

Endocytosis, recycling, and degradation of unoccupied Fc ϵ RI in human basophils

Donald W. MacGlashan Jr.¹

Johns Hopkins Asthma and Allergy Center, Baltimore, Maryland, USA

Abstract: Previous studies about basophils and mast cells identified the ability of IgE to up-regulate Fc ϵ RI expression by a process that depends on stabilization of the surface receptor by IgE. However, the mechanism of loss from the cell surface, when unoccupied, is not known. The current studies have examined whether unoccupied Fc ϵ RI on basophils is lost by shedding or endocytosis. IgE was dissociated partially from purified human basophils to augment loss of the unoccupied receptor, and comparisons were made between basophils $\pm$ IgE resensitization prior to 1-day culture. Incubation did not result in a detectable receptor in culture supernatants. However, in the presence of IL-3, although total cell surface expression decreased by 30% (relative to resensitized cells), Fc ϵ RI from whole cell lysates was not statistically different between the two conditions. Incubation for 18 h without IL-3 resulted in the same loss from the cell surface but equivalent loss in whole cell lysates. This degradation process was reversible with Bafilomycin A. There was also evidence that the internalized receptor could be recycled. After the initial 18-h down-regulation, the receptor could be found partially restored to the cell surface if IgE were added back to the culture $\pm$ cycloheximide. Loss of the unoccupied receptor, as well as accumulation of the receptor under the influence of IgE, was found to be insensitive to the presence of a src-family kinase inhibitor, PP1. These studies establish that the unoccupied receptor is lost by a process of endocytosis, partially recycled to the cell surface, and ultimately degraded by a lysosomal mechanism. *J. Leukoc. Biol.* 82: 000–000; 2007.

Key Words: mast cells · Fc receptors · allergy

INTRODUCTION

Expression of Fc ϵ RI on basophils and mast cells determines the ability of these cells to bind IgE and therefore respond to antigens. Various studies over the last two decades have demonstrated that monomeric IgE influences the expression of its high-affinity receptor (Fc ϵ RI) [1–5] as well as its low-affinity receptor (Fc ϵ RII, CD23) [6, 7]. This process of IgE-dependent regulation of Fc ϵ RI is considered a key reason that therapies

such as omalizumab treatment provide some therapeutic benefit [8, 9]. The mechanism of the regulation of Fc ϵ RI by IgE is not understood; however, there are data coming from studies of murine mast cells and human basophils in which the up-regulation of Fc ϵ RI induced by the presence of IgE is not the result of a signaling process, which induces receptor synthesis [10, 11]. IgE does not induce changes in mRNA for receptor components; neither are there changes in the synthetic rate of Fc ϵ RI α [10]. An indirect analysis of up-regulation also indicates that there are no changes in post-translational processing of the receptor [10]. IgE induces changes by interacting with the Fc ϵ RI rather than another IgE-binding protein [12]. Instead, IgE binding to the receptor appears to protect the exposed receptor from loss from the cell surface. Therefore, there is constitutive synthesis of Fc ϵ RI (at least with respect to the presence or absence of IgE), and IgE stabilizes its presence on the cell surface. None of these studies, however, have provided an indication for whether Fc ϵ RI is lost by shedding from the cell surface, as is found for the low-affinity IgE receptor [13], or whether the unoccupied Fc ϵ RI is internalized and ultimately degraded. This is an important component of the process to first understand, as any subsequent hypothesis for the mechanism of loss is guided by the nature of the loss process. The current studies were designed to determine if unoccupied Fc ϵ RI is lost by shedding or internalization in human basophils.

MATERIALS AND METHODS

Materials

The following were purchased: PIPES, BSA, EGTA, EDTA, D-glucose, NaF, Na₄P₂O₇, Na₃VO₄, 2-ME, Nonidet P-40 (NP-40), and fMLP (Sigma Chemical Co., St. Louis, MO, USA); crystallized human serum albumin (HSA; Miles Laboratories, Elkhart, IN, USA); FCS and RPMI 1640 containing 25 mM HEPES and L-glutamine (BioWhittaker, Walkersville, MD, USA); Percoll (Pharmacia, Piscataway, NJ, USA); Tris (hydroxymethyl)-aminomethane and Tween-20 (Bio-Rad, Hercules, CA, USA); leupeptin, DTT, and PMSF (Boehringer Mannheim, Indianapolis, IN, USA); biotinylated molecular weight markers (New England Biolabs, Beverly, MA, USA; now Cell Signaling); anti-syk mAb, 4D10, and rabbit anti-Fc ϵ RI α antibody (Upstate Biotechnology, Inc., Lake Placid, NY, USA); mouse anti-Fc ϵ RI α mAb, 22E7 and 15A5 (gifts from Hoffman-LaRoche, Nutley, NJ, USA); and HRP-conjugated donkey anti-rabbit Ig antibody, HRP-conjugated sheep anti-mouse Ig antibody, and protein G

¹ Correspondence: Johns Hopkins Asthma and Allergy Center, 5501 Hopkins Bayview Circle, Baltimore, MD 21224, USA. E-mail: dmacglas@jhmi.edu
Received February 9, 2007; revised May 5, 2007; accepted May 7, 2007.
doi: 10.1189/jlb.0207103

sepharose beads (Amersham Life Science, Arlington Heights, IL, USA). Goat anti-human IgE (hIgE) antibody was prepared as described previously [14], PPI was from Calbiochem (San Diego, CA, USA), and gp120-OVA was provided by Tanox, Inc. (Houston, TX, USA).

Several IgE antibodies were used in these studies. A gp120-specific monoclonal IgE was the gift of Tanox, Inc. This antibody was biotinylated and could be detected by flow cytometry by streptavidin-Alexa 647, which was calibrated with absolute IgE/basophil by methods described elsewhere [15].

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 was also 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. Lactic acid buffer was for removing endogenous, cell-bound IgE, 0.01 M lactic acid, 0.14 M NaCl, 0.005 M KCl, pH 3.9 [14, 16]. Novex electrophoresis sample buffer contained 5% 2-ME. Complete lysis buffer (CLB) is 20 mM Tris-HCl, pH 7.5, 100 $\mu\text{g}/\text{ml}$ aprotinin, 10 mM benzamidine, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride or 1 mM PMSF, 100 $\mu\text{g}/\text{ml}$ leupeptin, 50 mM NaF, 1 mM Na_3VO_4 , 1% NP-40, and 10% glycerol. Incomplete lysis buffer is CLB without the protease inhibitors NP-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 countercurrent flow elutriation, as described previously [17]. The cells were purified further by negative selection using MACS basophil isolation kit (Miltenyi Biotec, Auburn, CA, USA). More recently, we have used a cocktail of antibodies for negative selection from Stem Cell Technologies (Vancouver, BC, Canada; basophil purification kit) and columns from Miltenyi Biotec. The purity of basophils was determined by alcian blue staining [18], and basophils purified from leukapheresis packs generally exceeded 99% purity.

IgE dissociation and sensitization

For many of the experiments, basophils were treated with lactic acid (above) to dissociate endogenous IgE from its receptor. To minimize harm to the cells, the exposure times to lactic acid buffer ranged from 6 s to 20 s. To induce maximal loss of unoccupied receptor, 20 s was more commonly used, as it results from 50% to 80% removal of cell surface IgE. For studies of the loss of an unoccupied receptor, a comparison was made with cells with full occupancy of surface receptor. Therefore, after the dissociation step, a portion of the cells (usually 50%) was resensitized with IgE antibody. For many studies, the IgE used was anti-gp120 hIgE; it has been shown not to induce measurable signaling [19]. However, to minimize concerns that some low-level, but undetectable, signaling might occur during the sensitization, sensitization was performed at 4°C for 1 h (5 $\mu\text{g}/\text{ml}$ IgE), and the cells were washed once with ice-cold PAG and resuspended in cold media before warming prior to culture. Viability after lactic acid-induced dissociation was $92 \pm 4\%$.

Basophil cultures

Purified basophils were cultured at 2 million/ml in RPMI-1640 medium supplemented with 10 $\mu\text{g}/\text{ml}$ gentamycin and 300 $\mu\text{g}/\text{ml}$ HSA. In many experiments, except where noted, IL-3 was added at 10 ng/ml. In experiments where anti-IgE antibody was used to down-regulate receptor expression, the media included enough additional calcium to equal 1 mM and usually excluded IL-3. Several parameters of cell viability were enumerated for some experiments: recovery, erythrocine B positivity, and annexin V + propidium iodide positivity (by flow cytometry).

Biotinylation of cell surface proteins

For some experiments, a portion of the cells was biotinylated with EZ-Link Sulfo-NHS-SS-biotin (Pierce, Rockford, IL, USA). The cells were washed into PBS buffer, resuspended in 1 ml PBS, to which EZ-Link was added at 0.5 mg/ml for 10 min at room temperature, and the tube then flooded with cold

PIPES buffer, centrifuged, and washed twice with PAG buffer. In pilot experiments, cells were stripped of IgE before or after the biotinylation step.

Flow cytometry and its calibration

In previous studies, we have calibrated the measurement of cell surface Fc ϵ RI α , which is detected by flow cytometry using the anti-Fc ϵ RI α antibody, 22E7 (which detects occupied and unoccupied Fc ϵ RI α), to report absolute densities of the cell surface receptor in some experiments [10, 15]. Briefly, the flow cytometric measurements were calibrated by examining the fluorescence staining of basophils of six donors, which spanned a moderate range of staining intensities (ultimately calibrated to be 8000–140,000 Fc ϵ RI per basophil), and simultaneously assessing the receptor or IgE density by the acetate elution method described above. 22E7 labeling was compared with total Fc ϵ RI density by acetate elution (after sensitizing with PS myeloma IgE to fully sensitize all surface receptors so that IgE measured the total surface receptor effectively) and was linear, with $R = 0.963$. For some but not all of the experiments, the mAb specific for the unoccupied receptor (15A5) was also calibrated to absolute receptor densities. To cross-calibrate the 15A5 antibody with the 22E7 antibody, basophils $\pm$ lactic acid dissociation were sensitized with a couple levels of IgE antibody (fully or partially sensitized), and the cells were analyzed for IgE (anti-IgE antibody), total surface Fc ϵ RI α (22E7), or unoccupied Fc ϵ RI α (15A5). The fluorescence derived from anti-IgE labeling was used to calculate the fractional loading of the cell. For example, knowing the IgE loading was 50% and knowing the 22E7 and 15A5 signal allow one to deduce that the 15A5 signal represents 50% of the total receptors. From this information and the previous calibration of 22E7, 15A5 results could be converted to unoccupied receptors/basophil. Flow cytometry was carried out as described previously in detail [15].

Detection of the aggregated cell surface receptor

Prior to stimulation with anti-IgE antibody, the cells were sensitized with a biotinylated, anti-gp120-specific IgE. The receptor on the surface was monitored by three methods, using flow cytometric detection for each. In the first approach, the cell surface receptor was detected with an antireceptor antibody (22E7). In the second, the presence of the gp120-specific IgE antibody on the cell surface, acting as surrogate detector of all IgE, was detected by flow cytometry with an anti-idiotypic antibody. For the third method, the biotinylated, anti-gp120-specific IgE on the cell surface was detected by labeling with a streptavidin-Alexa 647 conjugate. Three methods were used because of a concern that epitopes on the receptor or its bound IgE might be masked in a maturing surface receptor aggregate.

Statistics

In general, data are shown as the mean $\pm$ SEM in text and figures. In some instances, the figure shows mean $\pm$ SEM, although the analysis is done with paired statistics (e.g., see Fig. 3).

RESULTS

Previous studies found that inhibitors of the metalloprotease, which induces shedding of CD23, had no effect on the loss of unoccupied Fc ϵ RI receptors [10], but a potential shedding mechanism for Fc ϵ RI could have been insensitive to these specific agents. The initial rationale for the current studies was to examine whole cell lysates for indications of receptor degradation and/or search for the receptor in culture supernatants during loss of an unoccupied receptor. However, natural rates of receptor loss are too slow to be sensitive indicators of receptor degradation or loss to the supernatants, as IgE has to dissociate from the receptor first. Previous studies suggested that if endogenous IgE were removed by dissociation with a brief exposure to lactic acid, pH 3.7, more unoccupied receptors become exposed and are therefore lost in shorter periods of time. In pilot studies, **Figure 1A** shows an example of loss of

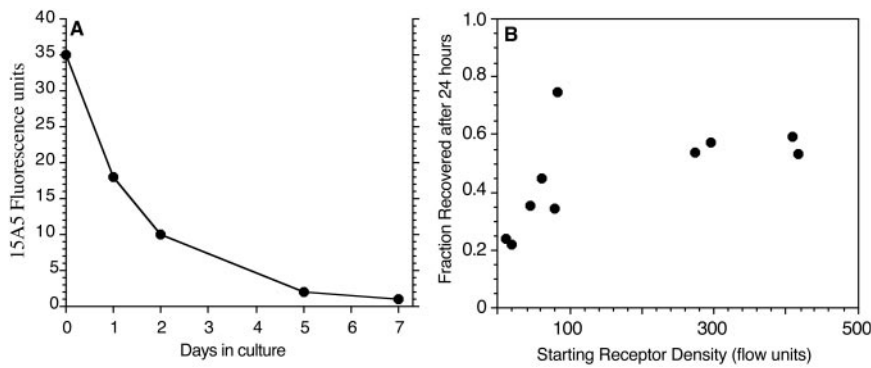


Fig. 1. Kinetics of the loss of unoccupied cell surface FcεRIα. Purified basophils were treated briefly with lactic acid to dissociate a fraction of cell surface IgE and cultured for the times shown. Flow cytometry was used to detect an unoccupied receptor. (A) A representative experiment: (●) 15A5-positive signal (unoccupied receptor) in cells cultured after stripping; the average loss after 24 h was $57 \pm 6\%$, $n = 10$. (B) The relationship between the decrease in an unoccupied receptor after 24 h and the starting receptor density on 10 different preparations of basophils.

kinetics using 15A5 antibody to detect only unoccupied receptors. On average, following a 24-h incubation, there remained $43 \pm 6\%$ of the Day 0 density of unoccupied receptors ($n=10$, $P<0.0001$). Figure 1B shows the relationship between the starting, unoccupied receptor density and the amount of loss in this 24-h period. This relationship is statistically flat (null hypothesis, slope=0; $P=0.0687$) across a 50-fold range of receptor density, giving the appearance of a pseudo first-order loss process, i.e., a process that is not saturable within the range of observed receptor densities, as might occur for an enzymatic process. These experiments were performed using an antibody for flow cytometry, which had been calibrated previously to express results in terms of absolute receptor densities (see Materials and Methods). Therefore, the absolute starting density of unoccupied FcεRIα for each of the 10 preparations used to obtain the data in Figure 1B could be estimated; unoccupied receptor density ranged from 2600 to 145,000 in these experiments. From an earlier set of experiments, also using 15A5 antibody but without the prior calibration, we also found this essentially flat relationship between loss and starting density ($n=10$; slope=0; $P>0.05$; data not shown).

Using the stripping method as a tool to accelerate loss, supernatants of cultured basophils were examined for the presence of FcεRIα-positive bands on electrophoresis, i.e., Western blots using 22E7 anti-FcεRIα or polyclonal anti-FcεRIα mAb. Purified basophils were treated with lactic acid to dissociate a portion of the cell-bound IgE; one-half of the cells were resensitized with 5 μg/ml IgE for 60 min at 4°C, and one-half were incubated without IgE. The cells were washed

free of IgE and cultured for 24 h. Supernatants were examined by Western blots for the appearance of FcεRIα-positive bands. A portion of the cells was examined by flow cytometry to detect the level of the cell surface receptor. The intent was to compare Western blot band patterns for cells cultured $\pm$ resensitization. There were some constraints for examining supernatants. It is not surprising that a Western blot of unconcentrated supernatants did not reveal positive bands, so the supernatants were adsorbed with polyclonal anti-FcεRIα antibody on a Fast-flow G bead. No FcεRIα-positive bands appeared in these preparations, which were related to the presence or absence of prior resensitization with IgE (data not shown). No bands appeared, which were not in the “no cells” antibody + bead controls. In some experiments, prior to culture, the cells treated with a biotinylation reagent, the supernatants immunoadsorbed with anti-FcεRIα (polyclonal), and Western blots developed using streptavidin-HRP to enhance detection. In these experiments, no differential banding was detected (data not shown).

Examining the cell pellets provided an explanation for the results with supernatants. When observed by flow cytometry, using 22E7 antibody to detect total cell surface FcεRIα, as Western blots necessarily used 22E7, the ratio for conditions of -IgE:+IgE was 0.72 ± 0.04 ($P<0.0001$ tested against null hypothesis=1.0), indicating loss of cell surface receptor (brief acid dissociation does not remove all surface IgE, so this ratio is greater than 0.5). In contrast, whole cell lysates on Western blot with 22E7 showed little or no loss, -IgE:+IgE ratio = 0.93 ± 0.04 ($P=0.12$). **Figure 2** shows an example of this experiment in Figure 2, A and B, and the average data in Figure 2C. Note that as we have found previously, basophils

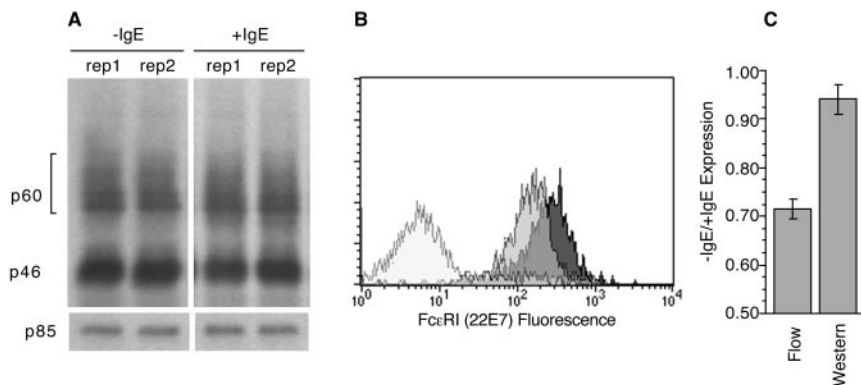
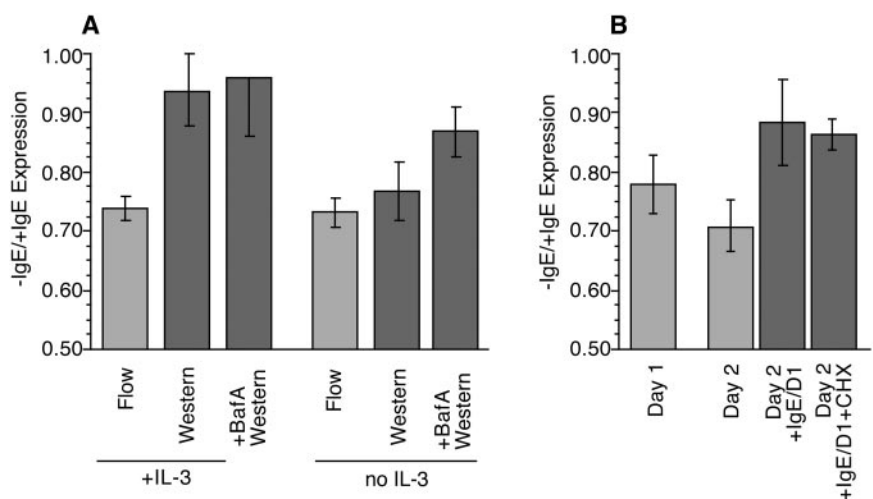


Fig. 2. Loss of cell surface FcεRIα compared with loss of whole cell lysate-derived FcεRIα. (A and B) Representative results for one experiment. (A) The Western blot results for the broad p58-p64 band (nominally, p60) in cells, which were treated with lactic acid first to dissociate a fraction of the cell surface IgE. A portion of the cells was cultured without resensitization with IgE (-IgE), and these compared with a portion, which was resensitized with IgE first (+IgE). The conditions were examined in duplicate, and the presence of the subunit of PI-3K, p85, was used as a control for lane loading. (B) The cell surface receptor determined by flow cytometry for the same experiment; light gray is the distribution using control IgG1 antibody, medium gray is the distribution using 22E7 on cells cultured without IgE sensitization, and dark gray is the distribution using 22E7 on cells cultured with IgE sensitization. (C) These experiments summarized ($n=10$). $P < 0.0001$ for the decrease measured by flow cytometry; $P > 0.05$ for the ratio measured by Western blot (both tested against a ratio=1.0).

tization, and dark gray is the distribution using 22E7 on cells cultured with IgE sensitization. (C) These experiments summarized ($n=10$). $P < 0.0001$ for the decrease measured by flow cytometry; $P > 0.05$ for the ratio measured by Western blot (both tested against a ratio=1.0).

Fig. 3. Effects of IL-3 on surface receptor or total cellular receptor. Basophils were treated with lactic acid first to dissociate a fraction of the cell surface IgE. A portion of the cells was cultured without resensitization with IgE (–IgE), and these compared with a portion, which was resensitized with IgE first (+IgE). (A) The average results for experiments in which a portion of each group of cells was cultured $\pm$ IL-3 (10 ng/ml) $\pm$ 200 nM Bafilomycin A (or DMSO equivalents; $n=5$). The dark bars represent relative band intensities from Western blots of whole cell lysates, and the light bars represent the flow cytometric results (all calculations were relative to the cells that had been resensitized with IgE). For the “no IL-3” group of conditions, no Bafilomycin A versus +Bafilomycin A was statistically different (*, $P=0.014$); there was no statistical difference between the values for flow cytometry and Western blots without Bafilomycin A. For the +IL-3 group of conditions (A, left side), the mean for the Western blot data was not statistically different than 1.0 ($P=0.394$) and not different from the +Bafilomycin condition. (B) $n=4$; the initial design was similar, but on Day 1, the cells cultured without prior resensitization were divided into those receiving no IgE (Day 2), receiving 0.5 μ g/ml IgE (Day 2 +IgE/D1), or receiving 0.5 μ g/ml IgE plus 2 μ M cycloheximide (Day 2 +IgE/D1+CHX). On Day 1, a portion of these cells was also analyzed by flow cytometry. On Day 2, all conditions were harvested and analyzed by flow cytometry. For comparison, a parallel set of cells, which had been resensitized on Day 0, was cultured for 1 and 2 days $\pm$ IgE on Day 1 or $\pm$ IgE+ cycloheximide and was analyzed by flow cytometry (as above). In a paired analysis, the difference between Day 1 and Day 2 +IgE/D1 was statistically significant ($P=0.039$) and between Day 1 and Day 2 +IgE/D1+CHX, $P=0.018$.



express two bands, which can be identified with any anti-Fc ϵ RI α antibody (monoclonal or polyclonal). There is a band that tracks nominally at p46, which we have demonstrated previously is likely to represent an immature, pre-golgi, form of Fc ϵ RI α , as its molecular weight can be reduced to $\sim$ 30 kD following treatment with endoglycosidase H [20]. It is also not immunoadsorbed (although p60 is) when anti-IgE antibody is used to capture the receptor from whole cell lysates. Then, there is a mature receptor, glycosylated heavily and heterogeneously, which migrates in a broad region centered around 60 kD. In these experiments, neither the p46 nor p60 bands were different between the two conditions. These results show that surface loss occurs, but in the short time-frame of these cultures, not enough time has elapsed to result in significant degradation of the receptor. This result indicates that the receptor was internalized but not degraded.

In studies unrelated to the current studies, it was noted that IL-3 appeared to change how proteins were processed in basophils. Therefore, we repeated the studies above, which were performed with IL-3 present but left IL-3 out of the medium for an 18-h culture. **Figure 3A** shows that under these conditions, loss of receptor was observed in flow cytometry and in whole cell lysates. This observation allowed testing of whether degradation of an internalized receptor, which could now be observed in a short culture, was sensitive to agents that modify internalized receptor processing. To test whether internalized Fc ϵ RI α was degraded in a lysosomal compartment, the cells were treated with 200 nM Bafilomycin A, an inhibitor of the acid pumps, which allows lysosomal degradation. In previous studies of human basophils, where Bafilomycin A was used, we found that the 200 nM concentration achieved $\sim$ 70% inhibition of protein processing, but at higher concentrations (600 and 2000 nM), some basophils were lost during culture. So, the choice of 200 nM represents a balance between strong inhibition and minimal cell loss (a study of basophil viability in the presence of Bafilomycin A at 200 nM showed $96\pm 3\%$ eryth-

rocin B viability with no Bafilomycin A and $92\pm 4\%$ viability with Bafilomycin A; $P>0.05$ for the difference). Figure 3A shows that Bafilomycin A partially inhibited the degradation of an internalized receptor, suggesting that once internalized, the receptor was ultimately lost through lysosomal processing.

An experiment suggested by these results is to return IgE to the culture of “stripped” cells after only 18 h of down-regulation (comparing the results with IgE returned to cultures of cells already resensitized with IgE on Day 0). As we have shown previously that IgE does not induce receptor synthesis beyond the slow, constitutive synthesis that occurs [10], a return of cell surface receptor would suggest recycling of an internalized receptor. Addition of cycloheximide would test whether the slow, constitutive synthesis contributed to any observed restoration of the cell surface receptor. The addition of IgE partially reconstituted the cell surface receptor, which was lost following 1 day of down-regulation (Fig. 3B). In the description of these experiments “loaded cells” refers to basophils, which were stripped and resensitized on Day 0 before being placed into culture. By Day 1, stripped basophils showed the expected decrease in total surface receptors (as detected by flow cytometry). Adding IgE after 1 day of loss led to an increase in cell surface receptor on Day 2 (on Day 2, without the addition of IgE, receptors had decreased further). Inclusion of 2 μ M cycloheximide resulted in restoration similar to its absence.

An attempt was made to identify an intermediate state of degradation of the receptor lost from the cell surface. Biotinylated basophils, cultured $\pm$ IgE overnight, were lysed and immunoadsorbed, the Western blots visualized with streptavidin-HRP and 22E7, and examined for the appearance of bands, which appeared in the absence of coincubation with IgE but did not appear when the cells were cultured with IgE (500 ng/ml). Only three biotinylated bands were observed (heavy and light chain of IgE and p60 Fc ϵ RI α), but these bands did not change their expression $\pm$ IgE. The re-blot with 22E7

showed the presence of the p46 band (which was not observed with the streptavidin blot); the ratio of this band intensity, +IgE:-IgE, was 1.1 ± 0.23 ($n=4$), and the total cell surface receptor expression (22E7 binding by flow cytometry compared with Day 0) was 0.77 ± 0.04 in cells incubated without IgE and 0.98 ± 0.02 in cells with IgE. Therefore, the appearance of the p46 22E7-positive band shows no relationship to loss of cell surface receptor, and no other lower molecular weight bands became apparent.

In murine mast cells, the ability of IgE to up-regulate FcεRIα expression was not impaired in mast cells derived from syk^{-/-} mice [21]. These results suggest that an early signaling component needed for the aggregated receptor response is not required for the stabilizing effect of IgE. The up-regulation in human basophils is a slow process in vitro, so that to determine if early kinase inhibitors influence up-regulation, the basophil donors must express low starting levels of surface receptor. Long-term cultures with some kinase inhibitors (notably some syk kinase inhibitors) lead to increased apoptosis, confounding the interpretation of the results. However, for a study of down-regulation, a shorter culture of 18 h was less problematic with a syk inhibitor. In addition, an equally important, early step in aggregated receptor signaling is the participation of src-family kinases upstream of syk activation, and we have found the longer cultures with PP1 were not problematic. Therefore, basophils were cultured for 2.5 days $\pm$ 500 ng/ml IgE $\pm$ 10 μ M PP1 (a src-family kinase inhibitor), a concentration shown in many previous studies to completely inhibit the downstream functions that follow aggregation of FcεRI [22–24] with an IC₅₀ of ~ 2 μ M. In pilot experiments, no index of basophil viability–recovery, erythrocin B positivity, propidium iodide positivity, or annexin V binding was altered by the presence of 10 μ M PP1 (**Table 1A**). At this concentration, PP1 did not inhibit the up-regulation of FcεRI, as determined by flow cytometry with 22E7, induced by IgE (Table 1C). At 10 μ M, PP1 also did not inhibit the loss of a cell surface, unoccupied receptor (Table 1B). For the shorter 18-h study, the syk family kinase inhibitor, NVP-QAB205, also did not influence recovery and viability significantly (Table 1A), and it did not influence the loss of an unoccupied receptor (Table 1B).

Basophils also down-regulate receptor expression if the receptor is aggregated. Early studies noted that down-regulation was not apparent after 2–3 h when a simple bivalent antigen hapten was used to aggregate receptor [25], but more recent studies suggested that some down-regulation does occur with a stronger aggregating stimulus, anti-IgE antibody [26]. In these recent studies, the timing of an aggregated receptor loss was not examined. We were interested in whether the Bafilomycin A-sensitive but PP1-insensitive loss of unoccupied receptors was also observed for aggregated receptor-dependent loss. For these experiments, basophils were incubated for up to 3 days with an optimal concentration of anti-IgE antibody. Previous studies established that some loss occurred in 18 h; **Figure 4, B and C**, shows that a maximum loss of $\sim 50\%$ occurs by 24 h, with little apparent loss at time-points less than 8 h, even for this stronger stimulus. Figure 4A shows that loss from the cell surface is only somewhat faster, with a T_{1/2} of ~ 12 h (see Materials and Methods for surface detection approaches). Al-

TABLE 1. Purified Basophils

A. Measurement	Ratio: Drug: No Drug			
	PP1	(P)	NVP-QAB205	(P)
Recovery ($n = 3$)	1.11 ± 0.08	n.s.	0.98 ± 0.11	n.s.
Erythrocin B+ ($n = 3$)	0.99 ± 0.06	n.s.	1.19 ± 0.11	n.s.
PI+/Annexin V+ ($n = 3$)	0.83 ± 0.12	n.s.	0.81 ± 0.08	n.s.
Total FcεRIα (+IgE, $n = 4$)	0.94 ± 0.07	n.s.	0.80 ± 0.21	n.s.
B. Condition (down-regulation)	Ratio: no IgE: +IgE			
	PP1	(P)	NVP-QAB205	(P)
No Drug	0.74 ± 0.03		0.78 ± 0.04	
+Drug	0.74 ± 0.02	n.s.	0.84 ± 0.12	n.s.
C. Condition (up-regulation)				
	PP1	(P)	NVP-QAB205	(P)
No Drug	1.29 ± 0.16		N.D.	
+Drug	1.53 ± 0.29	n.s.	N.D.	

Purified basophils were incubated in the absence and presence of the src-family kinase inhibitor PP1 at 10 μ M (or vehicle control DMSO at 0.05%) or the syk inhibitor NVP-QAB205 at 1 μ M (in A, note that values >1.0 would be considered a detrimental effect on viability). Experiments using PP1 or NVP-QAB205 were done with different preparations of basophils. (A and B) The data reflect results for 18 h cultures. In the DMSO control (18 h), cultured cells, the average erythrocin B positivity was $17 \pm 2\%$, and propidium iodide/annexin V positivity was $11 \pm 4\%$. (B) Cells were cultured $\pm$ drug, with and without prior sensitization to measure the extent of down-regulation; $n = 2$ for PP1, and $n = 4$ for NVP-QAB205. (C) For a 2.5-day experiment, the cells were cultured $\pm$ drug with IgE in the culture medium, and the ratio reflects the gain in receptor expression compared with Day 0 ($n = 2$). The ratio for cell recovery $\pm$ PP1 after 2.5 days was 1.07 ± 0.03 [nonsignificant (n.s.)]. The P values were all nonsignificant. (B and C) The P value reflects the comparison of no drug with +drug. N.D., Not done.

though degradation reached a maximum of $\sim 50\%$, loss from the cell surface reached 90% by Day 2. **Figure 5, A and B**, shows that receptor degradation following aggregation is also sensitive to Bafilomycin A, with $\sim 80\%$ inhibition of loss at 200 nM. However, unlike the insensitivity of the unoccupied receptor loss to the src-family kinase inhibitor PP1, PP1 did inhibit some loss of the receptor from the cell surface (Fig. 5C). Although the inhibition was only partial for surface loss, there was complete inhibition of degradation. The IC₅₀ for inhibition of surface loss and degradation was consistent with the IC₅₀ for inhibition of a functional endpoint such as histamine release.

DISCUSSION

These data resolve the question of whether the unoccupied receptor is internalized when lost from the cell surface. For the receptor to be lost from the cell surface but not in whole cell lysates, the receptor must be sequestered in a compartment not accessible to extracellular antibody. Numerous studies in other cell types would lead to the hypothesis that early in the process of loss from the cell surface, the receptor is located in an early endosomal structure. In other cells, such structures are often found to be a transient location for receptors, which might

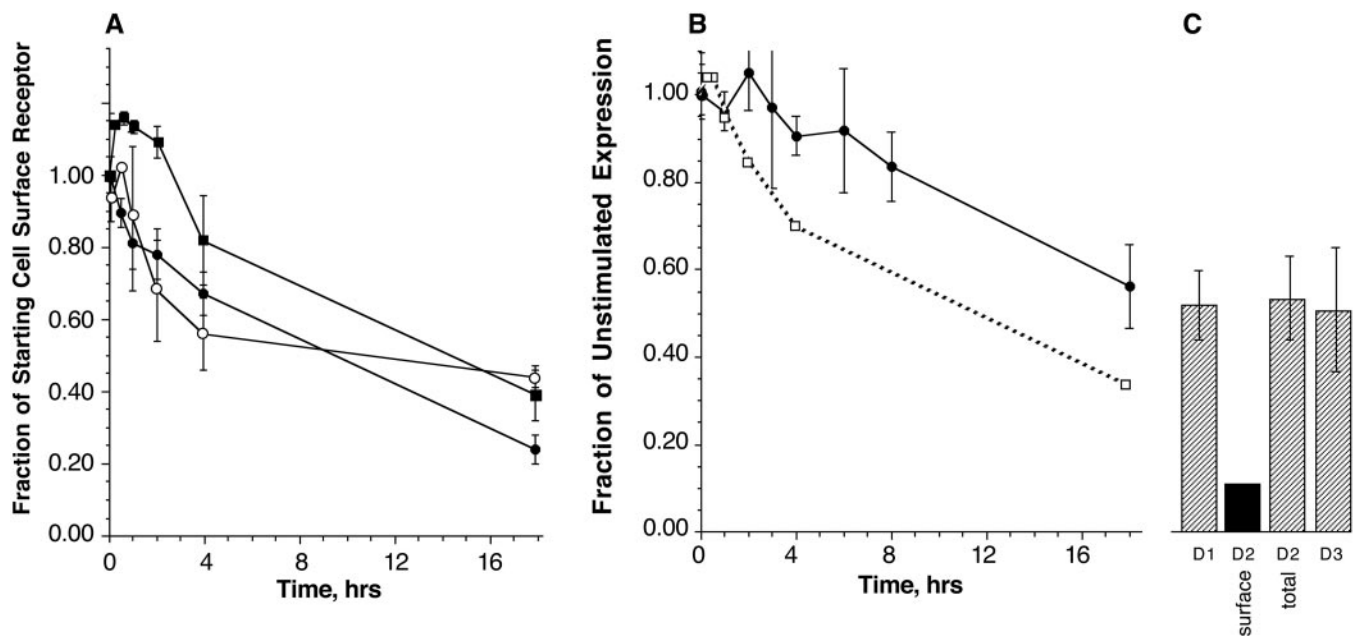


Fig. 4. Kinetics of receptor loss during aggregation with anti-IgE antibody. Purified human basophils were stimulated with a concentration of a polyclonal anti-IgE antibody, which is optimal for secretion (0.2 μ g/ml). The cells were incubated for the times shown and processed for whole cell receptor levels by Western blotting or for cell surface receptor and IgE detected by flow cytometry. (A) Flow cytometric detection of surface receptor. Basophils were sensitized with SE44B (biotinylated anti-gp120-specific IgE) prior to stimulation, stimulated with anti-IgE at 0.2 μ g/ml, and surface receptor-measured by flow cytometry; (○) detection with 22E7 (antireceptor antibody), $n = 4$; (●) detection with anti-idiotype, AB19-4 antibody, $n = 4$; (■) detection with streptavidin, $n = 3$. (B) (●) Western blot detection of the broad 22E7-positive band at p58-p64 (mature receptor), $n = 3$; (□) average of the three methods of surface receptor detection translated from A for comparison. (C) A separate set of experiments measuring the receptor in whole cell lysates (hatched bars) incubated for 1–3 days with anti-IgE antibody ($n = 3$); there was no statistically significant difference in the measurements among Days 1–3. The flow cytometric measurement of surface receptor was made only on Day 2 using 22E7 for detection (solid bar).

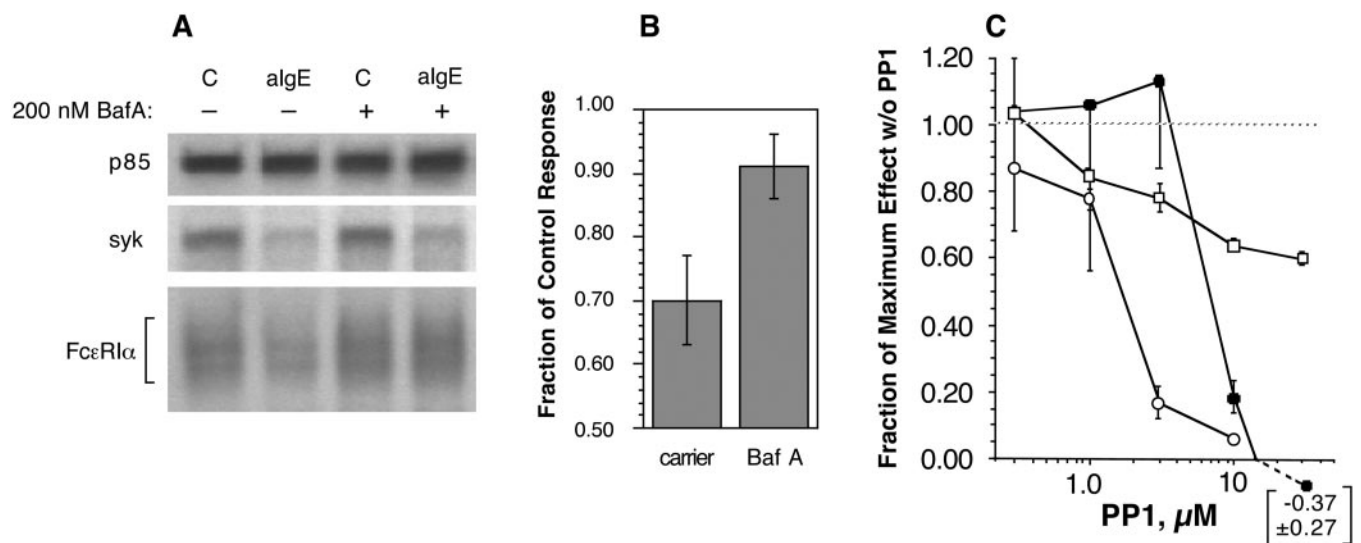


Fig. 5. Effects of Bafilomycin A or PP1 aggregated receptor loss. (A) A representative experiment; purified basophils were stimulated with 0.2 μ g/ml anti-IgE (algE) antibody in the presence or absence of 200 nM Bafilomycin A for 18 h. For reference, two additional signaling elements were analyzed by Western blot; p85 acts as the loading control (Lanes C), and syk is known to decrease under the conditions of stimulation. The blot also shows the mature, 22E7-positive FcεRIα (58–64 kD range) band. (B) Average of five experiments; $P = 0.006$ for the paired difference $\pm$ Bafilomycin A. (C) Purified basophils were incubated overnight with anti-IgE antibody $\pm$ the shown concentrations of PP1 (or vehicle control or cells without anti-IgE antibody $\pm$ PP1). The ability of PP1 to inhibit a measured effect (loss of total cell FcεRIα, loss of surface FcεRIα, or histamine release) was calculated relative to the observed effect in the absence of PP1, i.e., ratio of the magnitude of the anti-IgE-induced outcome with drug:the magnitude of the outcome without drug; (○) histamine release; (●) loss of total cell FcεRIα (p60) after 18 h; (□) loss of cell surface FcεRIα after 4 h of incubation. The highest concentration of PP1 resulted in a nonstatistically significant increase in FcεRIα (p60), the point located under the x-axis. For these experiments, the average changes in the absence of PP1 were histamine release, $29 \pm 6\%$; loss of total p60 receptor, $22 \pm 6\%$; loss of cell surface receptor, $45 \pm 6\%$.

recycle to the cell surface or finally be degraded by the lysosome. The basophil experiments suggest these two outcomes, as some receptor was found to recycle to the cell surface in the presence of IgE, and under conditions where the receptor was more rapidly degraded ($-IL-3$), the lysosomal proton pump inhibitor, Bafilomycin A, was found to inhibit degradation.

Although these studies do not identify a mechanism, they establish that the mechanism must involve an endocytotic process. These studies and previous observations suggest that IgE binding alters the receptor in a way that changes its recognition by a mechanism that targets the empty receptor for endocytosis. In basophils, this process is quite slow, with a $T_{1/2}$ of ~ 24 h. It also appears as almost a first-order in kinetics (Fig. 1B), the fraction lost being insensitive to the amount of starting receptor, as if there is a large reserve of interacting structure (protein, carbohydrate, lipid) to handle processing of the empty receptor. Recent studies by Kubota et al. [27] showed that the extracellular stalk length of the Fc ϵ RI α subunit determines the ability of this subunit to be processed for loss (although there is no indication in this study whether processing resulted from shedding or endocytosis). Studies by Charles and co-workers [28, 29] have suggested that there is a 28-kD protein, which associates with the unoccupied receptor in rat basophilic leukemia (RBL) and bone marrow-derived murine mast cells. This protein was discovered serendipitously when an antibody, which was thought to be specific for a phospholipid scramblase, was also found to coimmunoprecipitate Fc ϵ RI in the unoccupied state in RBL cells. The p28 protein associates with the unoccupied receptor and dissociates rapidly upon IgE binding, even if the unoccupied p28-Fc ϵ RI complex is immunoadsorbed and then exposed to IgE. The identity of this protein is not yet known, but these studies demonstrate the presence of a possible sensor protein of the unoccupied state.

We also found that degradation of aggregated receptors was sensitive to Bafilomycin A. The fact that more receptor was lost from the cell surface than lost by degradation indicates that there is also a holding pool of an aggregated receptor. However, there is no evidence yet that this pool of receptors can return to the cell surface. Unlike the loss of the unoccupied receptor, the loss of the aggregated receptor, not surprisingly, is sensitive to the activity of a src-family kinase (caveats notwithstanding about the selectivity of PP1).

In summary, the unoccupied receptor and aggregated receptor are lost from the cell surface by a slow process, which culminates in degradation by a Bafilomycin A-sensitive mechanism, suggesting a role for lysosomal degradation. The unoccupied receptor can recycle to the cell surface under the appropriate conditions. With this information in hand, it is possible to formulate hypotheses concerning the mechanism by which the unoccupied receptor is lost, which include endocytotic processes.

ACKNOWLEDGMENTS

These studies were supported by National Institutes of Health Grants AI02053 and AI070345. I thank Valerie Alexander for her expert technical assistance.

REFERENCES

1. Furuichi, K., Rivera, J., Isersky, C. (1985) The receptor for immunoglobulin E on rat basophilic leukemia cells: effect of ligand binding on receptor expression. *Proc. Natl. Acad. Sci. USA* **82**, 1522–1525.
2. Hsu, C., MacGlashan Jr., D. W. (1996) IgE antibody up-regulates high affinity IgE binding on murine bone marrow derived mast cells. *Immunol. Lett.* **52**, 129–134.
3. MacGlashan Jr., D. W., White-Mckenzie, J., Chichester, K., Bochner, B. S., Davis, F. M., Schroeder, J. T., Lichtenstein, L. M. (1998) In vitro regulation of Fc ϵ RI α expression on human basophils by IgE antibody. *Blood* **91**, 1633–1643.
4. Yamaguchi, M., Lantz, C. S., Oettgen, H. C., Katona, I. M., Fleming, T., Miyajima, I., Kinet, J. P., Galli, S. J. (1997) IgE enhances mouse mast cell Fc ϵ RI expression in vitro and in vivo: evidence for a novel amplification mechanism in IgE-dependent reactions. *J. Exp. Med.* **185**, 663–672.
5. Yamaguchi, M., Sayama, K., Yano, K., Lantz, C. S., Noben-Trauth, N., Ra, C., Costa, J. J., Galli, S. J. (1999) IgE enhances Fc ϵ RI expression and IgE-dependent release of histamine and lipid mediators from human umbilical cord blood-derived mast cells: synergistic effect of IL-4 and IgE on human mast cell Fc ϵ RI expression and mediator release. *J. Immunol.* **162**, 5455–5465.
6. Lee, W. T., Conrad, D. H. (1986) Murine B cell hybridomas bearing ligand-inducible Fc receptors for IgE. *J. Immunol.* **136**, 4573–4580.
7. Lee, W. T., Rao, M., Conrad, D. H. (1987) The murine lymphocyte receptor for IgE. IV. The mechanism of ligand-specific receptor upregulation on B cells. *J. Immunol.* **139**, 1191–1198.
8. Saini, S. S., MacGlashan Jr., D. W., Sterbinsky, S. A., Togias, A., Adelman, D. C., Lichtenstein, L. M., Bochner, B. S. (1999) Down-regulation of human basophil IgE and Fc ϵ RI α surface densities and mediator release by anti-IgE infusions is reversible in vitro and in vivo. *J. Immunol.* **162**, 5624–5630.
9. MacGlashan Jr., D. (2002) Anti-IgE antibody therapy. *Clin. Allergy Immunol.* **16**, 519–532.
10. MacGlashan Jr., D. W., Xia, H. Z., Schwartz, L. B., Gong, J. P. (2001) IgE-regulated expression of Fc ϵ RI in human basophils: control by regulated loss rather than regulated synthesis. *J. Leukoc. Biol.* **70**, 207–218.
11. Borkowski, T. A., Jouvin, M. H., Lin, S. Y., Kinet, J. P. (2001) Minimal requirements for IgE-mediated regulation of surface Fc ϵ RI. *J. Immunol.* **167**, 1290–1296.
12. MacGlashan Jr., D. W., Lichtenstein, L. M., McKenzie-White, J., Chichester, K., Henry, A. J., Sutton, B. J., Gould, H. J. (1999) Upregulation of Fc ϵ RI on human basophils by IgE antibody is mediated by interaction of IgE with Fc ϵ RI. *J. Allergy Clin. Immunol.* **104**, 492–498.
13. Marolewski, A. E., Buckle, D. R., Christie, G., Earnshaw, D. L., Flamberg, P. L., Marshall, L. A., Smith, D. G., Mayer, R. J. (1998) CD23 (Fc ϵ RII) release from cell membranes is mediated by a membrane-bound metalloprotease. *Biochem. J.* **333**, 573–579.
14. MacGlashan Jr., D. W., Peters, S. P., Warner, J., Lichtenstein, L. M. (1986) Characteristics of human basophil sulfidopeptide leukotriene release: releasability defined as the ability of the basophil to respond to dimeric cross-links. *J. Immunol.* **136**, 2231–2239.
15. MacGlashan Jr., D. W., Schroeder, J. T. (2000) Functional consequences of Fc ϵ RI α up-regulation by IgE in human basophils. *J. Leukoc. Biol.* **68**, 479–486.
16. Pruzansky, J. J., Grammer, L. C., Patterson, R., Roberts, M. (1983) Dissociation of IgE from receptors on human basophils. I. Enhanced passive sensitization for histamine release. *J. Immunol.* **131**, 1949–1953.
17. MacGlashan Jr., D. W., White, J. M., Huang, S. K., Ono, S. J., Schroeder, J., Lichtenstein, L. M. (1994) Secretion of interleukin-4 from human basophils: the relationship between IL-4 mRNA and protein in resting and stimulated basophils. *J. Immunol.* **152**, 3006–3016.
18. Gilbert, H. S., Ornstein, L. (1975) Basophil counting with a new staining method using alcian blue. *Blood* **46**, 279–286.
19. MacGlashan Jr., D. W. (2003) Two regions of down-regulation in the IgE-mediated signaling pathway in human basophils. *J. Immunol.* **170**, 4914–4925.
20. Saini, S. S., Richardson, J. J., Wofsy, C., Lavens-Phillips, S., Bochner, B. S., MacGlashan Jr., D. W. (2001) Expression and modulation of Fc ϵ RI α and Fc ϵ RI β in human blood basophils. *J. Allergy Clin. Immunol.* **107**, 832–841.
21. Kitaura, J., Xiao, W., Maeda-Yamamoto, M., Kawakami, Y., Lowell, C. A., Kawakami, T. (2004) Early divergence of Fc ϵ RI signals for receptor up-regulation and internalization from degradation, cytokine production, and survival. *J. Immunol.* **173**, 4317–4323.

22. Miura, K., MacGlashan Jr., D. W. (2000) Phosphatidylinositol-3 kinase regulates p21ras activation during IgE-mediated stimulation of human basophils. *Blood* **96**, 2199–2205.
23. Miura, K., Saini, S. S., Gauvreau, G., MacGlashan Jr., D. W. (2001) Differences in functional consequences and signal transduction induced by IL-3, IL-5 and NGF in human basophils. *J. Immunol.* **167**, 2282–2291.
24. MacGlashan Jr., D. W., Vilarino, N. (2006) Nonspecific desensitization, functional memory and the characteristics of SHIP phosphorylation following IgE-mediated stimulation of human basophils. *J. Immunol.* **177**, 1040–1051.
25. MacGlashan Jr., D. W., Mogowski, M., Lichtenstein, L. M. (1983) Studies of antigen binding on human basophils. II. Continued expression of antigen-specific IgE during antigen-induced desensitization. *J. Immunol.* **130**, 2337–2342.
26. MacGlashan Jr., D. W. (2007) Relationship between Syk and SHIP expression and secretion from human basophils in the general population. *J. Allergy Clin. Immunol.* **119**, 626–633.
27. Kubota, T., Mukai, K., Minegishi, Y., Karasuyama, H. (2006) Different stabilities of the structurally related receptors for IgE and IgG on the cell surface are determined by length of the stalk region in their α -chains. *J. Immunol.* **176**, 7008–7014.
28. Pastorelli, C., Veiga, J., Charles, N., Voignier, E., Moussu, H., Monteiro, R. C., Benhamou, M. (2001) IgE receptor type I-dependent tyrosine phosphorylation of phospholipid scramblase. *J. Biol. Chem.* **276**, 20407–20412.
29. Charles, N., Monteiro, R. C., Benhamou, M. (2004) p28, a novel IgE receptor-associated protein, is a sensor of receptor occupation by its ligand in mast cells. *J. Biol. Chem.* **279**, 12312–12318.