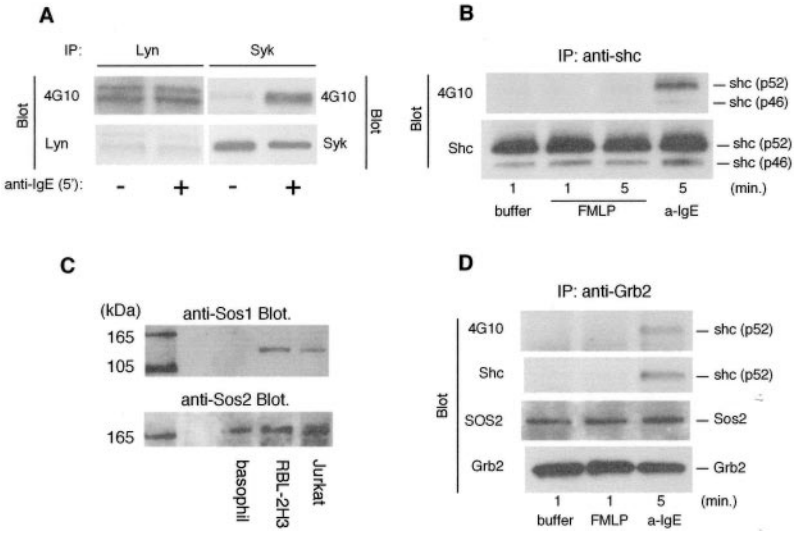

FIGURE 2. Tyrosine phosphorylation following stimulation with FMLP and anti-IgE Ab. Basophils were stimulated with or without anti-IgE Ab (0.2 μg/ml) or FMLP (1 μM) for the times indicated. Reactions were stopped with the addition of ice-cold PAG, and the cells were microfuged. Clarified lysates were immunoprecipitated with anti-*lyn* Ab or anti-*syk* Ab (*A*, *left* and *right*, respectively), anti-*shc* Ab (*B*), or anti-Grb2 Ab (*D*). The immunoprecipitated proteins were subjected to Western blotting analysis with the indicated Abs, as described in *Materials and Methods*. Each Western blot shown is representative of at least three separate experiments. *C*, Cells (5×10^6) of human basophils (>99%), RBL-2H3 cells, and Jurkat cells were lysed and subjected to Western blotting with anti-SOS1 Ab. The same membrane was stripped and reblotted using anti-SOS2 Ab. The Western blots shown are representative of two separate experiments.

Abs (data not shown). Likewise, basophils express Grb2, and anti-IgE Ab induces an association between Grb2 and *shc* (Fig. 2*D*). In these experiments, cells ± stimulation were lysed, and Grb2 was captured by immunoprecipitation on anti-Grb2/protein G beads. Proteins eluted from the beads were subjected to Western blot analysis. Shc was coimmunoprecipitated with Grb2 only in cells stimulated with anti-IgE Ab. Once again, stimulation with FMLP did not induce the association.

Basophils were tested for the presence of SOS protein. There are two forms of SOS (SOS1 and SOS2), and basophils were not found to express measurable quantities of SOS1 with two different anti-SOS1 Abs, as compared with RBL-2H3 cells and Jurkat cells (Fig. 2*C*). However, SOS2 was easily detected in these lysates. Using anti-Grb2 to immunoprecipitate proteins, Grb2 and SOS2 were found to be associated in resting cells, and stimulation with anti-IgE Ab for 5 min did not have a significant influence on the association of these two proteins (Fig. 2*D*). FMLP stimulation also did not affect the association of Grb2 and SOS2.

We have previously shown that stimulation with anti-IgE Ab results in activation of $p21^{ras}$ (26). This assay requires a large number of basophils even when there is a strong secretory response, so we were limited to basic observations concerning this signaling element. Fig. 3*A* demonstrates that $p21^{ras}$ was activated in basophils stimulated with either FMLP or anti-IgE Ab. Notably, an FMLP-induced transient activation of *ras* was observed. For these studies, cell lysates were adsorbed to agarose beads to which the RBD in *raf*-1 was linked. Activated *ras* protein (*ras*-GTP) bound to RBD on beads, and the bead-associated protein was eluted and analyzed by Western blotting with anti-*ras* Ab (see details in *Materials and Methods*). Fig. 3, *B* and *C*, demonstrates that both anti-IgE Ab and FMLP stimulated the appearance of the phosphorylated form of MEK-1. The bands detected by phospho-MEK Ab were also recognized by anti-MEK1 Ab, indicating a role for MEK-1 as a relevant MEK in human basophils (Fig. 3*B*). Previous studies have already established that stimulation with anti-IgE Ab or FMLP induces ERK1/2 phosphorylation (12). As shown in Fig. 3*C*, the kinetics of MEK-1 and ERK1/2 induced by stimulation with FMLP was transient with a peak at 1 min. Five minutes after FMLP, both MEK and ERK phosphorylation had decreased to <20% of the value at 1 min, and by 15 min, phosphorylation had returned to levels indistinguishable from resting cells.

To confirm that some of these steps in the activation cascade were sensitive to the activity of the earliest kinase, *lyn* kinase, the cells were treated with the *src* family kinase inhibitor PP1, 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (43), which we have recently shown to effectively inhibit *syk* phosphorylation (presumably by inhibiting *lyn*) in human basophils stimulated with anti-IgE Ab

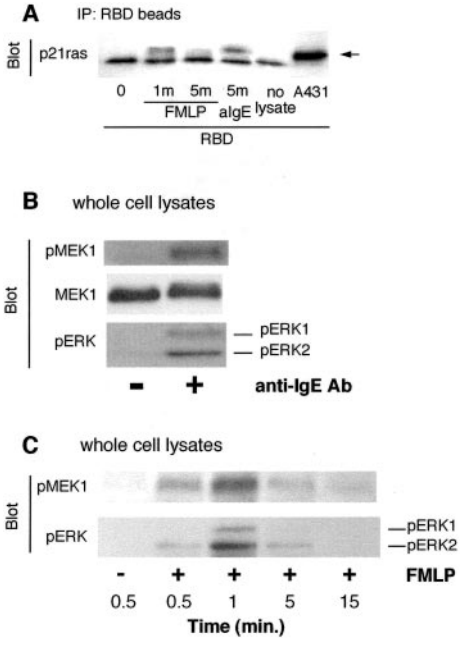

FIGURE 3. Activation of *ras*-related signaling elements following stimulation with FMLP and anti-IgE Ab. Basophils were stimulated with or without FMLP (1 μM) or anti-IgE Ab (0.2 μg/ml) for the times indicated. Reactions were stopped with the addition of ice-cold PAG, and the cells were centrifuged and lysed with either RBD lysis buffer or ESB. *A*, Clarified lysates were subjected to affinity precipitation with RBD-GST. Affinity-precipitated GTP-*ras* was detected by immunoblotting with anti-*ras* Ab. The immunoblot shown is representative of three separate experiments. An A431 cell lysate was used as a reference for *ras* protein, and RBD-agarose beads alone (without incubation with cell lysates) were also used as a negative control. These controls were important because the Western blots from this reagent include several nearby bands that are common to the RBD beads alone (e.g., a common nearby lower molecular mass band can be seen in Fig. 2*A*). *B* and *C*, Cell pellets were lysed with ESB and subjected to Western blotting analysis. The Western blots shown are representative of three separate experiments. *B*, The basophils were lysed 5 min after the addition of anti-IgE Ab at 0.2 μg/ml.

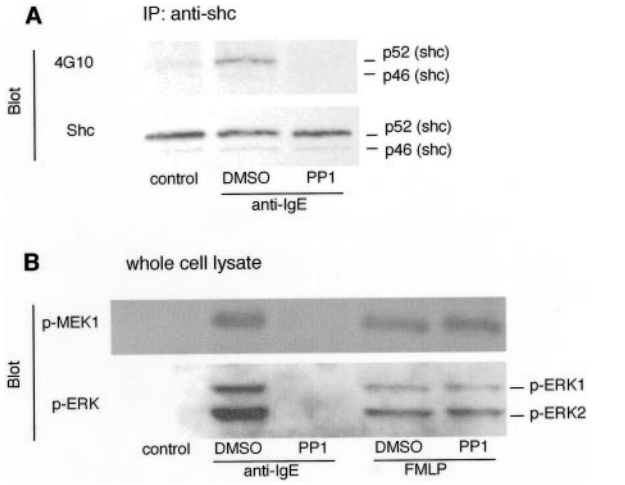

FIGURE 4. Effect of PP1 on phosphorylation of *shc* (*A*), MEK1, and ERKs (*B*) following stimulation with anti-IgE Ab. *A*, Basophils were preincubated with DMSO (0.1%) or PP1 (10 μM) for 10 min and were stimulated with or without anti-IgE Ab (0.2 μg/ml) for 5 min. Reactions were stopped with the addition of ice-cold PAG, the cells were centrifuged, and cell pellets were lysed with CLB. Clarified lysates were immunoprecipitated with anti-*shc* Ab. The immunoprecipitated proteins were subjected to Western blotting analysis with the indicated Abs, as described in *Materials and Methods*. Each Western blotting shown is representative of two separate experiments. *B*, The procedure was as described for *A*, with the exception that cells were lysed with ESB. FMLP was used at 1 μM. The anti-MEK1 Ab blot indicated essentially equal protein loading (data not shown). The Western blot shown is representative of two separate experiments.

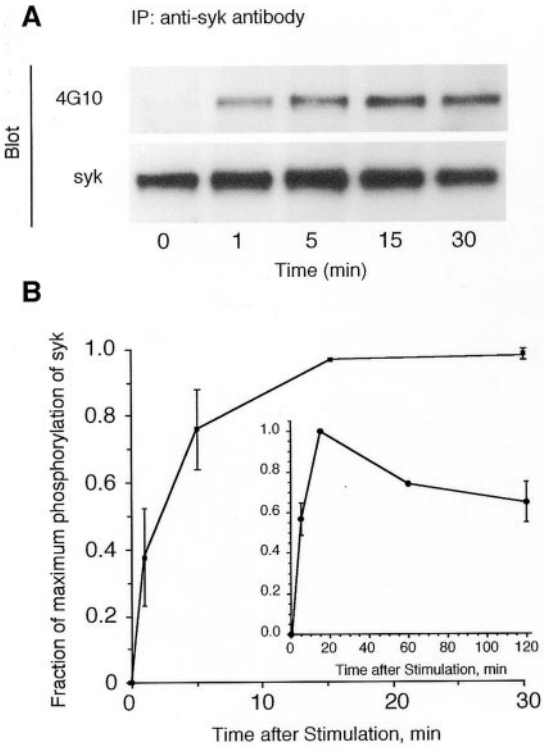

FIGURE 5. Kinetics of *syk* phosphorylation following stimulation with anti-IgE Ab. Basophils were incubated with 0.2 μg/ml anti-IgE Ab, reactions were stopped by centrifugation, and the cell pellets were lysed with CLB. Clarified lysates were immunoprecipitated with anti-*syk* Ab, and the immunoprecipitated proteins were analyzed by Western blotting. *A*, One example blot of the phosphorylation kinetics; *B*, Average of three experiments in which the band intensities were analyzed by digital imaging. *Inset*, Results for similar experiments with an extended reaction period ($n = 3$). Histamine release for the six experiments averaged 29 $\pm$ 5%.

(27). Fig. 4 shows that *shc*, MEK-1, and ERK1/2 phosphorylation were completely inhibited by PP1. Notably, when basophils were stimulated with FMLP, PP1 did not inhibit the phosphorylation of either MEK-1 or ERK1/2.

Kinetics of signaling element phosphorylation

Since 1) LTC_4 secretion (and histamine release) is complete within 15 min when the cells are stimulated with an optimal concentration of anti-IgE Ab, 2) the peak activation of *syk* phosphorylation occurs between 5 and 15 min for most preparations of basophils (as shown below), and 3) the kinetics of ERK phosphorylation was previously demonstrated (with a peak at 5 min) (12), we simplified, and made more practical, the analysis of all of the signaling elements by examining the relationship between phosphorylation or association states at two time points, 5 or 30 min poststimulation with anti-IgE Ab. However, to obtain a more complete sense of the kinetic time course for one of these signaling elements, we did focus more attention on *syk* phosphorylation. Fig. 5 demonstrates the kinetics of *syk* phosphorylation; it shows that *syk* phosphorylation is a sustained event, persisting not only for the primary period of interest in these studies (30 min), but showing marked elevation after 2 h. Fig. 6 summarizes the additional findings of these studies. Beginning at the top of Fig. 6, it can be seen that the ratio of *syk* and *shc* phosphorylation, Grb2/*shc* association, or Grb2/SOS2 association (30 min/5 min) was 0.96 $\pm$ 0.12, 0.99 $\pm$ 0.05, 1.12 $\pm$ 0.09, and 0.99 $\pm$ 0.04, respectively. In data not shown, we examined phosphorylation of *shc* at 1, 5, 15, and 30 min, and found that phosphorylation at 1 min was approximately 50% of the phosphorylation at 5 min, with a plateau beyond 5 min. These results indicated that there was sustained activity of the pathway extending from *syk* to the association of *shc*/Grb2 and SOS2. Parenthetically, we could not detect a gel shift of SOS2 following stimulation (under conditions in which electrophoresis was performed for a longer time period to tease out any differences in mobility). In contrast to the sustained activation of the nonshared signaling elements, the ratios (30 min/5 min) of activation/phosphorylation for *ras*, MEK1, ERK1, and ERK2 were 0.27 $\pm$ 0.09, 0.25 $\pm$ 0.04, 0.22 $\pm$ 0.14, and 0.14 $\pm$ 0.04, respectively. In addition to these experiments in which the various elements were measured simultaneously, we have accumulated many other studies that demonstrate the same results for each of the components shown in Fig. 6. For example, we have additional experience examining *syk* kinase and find that in addition to the results in Figs. 5 and 6, the average behavior of *syk* kinase phosphorylation, for the 30 min/5 min ratio is 1.20 $\pm$ 0.11 ($n = 21$). Likewise, for *shc* phosphorylation, the average 30 min/5 min is 0.91 $\pm$ 0.07 ($n = 6$), and for Grb2/*shc* association, 1.08 $\pm$ 0.06 ($n = 3$). For ERK phosphorylation, our experience also includes basophils isolated from selected donors by standard venipuncture; the average 30 min/5 min ratio is 0.42 $\pm$ 0.06 ($n = 12$). The clearest discrimination between ERK and *syk* phosphorylation kinetics is at 60 min, in which *syk* phosphorylation is still elevated (0.82 $\pm$ 0.09, $n = 11$, relative to the 5-min point) and ERK phosphorylation is quite low (0.09 $\pm$ 0.02, $n = 6$, relative to the 5-min point).

In data shown in Fig. 7, we demonstrated that the sustained phosphorylation of *shc* required the maintenance of antecedent signaling. These studies were done by adding PP1 to cells that were first stimulated with anti-IgE Ab for 60 min. Shc phosphorylation was significant even after 60 min of stimulation, but within 5 min

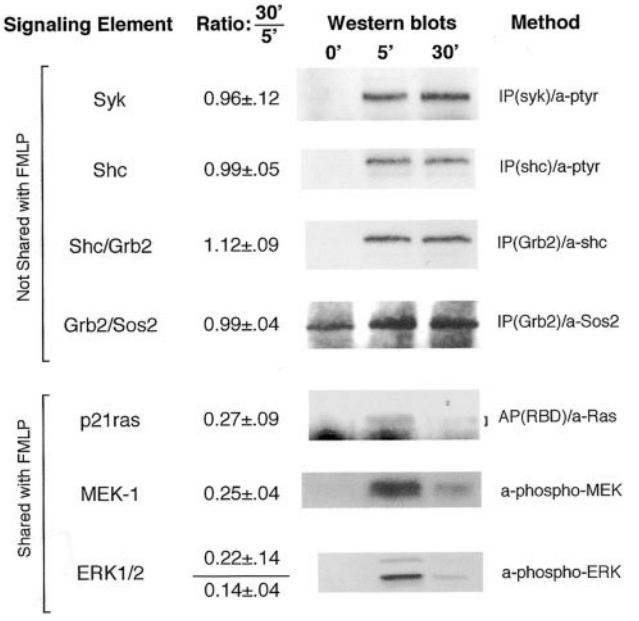

FIGURE 6. Kinetics of signaling elements involved in the pathway leading to ERK activation following stimulation with anti-IgE Ab. Basophils were stimulated with or without anti-IgE Ab (0.2 μg/ml) for the times indicated. Reactions were stopped with the addition of ice-cold PAG, the cells were centrifuged, and cell pellets were lysed with CLB or ESB. Activation, phosphorylation, and association of each element were examined, as described in *Materials and Methods*. The figure is representative of two separate experiments in which the various signaling elements were analyzed simultaneously. Histamine release for these experiments averaged 25%.

of the addition of PP1 (10 μM) *shc* phosphorylation was reduced to resting levels.

In the experiments shown in Fig. 1, sequential stimulation with anti-IgE Ab and then FMLP indicated that FMLP-induced release was essentially unaffected by the prior stimulation with anti-IgE Ab. From the data shown in Fig. 6, it appears that the transition from sustained to transient signaling occurs around or preceding the p21^{ras} signaling element. In Fig. 1*C*, we show that the ERK1/2 phosphorylation response that follows stimulation with FMLP is unaltered by prior stimulation with anti-IgE Ab. In this experiment, rather than stimulate with 1 μM FMLP to obtain the best additional mediator release, the FMLP concentration was reduced in anticipation that any effect of prior anti-IgE treatment might be easier to detect with a weaker second stimulus. At 50 nM FMLP, there was no significant effect of anti-IgE treatment on the FMLP response. At a concentration of 1 μM FMLP, there also was no difference ± prior anti-IgE Ab (data not shown).

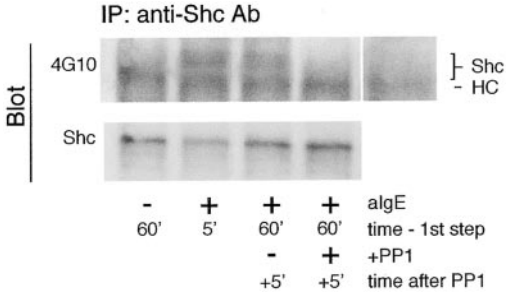

FIGURE 7. The sustained phosphorylation of Shc is sensitive to inhibition by PP1. Basophils were stimulated with anti-IgE Ab (0.2 μg/ml). Sixty minutes after the start of the reaction, PP1 was added to one sample (final concentration of 10 μM) and buffer to a second sample. After a further 5-min incubation, the reaction stopped by centrifugation and the cell pellet was lysed with CLB. Clarified lysates were immunoprecipitated with anti-Shc Ab, and the samples were analyzed by Western blotting with 4G10. Included in the figure is a lane showing the eluate from the fast flow beads alone, without cell lysates (*shc* migrates near the heavy chain that sometimes cross-reacts with the anti-mouse Ab). The blot shown is representative of three experiments.

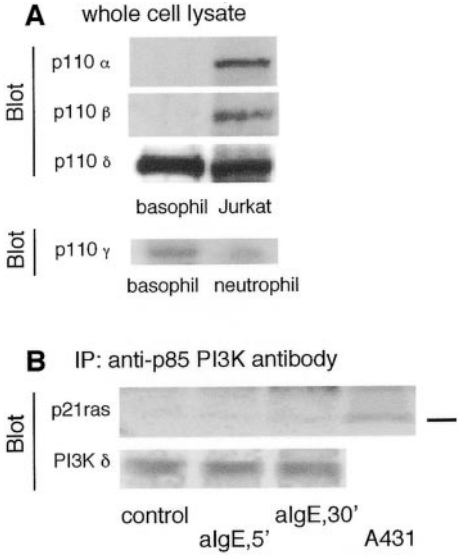

FIGURE 8. Expression of p110 isoforms in human basophils. *A*, Lysates from 5 × 10^6 basophils and Jurkat cells were Western blotted with PI3 kinase p110-specific Abs. For the test of p110 γ expression, the positive control was human neutrophils. *B*, Basophils were stimulated with 0.2 μg/ml anti-IgE Ab or buffer and after 5 or 30 min (5 min for buffer alone), the reaction stopped by centrifugation and cell pellets were lysed by CLB. Clarified lysates were immunoprecipitated with anti-p110 δ PI3 kinase Ab, and samples were analyzed by Western blotting with either anti-p21^{ras} Ab or anti-p110 δ Ab. The blot shown is representative of three experiments.

We have recently demonstrated that PI3 kinase activity appears to be required for the activation of p21^{ras} (26). It has been shown in other cells that some isoforms of PI3 kinase have RBD. We examined human basophils for the expression of the three known forms of p85-regulated PI3 kinase (α, β, δ) and, as shown in Fig. 8*A*, basophils appear to express only PI3 kinase δ. In two experiments, we have also detected in basophils the presence of the non-p85-regulated PI3 kinase, p110 γ. We next examined whether activation with anti-IgE Ab induced association between PI3 kinase δ (p110 δ) and p21^{ras}, and whether this association persisted for at least 30 min. Fig. 8*B* shows that stimulation with anti-IgE Ab did not induce any measurable increase in association between PI3 kinase and p21^{ras} for either the 5-min or 30-min time points.

Taken together, the current data and our previous studies suggest that regulation occurs between PI3 kinase and p21^{ras}. Since active PI3 kinase generates phosphatidylinositol-3,4,5-trisphosphate (PIP3) and this species can be regulated independently of PI3 kinase (see *Discussion*), we asked whether PIP3 was present transiently. The current direct methods of measurement of this molecule are not readily implemented for studies of human basophils,[5] so we turned to an indirect measure of the presence of PIP3: the phosphorylation of Akt. Fig. 9 shows that phosphorylation of Akt was transient on a time scale consistent with the p21^{ras}/ERK pathway activation profile. The 30 min/5 min ratio was

[5] We have recently attempted to measure the presence of PIP3 using the anti-PIP3 Ab developed by Echelon (Salt Lake City, UT), but have been unsuccessful detecting PIP3 this way for the IgE-mediated reaction.

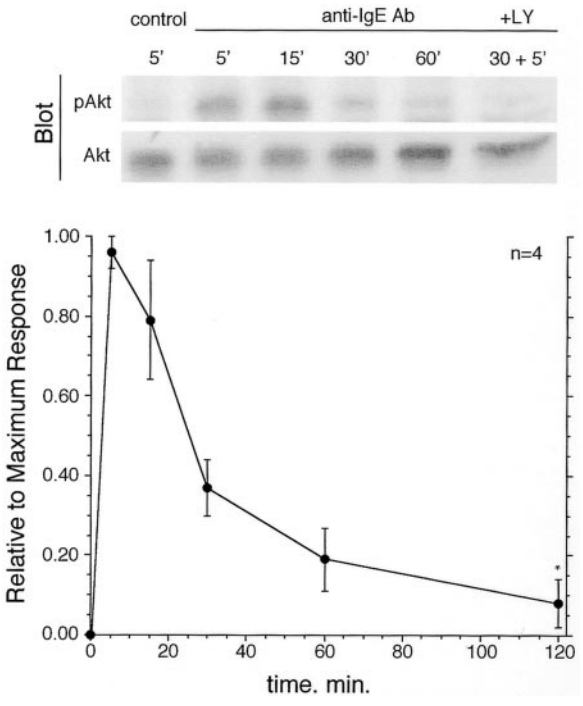

FIGURE 9. Kinetics of Akt phosphorylation. *A*, Basophils were stimulated with anti-IgE Ab (0.2 μg/ml) for the times shown, the cells were pelleted, and the pellet was treated with ESB. Following electrophoresis, the nitrocellulose was blotted with anti-phospho-Akt Ab. Stripping was then followed by blotting with anti-Akt Ab. In the *rightmost* lane (LY), the cells were stimulated for 30 min with anti-IgE Ab, after which 30 μM LY294002 was added, the reaction continued for another 5 min, and the cells were harvested. *B*, The average of four experiments, performed as in *A*, in which band intensities were measured from the individual blots. *, $n = 2$.

0.35 ± 0.07. Also shown in this figure is the observation that the addition of LY294002 at the 30-min time point reduced Akt phosphorylation to resting levels after only an additional 5 min of incubation. In data not shown, we found that the addition of LY294002 at any time up to 30 min (tested at 5–25 min at 5-min intervals) also reduced Akt phosphorylation to resting levels within 5 min. In two of these experiments, the kinetics of *syk* phosphorylation was studied in parallel and the results were as found in Fig. 5 (while Akt phosphorylation was transient). In addition, we found that simultaneous immunoblotting with both the anti-phospho-Akt and anti-phospho-ERK showed well-matched kinetic profiles for ERK and Akt phosphorylation at several concentrations of anti-IgE Ab (data not shown).

If the phosphorylation of Akt indicates the relative presence of PIP3, then PIP3 is transient on a time scale not compatible with the generation of IL-4. This is relevant because previous studies have shown that PI3 kinase activity is required to generate IL-4 and receptor aggregation must be maintained throughout IL-4 secretion for it to continue. Taken together, these various pieces of information suggest that PIP3 is not required later in the secretion of IL-4, but is required in the early stages of the reaction leading to IL-4 secretion. To test this hypothesis, basophils were stimulated for 3 h in RPMI 1640 (containing 1 mM Ca^{2+}) to measure IL-4 release. Thirty minutes after the addition of 0.2 μg/ml anti-IgE (the same concentration used in the studies above, but supraoptimal for IL-4 secretion), 30 μM LY294002 was added to the reaction (in parallel tubes, an equivalent amount of DMSO was added). Some cells were harvested at the 30-min time point, while others were harvested after an additional 2.5 h. In parallel, some cells received 30 μM LY294002 10 min before the addition of anti-IgE Ab and were incubated for 3 h. As expected, preincubation with LY294002 markedly inhibited IL-4 secretion (7 ± 4% of the release from untreated cells). In contrast, the addition of 30 μM LY294002 at 30 min, before the secretion of newly generated IL-4,[6] inhibited IL-4 secretion to only 53 ± 9% of the release from cells to which DMSO vehicle was added (in the absence of LY294002, net newly generated IL-4 was 94 ± 18 pg/10^6 basophils; average purity of the basophils was 99%). We also examined the ability of LY294002 to stop LTC_4 release, although the addition of LY294002 had to be made at 3.5 min, just before the majority of LTC_4 generation. In this study, LY294002 inhibited the release that normally occurred after this time point by 88% and the little additional release of LTC_4 that followed the addition of LY294002 occurred primarily in the first additional minutes (data not shown).

Discussion

These studies demonstrate that in the time frame of LTC_4 release (and histamine release), there are indeed signaling elements that display sustained phosphorylation or association. Fig. 10 summarizes our experience with various signaling elements in human basophils and, based on extensive studies in other cells types (in particular the RBL cell) and a few pharmacological studies in basophils, the cartoon makes tentative assignments for sequencing these events. By and large, those elements most likely involved in LTC_4 secretion and shared among the various secretagogues, notably FMLP in these studies, were transiently activated following an optimal stimulation with anti-IgE Ab. These elements are also shared with IL-3 (unpublished studies) and probably C5a (although we have not yet examined $p21^{ras}$ activation following C5a). The transient activity of the ERK pathway probably accounts for the cessation of LTC_4 following either stimulus (12), but as we have previously shown, the activity of ERK itself does not influence histamine or IL-4 secretion (12, 14, 37). It remains possible that signaling upstream of MEK-1, e.g., $p21^{ras}$, has an influence on histamine release with $p21^{ras}$ or Raf-1 mediating another signaling pathway. The interesting observation from these and our recent studies of PI3 kinase signaling is that events normally found to be upstream of $p21^{ras}$, with the possible exception of PIP3, are sustained following stimulation with anti-IgE Ab.

With reference to Fig. 10, we have now characterized three interrelated pathways with sustained function. First, our older studies of IgE-mediated elevations in cytosolic calcium have shown that it is sustained for a period that remains somewhat undefined (10). As noted in our previous studies, there are issues related to how the basophil processes the fura 2 probe used to detect cytosolic calcium levels that make it difficult to fully characterize the magnitude of the late elevation relative to the early elevation. However, it is clear that by 60 min, the cytosolic calcium response remains elevated. We have little information from studies of human basophils concerning the elements regulating the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) response. In studies yet to be published,

[6] We have noted previously, as have other laboratories, that there is a low level of IL-4 that appears to be prestored. This is especially true in the overnight cultured basophils used in these studies. Prestored IL-4 accounts for 3–15% of the final secreted IL-4 when compared with release from basophils challenged with a concentration of anti-IgE Ab optimal for IL-4 secretion (0.02–0.06 μg/ml, depending on the basophil preparation). The concentration for optimal histamine and LTC_4 release (i.e., that used in the signaling experiments, 0.2 μg/ml) induces less IL-4 secretion, but better release of the prestored IL-4, resulting in a ratio of newly synthesized to prestored of ~2:1. Thus, there is essentially equivalent release of IL-4 at 10 min and 30 min (reflecting the release of prestored IL-4 with granule release), but its release is not sensitive to cycloheximide, while the newly synthesized material is sensitive to cycloheximide. The IL-4 released from 30 to 180 min represents only newly synthesized IL-4, and it was this IL-4 secretion that was used in the calculations of inhibition by LY294002 during the later part of the reaction.

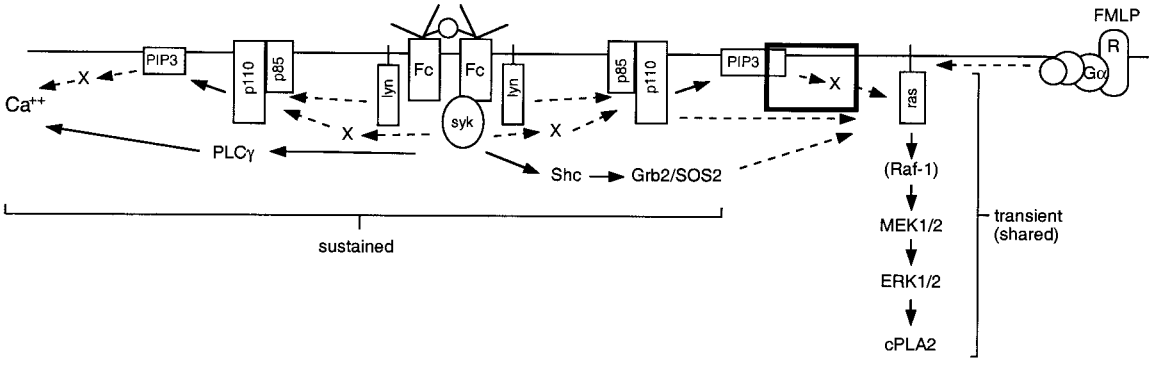

FIGURE 10. Schematic of IgE-mediated signaling in human basophils relevant to LTC_4 generation. Fc, FcεRI receptor (all subunits); X, unknown element or elements; p85/p110, PI3 kinase; PLCγ, phospholipase Cγ. Dotted arrows indicate linkages that may have additional elements and/or testing for cause and effect in human basophils has not been done. Solid arrows indicate linkages for which evidence of the proposed sequencing is found in other cell types or for which we have preliminary evidence in basophils.

we have observed the expected phosphorylation of phospholipase Cγ, and our more recent studies have found that there is some relationship between PI3 kinase activity and $[Ca^{2+}]_i$. However, unlike the characteristics of B cell receptor signaling in B cells, the influence of PI3 kinase on the $[Ca^{2+}]_i$ response appears only moderate for human basophils. A concentration of LY294002 that completely inhibits histamine, LTC_4, and IL-4 release inhibits the $[Ca^{2+}]_i$ response by only 50% (26).

In the current study, we find that *syk* and *shc* phosphorylation and the IgE-mediated association between *shc*, Grb2, and SOS2 is maintained for at least 30 min. Some of these experiments indicate that these elements remain active for far longer. The lengthy phosphorylation of *syk* appears to be an indication of sustained activity because shutting it off with PP1 immediately ablates the sustained phosphorylation of *shc*, a putative substrate of *syk*. Therefore, although *syk* has known inhibitory phosphorylation sites (44) and not all phosphorylation of *syk* can be equated with its activity (45), our results suggest that it is indeed active. As near as we can discern, this arm of early signaling is sustained and therefore should have had a sustained influence on the activity of $p21^{ras}$.

Sequencing of this arm of the early response is limited to our studies using PP1 (43). This drug has proven to be a specific inhibitor of IgE-mediated release in our hands and inhibits the phosphorylation of *syk*, as expected (27). The IC_{50} of 2 μM is high relative to its potency for isolated *src* family kinases, but consistent with all previous studies in intact cells (suggesting a permeability problem) (27, 43, 46). Our results indicate the linkage from *lyn* through *syk* and *shc* and *shc*/Grb2 association, and are also consistent with *lyn* being upstream of the *ras*-MEK-ERK pathway. However, this kinase probably acts as the first step in all FcεRI-associated cascades, so its inhibition would be expected to affect every pathway. We have examined the effects of piceatannol, a putative selective inhibitor of *syk* kinase. However, in studies to be reported in detail elsewhere (47), we found that while piceatannol is a reasonably potent and efficacious inhibitor of histamine release from human basophils (presumably indicating its ability to enter the cell), it has no effect on the IgE-mediated cytosolic calcium response or *shc* phosphorylation at relevant concentrations and also inhibits PMA-induced histamine release and FMLP-induced LTC_4 release. Therefore, its selectivity is suspect (an observation now reported by others using other cell types (see Ref. 47 for details)). The data either suggest that it does not inhibit *syk* kinase at the concentrations relevant for its inhibition of mediator release (and therefore must be acting on other signaling elements) or that *syk* kinase is not involved in phosphorylation of *shc* or the elevation of free cytosolic calcium in basophils. This latter possibility seems unlikely given recent indications that *syk* expression correlates well with the ability of the basophil to secrete in response to stimulation through FcεRI (24, 48).

The third partially characterized arm of the early response in basophils comes from our recent studies of PI3 kinase. The relative positioning of PI3 kinase in the sequence of events following stimulation varies considerably among cell types. As noted above, in basophils, we find that inhibition of PI3 kinase with wortmannin and LY294002 completely inhibits $p21^{ras}$ activation (26). This situation appears unique to basophils, and the reasons for this cause and effect relationship between PI3 kinase and *ras* activation are, as of yet, unclear. The RBD of the p110 δ subunit of PI3 kinase offers one possible connection between these two signaling elements (49–53). There have been in vitro studies that suggest that the binding of *ras* to p110 can influence the activity of p110, and there are indications that this occurs in vivo (49–52). However, the direction we need to understand is how PI3 kinase activity influences *ras*.

There is no evidence that PI3 kinase p110 binding to *ras* influences *ras* behavior in other cell types, but there are sequenced reactions that begin with PI3K (54), which by means unknown induce the activity of a c-Src kinase, which phosphorylates *shc*, thereby recruiting Grb2/SOS and activating $p21^{ras}$. However, the data in basophils are not consistent with such a scheme because PI3 kinase inhibitors do not block *shc* phosphorylation or its binding to Grb2/SOS2. Furthermore, we are trying to explain the transient activation of *ras*, and the data from the current studies show that *shc*/Grb2/SOS2 remain associated while $p21^{ras}$ is down-regulated. We find no stimulus-induced association between PI3 kinase and $p21^{ras}$, although there appears to be a weak constitutive association whose significance is not clear. Therefore, in Fig. 10, we have placed PI3K upstream of $p21^{ras}$ with some indeterminate connectors. Since our data indicate that $p21^{ras}$ activity is dependent on the activity of PI3 kinase, we ask whether there is any evidence that PI3 kinase activity is transient. Currently, this question can only be answered indirectly. Our previous data demonstrate that p85 phosphorylation is maintained for at least 30 min (26). The precise significance of p85 phosphorylation as it relates to the activity of the p110 catalytic subunit is not yet clear. The published literature provides only conflicting conclusions (55, 56). Further studies are necessary to understand the significance of the observation in human basophils. Recent studies have also shown an association between *shc* and PI3 kinase activation, through an interaction with Grb2, Gab2, and p85 (57, 58). Since we also find

sustained *shc* phosphorylation, even this potential pathway to PI3 kinase activation appears sustained.

Therefore, the current evidence indicates that the transient behavior of $p21^{ras}$ cannot be explained by either a transient activation of PI3 kinase (upon which it appears to depend) or a transient association between *shc*, Grb2, and SOS2 (for which we have no direct evidence that this is needed for $p21^{ras}$ activity). It seems likely that the regulation is exerted before *ras* because the addition of FMLP, after the IgE-mediated activation of this pathway has reached its near basal state, results in ERK stimulation that is similar to that observed without prior anti-IgE Ab stimulation. If there were active GTPase-activating protein (GAP)-like factors reducing its activity, one might expect some effect on FMLP stimulation, unless, of course, the pools of *ras* used by these two stimuli are distinct or FMLP utilizes alternative entry points into the ERK pathway. Therefore, the box in Fig. 10 indicating the point of control during activation is around a currently unknown element (or chain of elements) that leads from PI3 kinase to *ras*.

Just as it is unclear how PI3 kinase itself may interact with *ras*, so too is it unclear how the generation of PIP3 could influence *ras* activation. However, our data suggest that the presence of PIP3 is transient on a time scale that is consistent with the transient activity of *ras*. Of course, the observations are dependent on the assumption that Akt phosphorylation is an accurate indicator of the presence of PIP3. In support of this assumption, we found that the addition of LY294002 to the ongoing reaction rapidly reduced the phosphorylation of Akt. This suggests that Akt phosphorylation remains sensitive to the presence of PIP3 throughout the reaction and therefore, that its relatively rapid decline during stimulation with anti-IgE Ab could indicate the transient presence of PIP3. Taken together with the data discussed above concerning the possibly sustained activity of PI3 kinase, these results suggest that something is acting to eliminate newly generated PIP3. One obvious candidate that has currently received a great deal of attention as a gatekeeper in rodent mast cells is Src homology 2-containing inositol 5-phosphatase (59). This possibility is currently under study. However, even if PIP3 is only transiently present, we continue to need an explanation for the linkage between its presence and the activity of $p21^{ras}$. Alternatively, while *ras* activation may be a part of some PI3 kinase-dependent pathway, it remains possible (since we haven't shown the cause and effect relationship between *ras* activation and MEK activation in human basophils) that the linkage between PIP3 levels and activation of MEK-1/ERK/$cPLA_2$ occurs through some other non-*ras*-dependent pathway, and the *ras* data are a red herring in the context of LTC_4 release.

A surprising interpretation of the available data is that PI3 kinase activity is necessary for IL-4 secretion only in the earliest stages of the reaction. There has been no previous demonstration of an early reaction being required for a secretory endpoint only at the onset of the reaction. These results require further study to break down what is actually required. The increase in expression of mRNA for IL-4 significantly precedes the generation of the protein, so future studies may reveal that mRNA expression remains tightly associated with the activity of PI3 kinase, while protein generation does not. The only partial maintenance of IL-4 secretion after the addition of LY294002 at 30 min indicates that there continues to be some requirement for PI3 kinase activity even later in the reaction, but we know from previous studies that even increased expression of mRNA does not subside toward resting levels for 1–2 h. Since our previous studies have shown that cross-links are required throughout the reaction, we would tentatively conclude that some signaling is necessary throughout IL-4 secretion, just not the activity of PI3 kinase or its products.

The upstream events leading to *ras* activation following stimulation by G protein-coupled receptor are also complex (60–63). However, it was not the intent of the current studies to determine the upstream FMLP-driven events in basophils, but to delineate which IgE-mediated pathways did or did not have commonality to stimulation through a GTP-binding protein and to use this information to support interpretation of results for IgE-mediated signaling. In this context, the data in Fig. 1 demonstrated that there was no difference in the FMLP response, with respect to signaling (ERK phophorylation) or LTC_4 secretion, between cells having been treated with or without anti-IgE Ab. These data strongly suggest that the down-regulation imposed on the IgE-dependent signals occurred upstream of the otherwise shared pathway of $p21^{ras}$ through ERK1/2. Several pathways for activation of *ras* by a G protein-coupled receptor (e.g., *shc*/Grb2/SOS formation-dependent or independent *ras* activation) are reported using a variety of cell types and agonists. These findings are not consistent, suggesting that the pathway leading to activation may be dependent on certain cell types or stimuli. Activation of human basophils with FMLP did not result in phosphorylation of *shc* or the association of *shc* and Grb2. A similar inability for FMLP to induce *shc* phosphorylation was reported for FMLP receptor-transfected cells, and any tyrosine phosphorylation induced by FMLP is not required for $p21^{ras}$ activation in human neutrophils (as assessed using tyrosine kinase inhibitors) (63, 64), although this point remains controversial (65–67) and may be related to the presence and activity of PI3 kinase γ. Basophils clearly express this form of PI3 kinase, but the absence of inhibition of FMLP-induced secretion by wortmannin or LY294002 suggests that it does not play a role in either histamine or LTC_4 release. Despite the absence of *shc* phosphorylation in human basophils stimulated with FMLP, $p21^{ras}$ was activated to a degree equivalent to anti-IgE Ab when directly comparing stimulation with these two stimuli. Recently, Zheng et al. (63) have demonstrated that FMLP stimulation results in transient inhibition of *ras* p120-GAP with kinetics that correlated well with the kinetics of *ras* activation. Collectively, tyrosine kinase-dependent *ras* guanine nucleotide exchange factors (SOS) do not contribute to the FMLP-induced *ras* activation, while inhibition of p120-GAP is associated with *ras* activation. The same mechanism may exist for activation of *ras* following stimulation with FMLP in human basophils. However, further study of the events in human basophils is needed.

In summary, these studies identify a region of down-regulation during stimulation of human basophils with anti-IgE Ab. Stimulation of basophils through FcεRI is associated with transient activation of *ras* and its downstream signaling elements, while many events preceding *ras* activation are sustained. Notably, while PI3 kinase may remain active for long periods, one of its products, PIP3, appears present only transiently. When combined with results from other recent studies of IgE-mediated stimulation in human basophils, these results suggest that down-regulation of IgE-mediated LTC_4 secretion, initiated by strong stimuli like anti-IgE Ab, involves regulation of events between the activation of PI3 kinase, most probably regulation of PIP3 levels, and the activation of $p21^{ras}$.

Acknowledgments

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