# Spontaneous Phosphorylation of the Receptor with High Affinity for IgE in Transfected Fibroblasts

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*Received December 4, 2000; Revised Manuscript Received January 30, 2001*

ABSTRACT: Receptors with high affinity for IgE, FcεRI, which had been transfected into Chinese hamster ovary fibroblasts exhibit an over 20-fold greater spontaneous phosphorylation at physiological temperatures than the same receptors on the widely studied rat mucosal mast cell line, RBL-2H3. This enhanced phosphorylation was not accounted for either by changes in the src-family kinase responsible for the phosphorylation, by reduced activity of phosphatases, or by spontaneous association of the receptors with microdomains. A variety of approaches failed to detect evidence for stable spontaneous aggregates of the receptor. Whereas the altered posttranslational glycosylation of the receptor's principal ectodomain we detected could promote transient spontaneous aggregation and explain the observed effect, other changes in the membrane milieu cannot be excluded. The functional consequences of such spontaneous phosphorylation are considered.

The receptor with high affinity for IgE, FcεRI,[1] is one of a family of multichain receptors found on cells of the immune system (*1*). When cells are stimulated through such receptors, phosphorylation of tyrosines in characteristic motifs present on one or more of the cytoplasmic domains of the receptor itself is the earliest observed event (*2*−*4*). In those cells in which FcεRI have been studied most intensively, rat basophilic leukemia cells (RBL) (*5, 6*), there is persuasive evidence that the Src-family kinase Lyn mediates the phosphorylation (*7*). The phosphorylation that follows clustering of the receptors by a variety of procedures, but normally by interaction of the receptor-bound IgE with a multivalent antigen, has been explained by two, somewhat distinctive, but potentially complementary molecular mechanisms.

In one paradigm, phosphorylation is promoted because the aggregated FcεRI preferentially coalesce with specialized microdomains in the plasma membrane that are enriched in the kinase (*8*). In the alternative model we have forwarded, the role of aggregation is to promote an initial transphosphorylation of the receptors by the Lyn kinase constitutively associated with the FcεRI (*9, 10*). That is, the binding of multivalent antigen is proposed to shift the dynamic balance of phosphorylation and dephosphorylation that results from the spontaneous association/dissociation of receptors.

To examine the early events in isolation, we previously transfected Chinese hamster ovary (CHO) fibroblasts with FcεRI. Such cells, which have little endogenous Lyn kinase, were then additionally transfected with various constructs of Lyn to see how these affected the aggregation-induced phosphorylation of the receptors (*11*). Coincidentally, we observed that in the resting transfectants, that is, in cells whose FcεRI had not been deliberately aggregated, the receptors showed substantially more baseline phosphorylation than was observed on the RBL cells. No comparable increase in spontaneous phosphorylation of other cellular proteins in the CHO cells was noted. Because this implies a potential variable that is unaccounted for in the current molecular models, we decided to investigate this phenomenon.

## MATERIALS AND METHODS

The anti-DNP murine IgE has been described (*12, 13*). IgE conjugated with fluorescein isothiocyanate (FITC) was a kind gift from Prof. Richard Posner (North Arizona University). We conjugated IgE with tetramethylrhodamine isothiocyanate (TRITC) using a published method (*14*). Conjugation of IgE with the sulfoindocyanine succinimidyl ester, Cy3, employed a Cy3 Ab labeling kit (Amersham Pharmacia Biotech, Piscataway, NJ). Conjugated proteins were purified by a gel filtration column and extensively dialyzed. Fluorophor:protein ratios were 3.4 for FITC:IgE, 8.0 for TRITC:IgE, and 6.5 for Cy3:IgE. The DNP antigen used in these studies contained an average of 3.5 DNP-$\epsilon NH_2$ caproate groups per molecule of bovine Fab fragment (*15*). Rabbit anti-rat IgE and goat anti-mouse IgE were affinity-purified and rendered monospecific on appropriate columns of IgE. Mouse monoclonal IgG2b anti-phosphotyrosine 4G10 and the rabbit anti-Lyn antiserum were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Reagents for enhanced chemiluminescence analyses were obtained from Amersham Pharmacia Biotech; polyacrylamide gels were from NOVEX (San Diego, CA).

RBL cells of the 2H3 line were maintained as described previously (*6*). CHO cells, which had been transfected with FcεRI and Lyn, were grown as described in (*11*).

*Cell Lysates and Immunoprecipitation.* Cells were harvested with trypsin−EDTA, washed with buffer A [150 mM

[1] Abbreviations: FcεRI, high-affinity receptor for IgE; PY, *p*-phosphotyrosine; RBL, rat basophilic leukemia cells; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; FRET, fluorescence resonance energy transfer; FACS, fluorescence-activated cell sorting; CHO, Chinese hamster ovary; DTSSP, 3,3′-dithiobis-(sulfosuccinimidylpropionate); ITAM, immunoreceptor tyrosine activation motif.

10.1021/bi0027534 
Published on Web 03/06/2001

NaCl, 5 mM KCl, 5.4 mM glucose, 25 mM Pipes (pH 7.2) containing 0.1% BSA], and resuspended in buffer A at 6.2 $\times$ $10^6$ cells/mL. Two volumes of cell suspension was mixed with 1 volume of ice-cold 3$\times$ solubilization buffer, giving final concentrations of 0.5% TX-100, 50 mM Tris, 50 mM NaCl, 5 mM EDTA, 1 mM $Na_3VO_4$, 5 mM $Na_4P_2O_7$, 50 mM NaF, 2 mM iodoacetate, 1 mM phenylmethanesulfonyl fluoride, 10 $\mu$g/mL each of aprotinin, leupeptin, and pepstatin A (pH 7.6), and incubated for 30 min at 4 °C. After a 10 min centrifugation, Fc$\epsilon$RI were immunoprecipitated from the supernatant with polyclonal anti-IgE or monoclonal antibody to the Fc$\epsilon$RI $\beta$ chain (*16*), using protein A or protein G Sepharose beads (Amersham Pharmacia Biotech). In other experiments, cells were reacted with [$^{125}$I]-IgE, reacted with antigen or not, and lysed, and the lysate was reacted with excess anti-phosphotyrosine (4G10). Completeness of precipitation with the latter antibody was assessed by immunoprecipitating the supernatant with excess anti-IgE and comparing the residual phosphorylated receptors in those immunoprecipitates with the starting material. Under the conditions used, $\geq$95% of the phosphorylated receptors were precipitated by the 4G10.

*Phosphotyrosine Assay.* Immunoprecipitated proteins were extracted with hot sodium dodecyl sulfate sample buffer and electrophoresed on polyacrylamide gels. Phosphotyrosine was detected by Western blotting using antibody 4G10 under conditions that favored accurate quantitation as described previously (*11, 17*).

*Assay of Lyn Activity.* Incorporation of $^{32}$P into a Src kinase-specific cdc2-derived peptide was used to measure the activity of Lyn that had been immunoprecipitated from cell lysates (*18*). The precipitates were incubated with 2 mM peptide in kinase assay buffer (0.1 mM ATP, 0.5 mM $Na_3VO_4$, 2 mM CHAPS, 5 mM $MnCl_2$ in A-buffer). The assay was performed with Pierce phosphocellulose units (Pierce, Rockford, IL).

*Chemical Cross-Linking.* In certain experiments, cells were sensitized with an $\approx$1:1 mixture of rat IgE and $^{125}$I-labeled mouse IgE. Some aliquots were permeabilized (below). The cells suspended in assay buffer (25 mM Hepes, 119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, pH 8.0) were incubated with 4 mM 3,3′-dithiobis-(sulfosuccinimidylpropionate) (DTSSP) (Pierce) at 37 °C for 10 min. The amount of coprecipitated radioactive mouse IgE was measured in anti-rat IgE immunoprecipitates. Cross-linking of Lyn with receptor was done essentially as described previously (*9*). Cells that had been sensitized with IgE were washed and resuspended in assay buffer. The cells were then permeabilized by Streptolysin-O (Murex, Dartford, U.K.) (*19*). In some experiments, multivalent antigen was added to aggregate receptors. DTSSP was added to the cell suspension at 37 °C, and after 3 min, 40 mM glycine was added to stop the reaction. The cells were then lysed in solubilization buffer, and the receptors were immunoprecipitated. The precipitated proteins were reduced in order to cleave the cross-linking bonds, and separated on polyacrylamide gels. The proteins were then transferred to nitrocellulose membranes and analyzed for Lyn and for the $\beta$-chain of Fc$\epsilon$RI by Western blotting.

*Distribution of Lyn and Receptors on Plasma Membrane.* Cell lysate (1−1.4 mL), prepared by reacting 1 $\times$ $10^7$ cells/mL with 0.1% Triton-X 100, was mixed with an equal volume of 85% sucrose in Ultraclear centrifuge tubes (Beckman, Palo Alto, CA). This was overlaid with 6 mL of 30% sucrose and 3.5 mL of 5% sucrose. Gradients were then centrifuged at 200000*g*, at 37 °C, in a Beckman SW40Ti rotor for 4 h (*20*). One milliliter fractions were harvested from the top of the gradient. An opaque band representing the lipid rafts was always observed and harvested routinely in the fourth fraction. All fractions were counted in a gamma counter to assess the distribution of $^{125}$I-labeled IgE. Aliquots of these fractions were also subjected to SDS−PAGE followed by Western blotting for Lyn.

*Assay of Phosphatase Activity.* Phosphatase activity was measured against a bis-phosphorylated peptide (KSDAV-[pY]TGLNTRNQET[pY]ETLKHEK-OH) which represents the immunoreceptor tyrosine activation motif (ITAM) (*21*) of the Fc$\epsilon$RI $\gamma$ chain. The residual phosphopeptide was quantified by an enzyme-linked immunoassay using a kit distributed by Boehringer Mannheim (Indianapolis, IN) (*20*).

*Sequencing of Receptor Subunits.* The amino acid sequence of each of the receptor subunits was indirectly determined both at the mRNA and at the genomic DNA level by using the Big Dye kit obtained from Applied Biosystems (Foster City, CA).

*Fluorescence Microscopy.* Cells were sensitized with an $\approx$1:1 mixture of FITC- and TRITC-conjugated IgE. After staining and fixing cells with 3.5% paraformaldehyde at 37 °C, the distribution of IgE was monitored under a Leica fluorescence microscope. Mouse monoclonal anti-FITC antibody (Sigma, St. Louis, MO) was used to aggregate the FITC-IgE.

*Fluorescence Resonance Energy Transfer (FRET).* FRET was measured essentially as described (*22, 23*). To perform the measurement at $\approx$37 °C, the sheath fluid was warmed just before the measurement, and the cell suspension was kept at 37 °C by suspending the sample tube in 37 °C water during the measurement. Fluorescence-activated cell sorting was done by a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ). The 488 nm line of the argon ion laser was used for excitation, and the emitted fluorescence was detected at two wavelengths through a 530 nm or a 585 nm band-pass interference filter.

*Treatment of Cells with Neuraminidase.* For treatment with neuraminidase, the cells were suspended in 0.01 M phosphate-buffered 0.15 M NaCl (pH 7.2) with 0.1% BSA. Neuraminidase from *Vibrio cholerae* (Boehringer Mannheim) in a buffer containing 50 mM $NaCH_3COO$, 154 mM NaCl, 9 mM $CaCl_2$, 0.05% $NaN_3$, 25 mg/mL human serum albumin (pH 5.5) was added in a 1:2.33 volume:volume ratio for 30−60 min at 37 °C. Cells were washed after the enzyme reaction, and phosphorylation of receptor was analyzed.

*Surface Iodination and Treatment of Receptors with N-Glycanase.* Cells were surface-iodinated with Iodogen from Pierce (*24*). They were then solubilized with detergent (above), and the extract was incubated with Protein A beads that had been preloaded with ascitic fluid containing a monoclonal antibody to the $\alpha$ subunit of Fc$\epsilon$RI. Using the recommendations of the manufacturer of *N*-glycanase (Glyko, Novato, CA), the samples were then boiled with 1% sodium dodecyl sulfate for 5 min before addition of a reaction buffer, NP-40, and the recombinant enzyme. After incubation at 37 °C for 18 h, the samples, including controls to which no

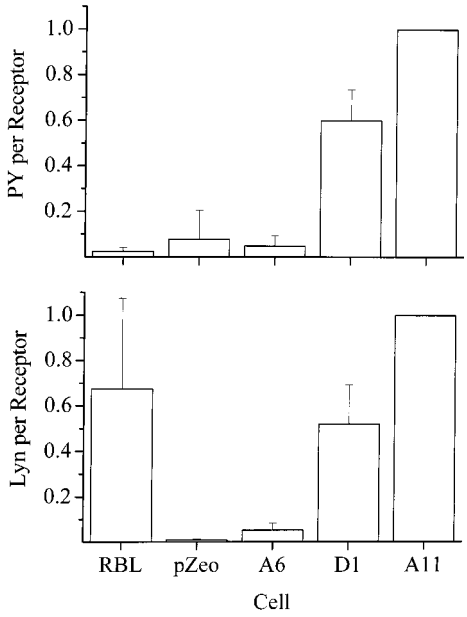


FIGURE 1: Spontaneous phosphorylation of receptors. Cells were incubated with IgE, washed, and lysed with 0.5% Triton X-100. Western blotting was used to assess the phosphorylation of the receptors and the amount of cellular Lyn. The bars show the amount of PY or Lyn relative to that observed on the A11 cells analyzed on the same gel. The error bars show the standard deviations in the four to six experiments in which the clones were analyzed. For the number of FcεRI associated with each clone, see the text. Top panel: The IgE−receptor complexes were immunoprecipitated with anti-IgE and analyzed for phosphotyrosine (PY) associated with the $\beta$ and $\gamma$ subunits of the receptor. The data were corrected for the number of receptors by blotting with anti-$\beta$ subunit. Bottom panel: An aliquot of the lysate was analyzed by blotting with anti-Lyn.

enzyme had been added, were processed for electrophoresis on polyacrylamide gels in sodium dodecyl sulfate.

## RESULTS

*FcεRI in CHO Cells Are Spontaneously Phosphorylated.* The upper panel in Figure 1 shows the basic finding we decided to explore. It summarizes the data from four to six experiments in which each of several transfected CHO cell lines was compared to the RBL cells with respect to the amount of phosphotyrosine on the receptors of unstimulated cells. All of the CHO cells had been transfected with FcεRI. The transfectant "pZeo" had in addition been transfected with vector containing the antibiotic marker Zeocin. Transfectants "A6", "D1", and "A11" had been transfected with vector containing, in addition to the Zeocin marker, the open reading frame for the "short" form of rat Lyn kinase, and expressed that kinase to a variable extent (*11*).

The bar graph presents the results as phosphotyrosine per receptor. These ratios were determined from quantitative analyses of Western blots using anti-phosphotyrosine and antibodies to the $\beta$ subunit of the receptor. The figure shows the substantial difference between the cells with respect to the extent to which their FcεRI are phosphorylated. The ratio of the spontaneously formed phosphotyrosine on the dimer of $\gamma$ chains relative to the amount on the $\beta$ chain was approximately 2:1, similar to the ratio observed on the aggregated receptors of RBL cells and the CHO transfectants (*25*). Therefore, in the results depicted in the figure, we combined the amount of phosphotyrosine expressed on both the $\beta$ chain and the dimer of $\gamma$ chains.

Table 1: Fraction of Tyrosine-Phosphorylated FcεRI in Resting and Stimulated RBL Cells and CHO Transfectants As Assessed by Precipitation with Anti-Phosphotyrosine[a]

| cell | antigen | % FcεRI precipitated |
|---|---|---|
| RBL | − | 0.62 (0.074) |
| | +[b] | 12 (10) |
| CHO-A11 | − | 11 (0.92) |
| | + | 33 (4.1) |

[a] Cells were saturated with [$^{125}$I]-IgE, reacted with antigen (or not), and lysed. The lysate was reacted with excess anti-phosphotyrosine (see Materials and Methods), and the percent of labeled IgE precipitated was determined. The data are from three separate experiments in which resting and stimulated RBL and CHO cells were compared directly. Quadruplicate samples of each condition were assayed, and the receptors in duplicate aliquots were solubilized with or without hapten in the solubilization buffer (*19*). Because there was no consistent trend between the two methods of solubilization, the data for the quadruplicates have been pooled. The values shown are the averages for all the experiments with the standard deviation shown in parentheses. [b] Cells were reacted with 50 ng/mL [DNP-cap]$_{3.5}$-Fab for 3 min prior to solubilization.

In prior studies, we observed that the extent of phosphorylation of the aggregated FcεRI was sensitive to the relative amount of Lyn (*11*, *17*, *18*). The lower panel in Figure 1 presents the ratio of total cellular Lyn kinase per receptor in the several transfectants as determined by quantitative analysis of Western blots using antibodies to Lyn and to the $\beta$ subunit. It is apparent that the variation in the ratios of total kinase to receptor is insufficient to explain the marked differences in spontaneous phosphorylation. It is also clear that whereas there is some proportionality between the amount of Lyn expressed in the transfectants and the extent of the phosphorylation, this is not the case with the RBL cells, where the spontaneous phosphorylation is virtually undetectable despite a substantial amount of Lyn per receptor.

In one experiment, the absolute number of receptors for each cell type was determined and found to be $3.5 \times 10^5$ for the RBL cells, and $1.3 \times 10^5$, $1.3 \times 10^5$, $1.0 \times 10^5$, and $1.1 \times 10^5$, respectively, for the pZeo, A6, D1, and A11 CHO transfectants. These values are so close to what we had previously found (*11*), that this analysis was not repeated for each experiment.

*Spontaneous versus Induced Phosphorylation.* Table 1 summarizes several experiments in which the fractions of tyrosine-phosphorylated receptors in resting and stimulated cells were compared. In resting RBL cells whose receptors have been filled with monomeric IgE, less than 1% of the FcεRI are tyrosine-phosphorylated at any instant in time by this criterion. Upon incubation with 50 ng/mL of a paucivalent DNP conjugate ([DNP-cap]$_{3.5}$-Fab) for 3 min—a modest stimulus although one sufficient to aggregate a substantial fraction of the receptors—there is an almost 20-fold increase in the number of receptors precipitable by anti-phosphotyrosine.

By contrast, in the resting A11 clone roughly 10% of the receptors were precipitated by anti-phosphotyrosine. When the latter cells were stimulated with antigen, there was a further substantial increase, the differential between the resting and stimulated cells (in absolute terms) being certainly no less than that observed for the RBL cells.

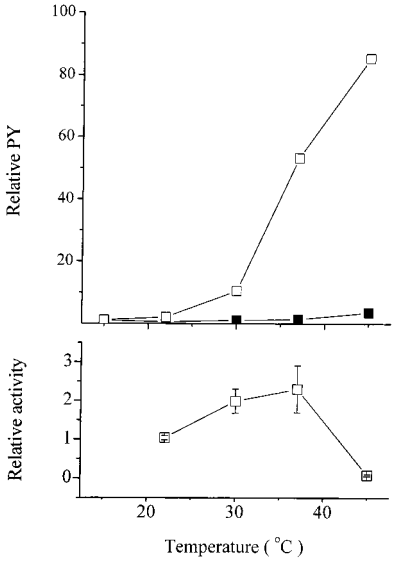


FIGURE 2: Temperature effect on spontaneous phosphorylation and activity of Lyn kinase. Top panel: Cells were incubated with IgE, washed, and maintained at the indicated temperature for 10 min. The cells were then lysed in 0.5% Triton X-100, and the IgE−receptor complexes were immunoprecipitated with anti-IgE and analyzed for phosphotyrosine (PY) associated with the $\beta$ and $\gamma$ subunits of the receptor by Western blotting. (■) RBL cells; (□) A11 cells. The ordinate shows the amount of PY relative to that observed on the RBL cells incubated at 15 °C. Bottom panel: Immunoprecipitates of Lyn kinase were assayed for kinase activity using a peptide relatively specific for Src-family kinases. The graph shows the results of two independent experiments in each of which triplicate determinations were performed at each temperature. The data from each experiment were normalized to one of the three samples assayed at 22 °C. Where not seen, the error bars (standard deviations) were smaller than the size of the symbol. Note that the ordinate scale in this panel is 13-fold-expanded compared to the top panel.

*Temperature Dependence.* Early in our studies we observed that the spontaneous phosphorylation was markedly dependent on temperature (top panel, Figure 2). Little spontaneous phosphorylation was detected at 15 °C, but at higher temperatures, the level of phosphorylation was dramatically increased in the A11 cells. Virtually no phosphorylation was observed in resting RBL cells within this range of temperatures.

*Role of Kinase.* We investigated whether the spontaneous phosphorylation results from Lyn being unusually active in these transfectants. To some extent, this appears unlikely because we had previously noted that the transfectants exhibited no generalized increase in phosphotyrosine-containing proteins (*11*) and this was confirmed in the current studies (data not shown). Direct assessment of the activity of Lyn confirmed the indirect data. Table 2 shows the results of experiments in which Lyn was immunoprecipitated from cell lysates. We quantitated both the amount of immunoreactive protein (column 2) and the kinase activity against a src-family specific peptide substrate (column 3). The control immunoprecipitates with normal rabbit IgG from the lysates of both RBL and A11 cells contained only a small amount of putative Lyn by Western blotting and a small amount of kinase activity (footnote *a* in Table 2). Compared to the control precipitates, the precipitates prepared with anti-Lyn showed over 100-fold more Lyn protein and roughly 200-fold more activity. Significantly, within experimental variation, there was no consistent difference in the results between the A11 and RBL cells, and the calculated ratio for the specific activities of Lyn in the specific immunoprecipitates from the two cell types (value in boldface type at the bottom of data in column 4) was remarkably close to 1. RBL cells express both the long and short forms of Lyn in roughly equivalent amounts, whereas the A11 clone had been transfected with the short form only. The results of the kinase assays suggest that the long and short forms of Lyn have the same or similar specific activities.

Table 2: Relative in Vitro Kinase Activity of Immunoprecipitated Lyn from Resting RBL Cells and CHO Transfectants[a]

| cell type | amount | relative[b] activity | specific activity[c] |
|---|---|---|---|
| RBL-2H3 | 14.6 (1.26) | 233 (23.6) | 16.2 (3.01) |
| CHO-A11 | 11.1 (0.20) | 183 (53.5) | 16.5 (3.92) |
| CHO/RBL | 0.76 | 0.78 | **1.02** |

[a] The data are from two separate experiments in which resting RBL and CHO cells were compared directly. Duplicate samples of each cell extract were immunoprecipitated either with nonspecific rabbit IgG or with anti-Lyn antibodies. The washed precipitates were analyzed either for Lyn protein (by Western blotting) or for tyrosine kinase activity. The values shown are the averages for the two experiments with the range shown in parentheses and are for the specific precipitates that have been corrected for the values associated with the nonspecific precipitates. For the RBL-2H3 cells, these nonspecific precipitates contained amounts and activity of 0.127 (±0.013) and 0.939 (±0.0123), respectively. The corresponding values for the A11 cells were 0.089 (±0.015) and 0.856 (±0.303). [b] To simplify the table, the experimental readings have been multiplied by arbitrary factors: The densitometric readings from the autophotographs of Western blots used to quantify the amount of Lyn have been multiplied by $10^{-2}$, the counts incorporated in the enzyme assay by $10^{-4}$, and the specific activity therefore by $10^{-2}$. [c] These values are the average values from the individual experiments and therefore differ slightly from the values obtained by simply dividing the values shown in column 3 by those in column 2.

In view of the marked effect of temperature on the spontaneous phosphorylation of FcεRI in the CHO cells, we directly assessed the temperature dependence of the activity of Lyn. As shown in the lower panel of Figure 2, the activity of Lyn against a Src kinase-specific peptide is much lower at 45 °C than that at 37 °C, whereas spontaneous phosphorylation of the receptors still increases over this temperature range (top panel, Figure 2).

In previous studies on both RBL cells and the CHO cell transfectants, we concluded that the extent of phosphorylation of tyrosines on the receptor was governed by the availability of Lyn kinase to the receptors. For example, in RBL cells, direct analyses with chemical cross-linking reagents revealed that only 3−4% of the receptors are constitutively associated with Lyn (*9*), and with appropriate protocols, we documented competition between clustered receptors for the kinase (*26*). Likewise, as already noted, CHO cells transfected with FcεRI and expressing increasing amounts of transfected Lyn exhibit correspondingly greater responses to equivalent stimuli.

It was possible, therefore, that in the spontaneously phosphorylating transfectants, the percent of receptors associated with Lyn was enhanced. One such analysis using Western blotting is shown in Figure 3A. Resting cells that had been incubated with monomeric IgE were permeabilized and reacted with cross-linking reagent, lysed, and immunoprecipitated with anti-IgE. The upper bands were revealed in a Western blot utilizing anti-Lyn. In the RBL cells (two left lanes), the long and short forms of Lyn are clearly seen.

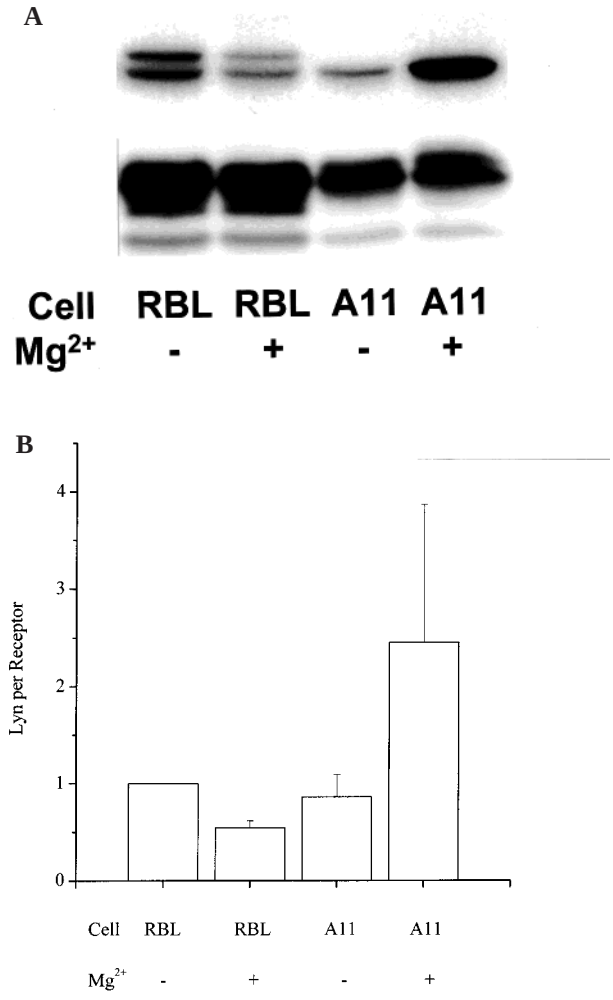


FIGURE 3: Association of Lyn with unaggregated receptors. The association of the kinase with the receptors was assessed under conditions in which kinase was inhibited ($-Mg^{2+}$) or not inhibited ($+Mg^{2+}$). Cells were incubated with monomeric IgE, permeabilized, and reacted with the chemical cross-linking reagent for 3 min at 37 °C (Materials and Methods). After the reaction was quenched, the cells were lysed, and the immunoprecipitated receptors from each sample were loaded onto a single gel and transferred. The membrane was then cut into an upper portion for blotting with anti-Lyn, and a lower portion for blotting with antibody to the $\beta$ subunit of the receptor. All the cells had been permeabilized. (A) Example of one experiment. (B) Summary of four experiments. For each experiment, the amount of Lyn associated with the receptor was normalized to the amount associated with the receptors from RBL cells exposed to $Mg^{2+}$-free buffer. The error bars show the standard deviations.

The A11 cells (two right lanes) show only the short form with which the cells had been transfected. (The reaction of the antibody with the endogenous Lyn is too weak to be observable using the time of exposure shown.) The bands at the bottom were seen on the same membrane after blotting with anti-$\beta$ antibodies. Because phosphorylated receptors recruit additional molecules of Lyn (*9*, *10*), we also examined a parallel set of permeabilized cells that were suspended in a buffer free of $Mg^{2+}$ conditions that inhibit the activity of Lyn kinase (*9*, *27*). The receptors in such preparations should be associated only with the amount of Lyn with which they were constitutively associated. Panel B of Figure 3 summarizes our findings from six experiments performed in the presence and four in the absence of $Mg^{2+}$. For each comparison, the relative value of Lyn/$\beta$ was normalized to the value obtained for the permeabilized RBL cells exposed to $Mg^{2+}$-free buffer. The composite data show no evidence for enhanced constitutive association of the Lyn kinase with the receptor in the A11 transfectants. They also confirm that phosphorylated Fc$\epsilon$RI recruit additional molecules of Lyn.

In earlier work we had evidence that the spontaneous dephosphorylation of aggregated receptors in RBL cells (see below) is accompanied by a dissociation of Lyn kinase from the aggregates [see Figure 4 in (*26*)]. There are also a variety of experimental results that we have interpreted as resulting from such dissociation being due to interaction of the aggregated receptors with cytoskeletal components (manuscript in preparation). Therefore, we exposed the cells to conditions that stabilize the phosphorylation of aggregated Fc$\epsilon$RI in RBL cells. One such manipulation is inhibition of F-actin, e.g., by cytochalasin (*28*); another is allowing the cells to adhere to a surface (*29*). We observed no enhancement of spontaneous phosphorylation of receptors in the A11 cells with up to 5 $\mu$M cytochalasin D, and, if anything, adherent A11 cells showed a slight decrease in spontaneous phosphorylation (data not shown).

*Role of Phosphatases.* The phosphorylation of aggregated Fc$\epsilon$RI reflects a dynamic balance between the action of kinase and phosphatase(s). Thus, if kinase activity is inhibited, aggregated receptors that had become tyrosine-phosphorylated are promptly dephosphorylated (*27*). Contrariwise, in the presence of phosphatase inhibitors, the extent of phosphorylation of aggregated receptors is enhanced, and a small amount of "spontaneous" phosphorylation of unaggregated receptors is observed (*30*, *31*). There are no compelling data in the literature to suggest that cells utilize changes in the specific activity of phosphatases to regulate phosphorylation of Fc$\epsilon$RI. Nevertheless, we wished to determine if alterations in phosphatase activity could account for the spontaneous phosphorylation of the Fc$\epsilon$RI in the CHO cells transfected with Lyn kinase.

*(i) Activated Cells.* Particularly in RBL cells studied in suspensions, the aggregation-induced phosphorylation of their Fc$\epsilon$RI is relatively transient. That a similar though less rapid dephosphorylation is observed in stably aggregated receptors (*27*) suggests a time-dependent shift in the dynamic equilibrium between phosphorylation and dephosphorylation. The basis of this shift is only partially understood, but, as noted above, may in part be related to spontaneous dissociation of Lyn kinase from the receptors. We examined the CHO transfectants to see if a similar shift was observed in them. The experiment shown in Figure 4 compares the time course of receptor phosphorylation in A11 and RBL cells bearing anti-DNP IgE and incubated with 50 ng of a paucivalent DNP-antigen. It is apparent that the spontaneous dephosphorylation observed after stimulation with antigen is as prominent in the A11 cells as in the RBL cells. Dephosphorylation is also seen when antigen-clustered Fc$\epsilon$RI are disaggregated, e.g., by addition of hapten (*4*, *31*). In either of the two mechanistic models describing how aggregation of the receptors promotes phosphorylation of the receptors (see the introduction), the disaggregation induced by the hapten leads to decreased exposure of the Fc$\epsilon$RI to the Lyn kinase whereas the action of omnipresent phosphatase(s) remains unabated. In the experiment shown in Figure 5 we followed the time course of hapten-induced receptor dephos-

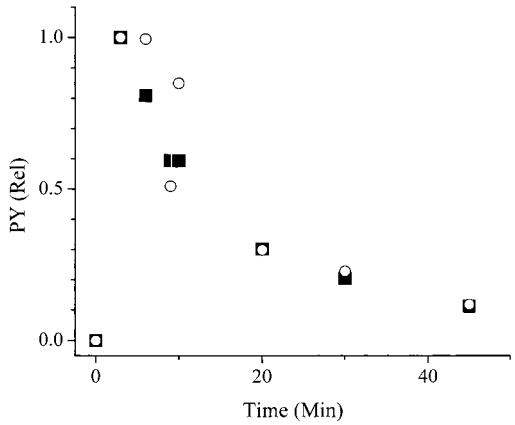


FIGURE 4: Spontaneous dephosphorylation of receptors. Cells bearing DNP-specific IgE were stimulated with 50 ng/mL antigen, and detergent extracts were prepared after increasing times. The phosphotyrosine on the receptors was assessed by Western blotting. The results from two independent experiments are shown, and the data were analyzed as follows. For each experimental group, the "spontaneous" amount of phosphorylation observed at time zero (actually in the samples that were not stimulated with antigen) was subtracted from all of the subsequent time points. In the case of the RBL cells (■), this equaled about 2% of the maximum observed; for the A11 cells (○), about 9%. The net values were then normalized to the maximum value observed for the RBL and A11 cells, respectively.

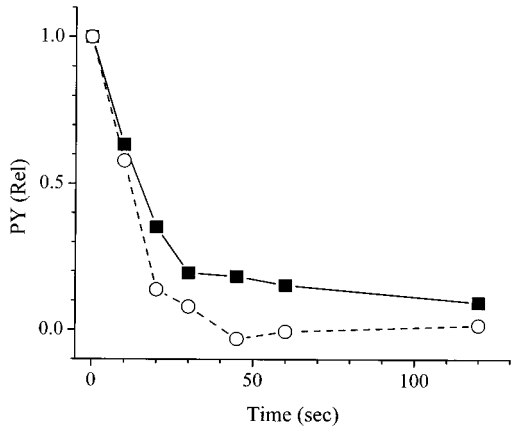


FIGURE 5: Dephosphorylation induced by disaggregation of receptors. Cells bearing DNP-specific IgE were stimulated with 50 ng/mL antigen for 3 min. At that point (time zero in the graph), one aliquot was treated with detergent directly, and the hapten DNP-caproate was added to a second aliquot (final concentration 0.1 mM). Periodically thereafter, samples of the hapten-containing suspension were exposed to detergent. The phosphotyrosine on the subunits of the FcεRI immunoprecipitated from the detergent extracts was quantitated by Western blotting. The results shown are from a single experiment. The data were analyzed as follows: For each experimental group, the phosphorylation observed in the samples that were not stimulated with antigen was subtracted from all of the samples. The resulting net phosphorylation was then normalized to the value obtained for the samples treated with antigen but no hapten and sampled at 3 min. (■) RBL; (○) A11.

phorylation in RBL and A11 cells. Again, no significant difference was observed in this and a second experiment.

*(ii) Resting Cells.* We also examined the status of the phosphatases in the resting cells. We performed in vitro assays under conditions that maximized the possibility that we could detect differences in phosphatases that may be particularly directed to the receptor. We therefore utilized a bis-phosphorylated peptide corresponding to the ITAM of the FcεRI $\gamma$ chain, and compared the phosphatase activity in the total cell lysate of resting RBL and A11 cells. In five such experiments, we observed no significant difference in the relative phosphatase activity toward the receptor-related peptide (data not shown). We also checked the dependence on temperature of the cellular phosphatases against a bis-phosphorylated ITAM peptide of the FcεRI $\gamma$ chain. Above 30 °C, their activity was modestly *increased* and therefore could not explain the large increase of spontaneous phosphorylation.

Table 3: Coprecipitation of Receptors[a]

| no. of expt | cell | aggreg. | chemical cross-linking | permeabilized | % coprecipitation[b] |
|---|---|---|---|---|---|
| 1 | RBL | Ag | − | − | 0.109 |
| 1 | RBL | Ab | − | − | 0.181 |
| 1 | A11 | Ab | − | − | 0.113 |
| 3 | RBL | Ag | + | − | 1.672 (0.126) |
| 3 | A11 | Ag | + | − | 0.990 (0.080) |
| 2 | RBL | − | + | − | 1.326 (0.262) |
| 2 | A11 | − | + | − | 0.733 (0.242) |
| 1 | RBL | Ag | + | + | 0.694 |
| 1 | A11 | Ag | + | + | 0.659 |
| 1 | RBL | − | + | + | 0.576 |
| 1 | A11 | − | + | + | 0.645 |

[a] Cells were incubated with an approximately 1:1 mixture of rat and $^{125}$I-labeled mouse IgE, then permeabilized (or not), exposed to antigen ("Ag") or anti-rat IgE ("Ab") (or not), and then exposed to the chemical cross-linker (or not). Duplicate specimens were used in each experiment. After solubilization, all the lysates were immunoprecipitated with anti-rat IgE. [b] In all cases, the results of the duplicate samples were averaged; where multiple experiments were performed, the average and the standard deviation (in parentheses) for all the experiments are shown.

*Role of Aggregation.* Because we found no evidence for unusual alterations in either the Lyn kinase or the phosphatases in the transfectants, and because in the principal mechanistic models phosphorylation of FcεRI is initiated by aggregation, we looked for evidence of spontaneous aggregation as well as for factors that might favor such associations.

*(i) Direct Studies.* Using the same approach that had led to the demonstration that the receptors on RBL cells are univalent (*32*), receptors were loaded with FITC- or TRITC-labeled IgE in an ≈1:1 ratio, and after washing, bivalent anti-FITC antibodies were added. The distribution of receptors was monitored by fluorescence microscopy. Under conditions where FITC-IgE-bound receptors formed capping structures, TRITC-IgE-bound receptors were uniformly distributed, and no co-capping was observed (data not shown). This result showed that no substantial fraction of receptors stably interacts on either RBL or A11 cells.

Next we attempted to identify spontaneously formed oligomers of receptor by using the chemical cross-linker DTSSP to stabilize such associations. Cells that had been sensitized with rat IgE and $^{125}$I-labeled mouse IgE in an ≈1:1 ratio were reacted with antigen or not reacted and then exposed to the chemical cross-linker. Since DTSSP is impermeant, some aliquots of the cells were permeabilized in order to enhance the likelihood that spontaneous aggregates of the FcεRI would become cross-linked. Coprecipitation of $^{125}$I-labeled mouse IgE in anti-rat IgE immunoprecipitates was measured. As shown in the last column of Table 3, we observed no significant difference in the amount of coprecipitation between RBL and A11 cells.

Table 4: Fluorescence Resonance Energy Transfer[a]

| cell | frac. occup.[b] | no. of expt | antigen | anti-IgE ($\mu$g/mL) | FRET (ratio) |
|---|---|---|---|---|---|
| RBL | 1 | 4 | − | − | 0.390 (0.043)[c] |
| | 0.33 | 4 | − | − | 0.423 (0.056) |
| | 0.67 | 1 | − | − | 0.441 |
| | 1 | 3 | +[d] | − | 0.669 (0.187) |
| | 1 | 4 | − | 9 | 0.518 (0.055) |
| | 1 | 1 | − | 2.9 Cy3[e] | 2.738 |
| | 1 | 2 | − | 9 Cy3 | 20.75 (6.03) |
| A11 | 1 | 7 | − | − | 0.386 (0.036) |
| | 0.33 | 1 | − | − | 0.409 |
| | 0.67 | 1 | − | − | 0.427 |
| | 1 | 3 | +[d] | − | 0.492 (0.166) |
| | 1 | 4 | − | 9 | 0.429 (0.053) |
| | 1 | 1 | − | 2.9 Cy3 | 2.664 |
| | 1 | 2 | − | 9 Cy3 | 6.53 (0.164) |

[a] The results are based on measurements of 20 000 cells per specimen in each experiment. [b] Refers to occupancy with labeled IgE in those experiments in which the labeled IgEs were diluted with unlabeled IgE. [c] Where applicable, the values shown are the averages for the separate experiments and (in parentheses) the standard deviation or range. [d] Stimulation was with 50 ng/mL $[DNP\text{-}cap]_{3.5}$-Fab for 2 min. [e] In these instances, the anti-IgE had been conjugated with Cy3.

We also looked for spontaneous association of receptors using the method of fluorescence resonance energy transfer (FRET). Cells were loaded with FITC- and Cy3-labeled IgE in an ≈1:1 ratio, and energy transfer was measured as the ratio of fluorescence intensity of the acceptor fluorophore, Cy3, to that of the donor fluorophore, FITC (*22, 23*). Individual cells were evaluated by FACS. We observed no resonance energy transfer in either cell type (Table 4). We used three conditions as potential positive controls. In one case, 2.9 or 9 $\mu$g/mL Cy3-labeled anti-IgE antibody was added to the cells loaded with an ≈1:1 ratio of FITC- and Cy3-labeled IgE. Substantial energy transfer was seen within 2 min. We also added unlabeled anti-IgE to cells bearing receptor-bound FITC- and Cy3-IgE. In this instance, the increased energy transfer was only ≈33% in RBL cells and ≈11% in the A11 cells. Aggregation by antigen gave a similarly negligible efficiency of energy transfer.

*(ii) Indirect Studies.* Individual A11 cells express 3-fold fewer FcεRI and on average have a similar surface area as the RBL cells, so that their augmented spontaneous phosphorylation cannot be explained by enhanced spontaneous associations based simply on differences in surface density. However, we examined a number of aspects that might promote such spontaneous aggregation.

In virtually all of the studies already described in which the RBL and transfected clones were compared, the same preparations of IgE were used. Thus, it was unlikely that the phosphorylation we observed only with the resting transfected cells could have been due to adventitious clustering of receptors by small amounts of aggregated IgE. Nevertheless, we directly excluded a role for IgE by exposing the receptors to IgE only in vitro *after* they had been extracted from the cells. A similar level of spontaneous phosphorylation was observed as when the transfectants had bound IgE in vivo (data not shown).

Substitution of $Thr_{52}$ by Ala in the $\gamma$ chain promotes spontaneous phosphorylation of ITAM tyrosines on the $\gamma$ chain by an unknown mechanism (*33*). It was possible that a spontaneously occurring mutation in the transfected FcεRI had led to the same or to an alternative substitution. Therefore, we sequenced the mRNA for each chain of the transfected receptors but found the sequences to be identical to those previously reported (*34*−*36*).

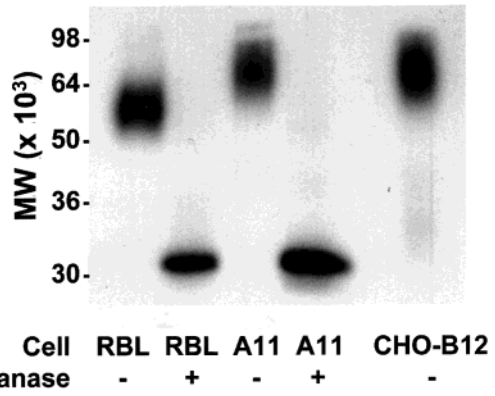


FIGURE 6: Posttranslational differences in receptors. The surface proteins of RBL and CHO transfectants were oxidatively iodinated. The cells were then lysed with Triton X-100, the FcεRI were immunoprecipitated with Protein A beads preincubated with ascitic fluid containing a monoclonal antibody to the α subunit of FcεRI, and the immunoprecipitates were denatured with sodium dodecyl sulfate prior to being treated or not treated with *N*-glycanase. The products were then analyzed on a 12% polyacrylamide gel. A radioautograph of the gel is shown. B12 refers to the clone CHO-B12, a clone derived from CHO cells that had been transfected only with FcεRI (*11*).

Variations in glycosylation of cell-surface proteins can affect their clustering or diffusion (*37*−*39*). Therefore, we examined the α chain of FcεRI, the subunit that is normally glycosylated (*40, 41*). As shown in Figure 6, the α chains on the A11 cell had a higher apparent molecular weight than the α chains on RBL cells. After removing *N*-linked carbohydrate by treatment with *N*-glycanase, the α chains from both cells showed the same molecular weight. This indicates glycosylation accounts for the difference in apparent molecular weight of the α chains on RBL and A11 cells. The α chain of the parental cell line CHO-B12, which was transfected only with receptors and an antibiotic marker, also showed the apparent increase in molecular weight (Figure 6). These results show that the posttranslational modification of the receptor α chains is cell-specific.

We attempted to assess the role of *N*-glycans by treating both RBL and A11 cells with neuraminidase to remove sialic acids from *N*-glycans and monitored the spontaneous receptor phosphorylation in the treated cells. We determined that there was no loss of receptors by the enzyme treatment by quantitating the amount of bound IgE. In five experiments involving resting or both resting and stimulated cells, no reproducible changes in the extent of phosphorylation of FcεRI induced by treatment of the cells with neuraminidase were observed (data not shown).

*Distribution of Lyn and FcεRI in Microdomains*. The Src-family kinases including Lyn preferentially congregate in plasma membrane "microdomains" that resist solubilization by certain detergents (*42*). As noted in the introduction, a model that emphasizes the critical role of such domains in the initial phosphorylation of the FcεRI has been proposed (*8*). We therefore investigated whether the Lyn, or FcεRI, or both, were differently distributed in the transfected CHO cells than in the RBL cells, using conditions (Materials and

Table 5: Distribution of FcεRI and Lyn in Microdomains, and the Effect of Cyclodextrin[a]

| | | FcεRI fractions | | Lyn fractions | | |
|---|---|---|---|---|---|---|
| cell | cyclodex.[b] | 3−5 | 10−12 | 3−5 | 10−12 | PY/FcεRI[c] |
| RBL | − | 5.7 (±2.0)[d] | 93 (±2.0) | 22 (±0.60) | 77 (±0.6) | 0.58 (±0.05) |
| A11 | − | 8.3 (±5.7) | 89 (±6.9) | 25 (±3.2) | 73 (±3.8) | 13.8 (±1.9) |
| RBL | + | 1.4 | 97 | 7.6 | 81 | 2.8 (±0.2) |
| A11 | + | 1.9 | 97 | 14 | 73 | 12 (±2.4) |

[a] Detergent extracts that had been prepared at 37 °C were fractionated on sucrose step gradients and centrifuged at 37 °C for 4 h. Successive fractions were assayed for [$^{125}$I]-IgE by counting of radioactivity, and for Lyn by Western blotting. Aliquots of the extracts were also immunoprecipitated with anti-IgE and the immunoprecipitates analyzed for PY. Fractions 3−5 represent the microdomains; 10−12 the cytoplasmic and detergent-extractable proteins. [b] Where indicated, cell suspensions were incubated with 10 mM cyclodextrin at 37 °C for 1 h (*44*). [c] The ratio of relative intensities from the Western blots using anti-PY and anti-$\beta$ subunit of FcεRI. [d] Average and range (in parentheses) of results from two independent experiments.

Methods) under which we had independently confirmed the movement of aggregated receptors into the lipid rafts (*20*).

In two experiments we observed that in the RBL cells, ≈22% of the Lyn was in the sucrose density gradient fractions containing the Triton-X-resistant microdomains. The corresponding value for the A11 clone was 25% (Table 5). A similar analysis, in which the distribution of the FcεRI was determined, again showed no significant difference between the RBL cells and the A11 cells.

Another way of probing this question is to inhibit the formation of the microdomains using a cholesterol-extracting drug such as cyclodextrin (*43, 44*). We reacted RBL and A11 cells with 10 mM cyclodextrin for 1 h and then quantitated the residual cell-bound IgE and the phosphotyrosine *per* extracted FcεRI. Notably, whereas there was an ≈60% loss of receptors from the treated RBL cells [cf. (*45*)], there was little or no loss from the A11 cells. In duplicate experiments, the remaining receptors from the cyclodextrin-treated RBL cells actually showed a 4.7-fold increase in "spontaneous" phosphorylation compared to those recovered from the untreated RBL cells. However, the exalted phosphorylation of the receptors from the A11 cells was unaffected by the treatment with cyclodextrin; the treated cells showed the same high level of spontaneous phosphorylation as the untreated ones (Table 5).

## DISCUSSION

Following their aggregation, FcεRI and closely related receptors of the immune system become phosphorylated on canonical tyrosines in their cytoplasmic domains, the initiating event in the cellular responses they stimulate. The central role played by this mechanism has prompted extensive investigations of the factors that influence it. Although relatively simple compared to other members of this family such as the antigen receptor on T lymphocytes (*46*), even FcεRI engage in complex interactions. They have been proposed to involve associations with one or more kinases (*47, 48*), phosphatases (*49, 50*), membrane "microdomains" (*8*), and cytoskeletal components (*51*).

To isolate some of these early events for more detailed study, we transfected FcεRI and Lyn into CHO fibroblasts. Surprisingly, the receptors on unstimulated transfectants exhibited phosphorylation of their tyrosines many-fold greater than that observed on the rodent mast cell line from which the transfected genes for the receptors had been derived. This report describes our investigation of this spontaneous phosphorylation.

Lyn is thought to be the kinase that normally phosphorylates the receptors after they are clustered. One obvious point of interest is that the spontaneous phosphorylation correlated with the level of Lyn kinase with which the cells had been cotransfected. This is consistent with the prior observation that although the CHO cells transfected only with FcεRI must obviously contain numerous other kinases, they are deficient in Src-family kinases, and in the absence of transfected Lyn, they respond only weakly to aggregation of the FcεRI (*11*). Together, these results suggest that the mechanisms for the spontaneous and induced phosphorylation are analogous. With that in mind, we explored each factor currently known to influence that mechanism and were able to exclude all but one. Thus, the spontaneous phosphorylation could not be accounted for by the binding of IgE to the receptor, by an unusually high constitutive association of Lyn with the receptor, nor by a heightened specific activity of the transfected Lyn. Of course we cannot rule out the remote possibility that somehow in vivo the activity of the transfected Lyn is amplified by a mechanism that would not be discernible in vitro. We found no evidence for a reduced phosphatase activity, nor an enhanced association of the unaggregated receptors with detergent-resistant microdomains. The marked augmentation of the anomalous phosphorylation at physiological or higher temperatures, conditions that would promote hydrophobic associations, made attractive the possibility that the receptors were spontaneously translocating to microdomains with their specialized lipids (*42*). It also seemed plausible that the concentration of particular glycoproteins in the microdomains might have attracted the anomalously glycosylated FcεRI. However, direct analysis provided no support for such a mechanism, and disrupting the microdomains with cyclodextrin failed to diminish the spontaneous phosphorylation.

There was no evidence for a spontaneously arising mutation leading to an alteration in the amino acid sequence which could have led to spontaneous phosphorylation (*33*), possibly as a result of spontaneous aggregation, but we did observe a change, presumptively an enhancement, in the glycosylation of the receptors' α subunit (Figure 6). Spontaneous aggregation of this and other receptors have been seen as a result of deficient glycosylation, although not to our knowledge as a consequence of enhanced or otherwise altered posttranslational conjugations (*37*−*39*). Differences in the glycosylation of the ectodomains of proteins such as transplantation antigens can give changes in their diffusibility although this may depend on the particular cellular milieu (*52, 53*). Ironically, in the present context, CHO transfectants

showed only minimal effects of such alterations(*53*). We used a variety of approaches to obtain evidence for spontaneous aggregation of the receptors: co-immunoprecipitation, chemical cross linking, comigration, and energy transfer procedures, but failed to detect any evidence for such associations. However, even with the least perturbing and perhaps most sensitive assay, FRET, approximations may fail to be detected. This can be seen from the results shown in data rows 4,5 and 11,12 in Table 4. Despite the functionally significant aggregation produced by the antigen and unlabeled anti-IgE, the energy transfer was negligible.

Thus, the only difference related to the Fc$\epsilon$RI we have observed between those cells in which a significant proportion of those receptors are spontaneously phosphorylated versus those cells in which they are not, was the altered glycosylation of the ectodomain of the $\alpha$ chain of the Fc$\epsilon$RI. Although it is plausible that this difference induces spontaneous transient aggregation, we cannot rule out other changes in the membranes that could in whole or in part contribute to the phenomenon.

*Concluding Remarks*. For receptors that are largely "activated" by approximation, the transient associations between such receptors that result from their rapid diffusibility (*54*) might be expected to engender spontaneous activation with greater or lesser frequency even in the absence of ligand (*55*), and fortuitously arising subtle structural changes could enhance such background "noise". It is interesting to speculate how cells protect themselves from such spurious initiation of signaling cascades. One such safeguard that can limit the propagation of unwanted initial signals is a kinetic proofreading regimen (*56*). In such a regimen, the *lifetime* of the initiating component and not just its concentration becomes critical. We have presented evidence that at least some of the cellular responses generated by Fc$\epsilon$RI are constrained by just such a regimen (*15*).

## ACKNOWLEDGMENT

Ms. Hana-Haleem-Smith prepared the mRNA library from CHO cells and performed the nucleic acid sequence analysis of each of the subunits of Fc$\epsilon$RI. Dr. Matthew Peirce performed several pilot studies on the distribution in the membrane of receptors and Lyn on the CHO cells, as well as on the in vitro phosphatase activity in the CHO cells. We thank Dr. Richard Posner for the FITC-labeled IgE.

## REFERENCES

1. Kinet, J.-P. (1999) *Annu. Rev. Immunol. 17*, 931−972.
2. Reth, M. (1989) *Nature 338*, 383−384.
3. Flaswinkel, H., Barner, M., and Reth, M. (1995) *Semin. Immunol. 7*, 21−27.
4. Paolini, R., Jouvin, M.-H., and Kinet, J.-P. (1991) *Nature 353*, 855−858.
5. Kulczycki, A., Jr., Isersky, C., and Metzger, H. (1974) *J. Exp. Med. 139*, 600−616.
6. Barsumian, E. L., Isersky, C., Petrino, M. G., and Siraganian, R. P. (1981) *Eur. J. Immunol. 11*, 317−323.
7. Eiseman, E., and Bolen, J. B. (1992) *Nature 355*, 78−80.
8. Sheets, E. D., Holowka, D., and Baird, B. (1999) *Curr. Opin. Chem. Biol. 3*, 95−99.
9. Yamashita, T., Mao, S.-Y., and Metzger, H. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11251−11255.
10. Pribluda, V. S., Pribluda, C., and Metzger, H. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11246−11250.
11. Vonakis, B. M., Chen, H., Haleem-Smith, H., and Metzger, H. (1997) *J. Biol. Chem. 272*, 24072−24080.
12. Liu, F. T., Bohn, J. W., Ferry, E. L., Yamamoto, H., Molinaro, C. A., Sherman, L. A., Klinman, N. R., and Katz, D. H. (1980) *J. Immunol. 124*, 2728−2737.
13. Holowka, D., and Metzger, H. (1982) *Mol. Immunol. 19*, 219−227.
14. Goding, J. W. (1976) *J. Immunol. Methods 13*, 215−226.
15. Torigoe, C., Inman, J. K., and Metzger, H. (1998) *Science 281*, 568−572.
16. Rivera, J., Kinet, J.-P., Kim, J., Pucillo, C., and Metzger, H. (1988) *Mol. Immunol. 25*, 647−661.
17. Wofsy, C., Vonakis, B. M., Metzger, H., and Goldstein, B. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 8615−8620.
18. Vonakis, B. M., Haleem-Smith, H., Benjamin, P., and Metzger, H. (2001) *J. Biol. Chem. 276*, 1041−1050.
19. Mao, S.-Y., and Metzger, H. (1997) *J. Biol. Chem. 272*, 14067−14073.
20. Peirce, M. J., and Metzger, H. (2000) *J. Biol. Chem. 275*, 34976−34982.
21. Cambier, J. C. (1995) *Immunol. Today 16*, 110−110.
22. Kenworthy, A. K., and Edidin, M. (1999) *Methods Mol. Biol. 116*, 37−49.
23. Guo, C., Dower, S. K., Holowka, D., and Baird, B. (1995) *J. Biol. Chem. 270*, 27562−27568.
24. Fraker, P. J., and Speck, J. C., Jr. (1978) *Biochem. Biophys. Res. Commun. 80*, 849−857.
25. Pribluda, V. S., Pribluda, C., and Metzger, H. (1997) *J. Biol. Chem. 272*, 11185−11192.
26. Torigoe, C., Goldstein, B., Wofsy, C., and Metzger, H. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 1372−1377.
27. Kent, U. M., Mao, S.-Y., Wofsy, C., Goldstein, B., Ross, S., and Metzger, H. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 3087−3091.
28. Frigeri, L., and Apgar, J. R. (1999) *J. Immunol. 162*, 2243−2250.
29. Hamawy, M. M., Oliver, C., Mergenhagen, S. E., and Siraganian, R. P. (1992) *J. Immunol. 149*, 615−621.
30. Adamczewski, M., Paolini, R., and Kinet, J.-P. (1992) *J. Biol. Chem. 267*, 18126−18132.
31. Pribluda, V. S., and Metzger, H. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 11446−11450.
32. Mendoza, G. R., and Metzger, H. (1976) *Nature 264*, 548−550.
33. Swann, P. G., Odom, S., Zhou, Y. J., Szallasi, Z., Blumberg, P. M., Draber, P., and Rivera, J. (1999) *J. Biol. Chem. 274*, 23068−23077.
34. Kinet, J.-P., Metzger, H., Hakimi, J., and Kochan, J. (1987) *Biochemistry 26*, 4605−4610.
35. Kinet, J.-P., Blank, U., Ra, C., Metzger, H., and Kochan, J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 6483−6487.
36. Blank, U., Ra, C., Miller, L., Metzger, H., and Kinet, J.-P. (1989) *Nature 337*, 187−189.
37. Blank, U., Ra, C., and Kinet, J.-P. (1991) *J. Biol. Chem. 266*, 2639−2646.
38. Tsuda, T., Ikeda, Y., and Taniguchi, N. (2000) *J. Biol. Chem. 275*, 21988−21994.
39. Rajan, N., Tsarbopoulos, A., Kumarasamy, R., O'Donnell, R., Taremi, S. S., Baldwin, S. W., Seelig, G. F., Fan, X., Pramanik, B., and Le, H. V. (1995) *Biochem. Biophys. Res. Commun. 206*, 694−702.
40. Hempstead, B. L., Parker, C. W., and Kulczycki, A., Jr. (1981) *J. Biol. Chem. 256*, 10717−10723.
41. Kanellopoulos, J. M., Liu, T. Y., Poy, G., and Metzger, H. (1980) *J. Biol. Chem. 255*, 9060−9066.
42. Hooper, N. M. (1999) *Mol. Membr. Biol. 16*, 145−156.
43. Pitha, J., Irie, T., Sklar, P. B., and Nye, J. S. (1988) *Life Sci. 43*, 493−502.
44. Ilangumaran, S., and Hoessli, D. C. (1998) *Biochem. J. 335*, 433−440.
45. Sheets, E. D., Holowka, D., and Baird, B. (1999) *J. Cell Biol. 145*, 877−887.

46. Davis, M. M., and Chen, Y.-H. (1998) in *Fundamental Immunology* (Paul, W. E., Ed.) pp 341−366, Lippincott-Raven, Philadelphia.
47. Siraganian, R. P., Zhang, J., and Kimura, T. (1999) in *Signal Transduction in Mast Cells and Basophils* (Razin, E., and Rivera, J., Eds.) pp 115−133, Springer, New York.
48. Swann, P. G., Odom, S., and Rivera, J. (1999) in *Signal Transduction in Mast Cells and Basophils* (Razin, E., and Rivera, J., Eds.) pp 152−170, Springer-Verlag, New York.
49. Swieter, M., Berenstein, E. H., and Siraganian, R. P. (1995) *J. Immunol. 155*, 5330−5336.
50. Kimura, T., Zhang, J., Sagawa, K., Sakaguchi, K., Appella, E., and Siraganian, R. P. (1997) *J. Immunol. 159*, 4426−4434.
51. Oliver, J. M., Pfeiffer, J. R., and Wilson, B. S. (1997) in *IgE Receptor (FceRI) Function in Mast Cells and Basophils* (Hamawy, M. M., Ed.) pp 139−172, R. G. Landes Co., Austin.
52. Wier, M. L., and Edidin, M. (1988) *Science 242*, 412−414.
53. Barbour, S., and Edidin, M. (1992) *J. Cell. Physiol. 150*, 526−533.
54. McCloskey, M. A., Liu, Z.-Y., and Poo, M.-M. (1984) *J. Cell Biol. 99*, 778−787.
55. DeLisi, C. (1981) *Nature 289*, 322−323.
56. McKeithan, T. W. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 5042−5046.

BI0027534