Activated *N*-Formyl Peptide Receptor and High-Affinity IgE Receptor Occupy Common Domains for Signaling and Internalization

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Immune cells display multiple cell surface receptors that integrate signals for survival, proliferation, migration, and degranulation. Here, immunogold labeling is used to map the plasma membrane distributions of two separate receptors, the *N*-formyl peptide receptor (FPR) and the high-affinity IgE receptor (FcεRI). We show that the FPR forms signaling clusters in response to monovalent ligand. These domains recruit G_i, followed by the negative regulatory molecule arrestin2. There are low levels of colocalization of FPR with FcεRI in unstimulated cells, shown by computer simulation to be a consequence of receptor density. Remarkably, there is a large increase in receptor coclustering when cells are simultaneously treated with *N*-formyl-methionyl-leucyl-phenylalanine and IgE plus polyvalent antigen. The proximity of two active receptors may promote localized cross-talk, leading to enhanced inositol-(3,4,5)-trisphosphate production and secretion. Some cointernalization and trafficking of the two receptors can be detected by live cell imaging, but the bulk of FPR and FcεRI segregates over time. This segregation is associated with more efficient internalization of cross-linked FcεRI than of arrestin-desensitized FPR. The observation of receptors in lightly coated membrane invaginations suggests that, despite the lack of caveolin, hematopoietic cells harbor caveolae-like structures that are candidates for nonclathrin-mediated endocytosis.

INTRODUCTION

Immune cells display a range of receptors on their cell surface that integrate signals for cell survival, differentiation, and proliferation as well as regulated responses such as degranulation and cytokine/chemokine production. One large class of these receptors is the G protein-coupled receptor (GPCR) family that signals by coupling to heterotrimeric G proteins and their downstream effectors. GPCRs expressed on the surface of immune cells include members of the CXCR and CCR chemokine receptor family, the complement receptors C3a and C5a, and receptors that mediate innate immunity, such as the *N*-formyl peptide receptor (FPR). Another large class is represented by receptor tyrosine kinases that signal through integral tyrosine kinase activity, such as the receptors for colony stimulating factor, granulocyte macrophage–colony-stimulating factor, and stem cell factor. Functionally related to this class is a large

group of receptors that signal by coupling to cytoplasmic tyrosine kinases. This group includes the family of multichain immunoreceptors, such as the T cell receptor (TCR), B cell receptor (BCR), and Fc receptors, as well as the heterodimeric cytokine receptors.

Here, we explore the potential for signal cross-talk between two distinct classes of receptors to take place in specialized regions of membrane. We focus first on the *N*-formyl peptide receptor (FPR), a member of the G_i-coupled GPCR family and an important chemotactic receptor on neutrophils and macrophages. FPR activation is initiated by the binding of small, monovalent bacterial peptides, including *N*-formyl-methionyl-leucyl-phenylalanine (fMLF). Ligand–receptor interaction induces a conformational change in the receptor, stimulating guanine nucleotide exchange and dissociation of the heterotrimeric G protein into its α_i and βγ components, both of which can activate downstream effectors such as the class 1B isoform of phosphatidylinositol 3-kinase (PI3K) and multiple isoforms of phospholipase C (PLC)β (Quehenberger *et al.*, 1993; Vines *et al.*, 2002). FPR desensitization is initiated by receptor serine and threonine phosphorylation by G protein-receptor kinases (GRKs) (Prossnitz *et al.*, 1995; Prossnitz, 1997). Arrestin binding to the ligand-activated, GRK-phosphorylated FPR completes the process of signal termination and targets the receptor to a predominantly clathrin-independent internalization pathway for degradation or recycling (Hsu *et al.*, 1997; Maestes *et al.*, 1999; Bennett *et al.*, 2000; Prossnitz, 2004; Xue *et al.*, 2004).

The FPR is naturally coexpressed on the surface of human basophils with the high-affinity IgE receptor (FcεRI). Work by Dahinden and others has shown that IgE-dependent

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Abbreviations used: BCR, B cell receptor; DNP-BSA, dinitrophenol-conjugated bovine serum albumin; GFP, green fluorescent protein; FcεRI, high-affinity receptor for IgE; fMLF, *N*-formyl-methionyl-leucyl-phenylalanine; FPR, *N*-formyl peptide receptor; TCR, T-cell receptor.

secretory and other responses are enhanced when the FPR is costimulated (Ochensberger *et al.*, 1996; de Paulis *et al.*, 2002). A similar synergy between these distinct receptors has been demonstrated in studies with rat basophilic leukemia cells stably expressing the FPR (rat basophilic leukemia [RBL]^{FPR} cells; Hall *et al.*, 1997; Lee *et al.*, 1997). The synergy translated to stronger and more sustained calcium responses as well as enhanced degranulation when both receptors were ligated compared with responses simulated by either single ligand. Armed with the knowledge that antigen-stimulated FcεRI forms signaling patches that are populated with multiple signaling partners (Syk, PLCγ2, PI3K, Grb2, Gab2, Cbl, and others; Wilson *et al.*, 2000, 2001; Oliver *et al.*, 2004), we speculated that GPCRs might also localize to distinct domains during signaling, and we tested this hypothesis by using RBL-2H3 cells transfected with FPR-green fluorescent protein (GFP) (RBL^{FPR-GFP} cells). We show that activated FPRs form large clusters that sequentially recruit G_i and arrestin. Clustering and internalization are accelerated at high ligand concentration. If stimulated individually, FPRs and FcεRI are not spatially related. However, within 30 s of simultaneous stimulation, a significant portion of the two receptors occupy the same signaling domain. When visualized by conventional ultrathin section transmission electron microscopy (TEM) methods, both receptors can be seen in membrane invaginations with a characteristic light coat. Using both TEM and live cell microscopy, we demonstrate that some of both species of agonist-bound receptors internalize in the same endocytic vesicles. However, the bulk of receptors follows separate pathways to internalization, likely through a combination of clathrin and nonclathrin-mediated budding of vesicles.

MATERIALS AND METHODS

Chemicals and Antibodies

fMLF and hexapeptide (*N*-formyl-norleucyl-leucyl-phenylalaninyl-norleucyl-tyrosinyl-lysine; 6-pep) were purchased from Sigma-Aldrich (St. Louis, MO). 6-pep-FITC was from Invitrogen (Carlsbad, CA). Minimal essential medium (MEM) was obtained from Invitrogen. Mouse monoclonal DNP-specific IgE was prepared by affinity purification from ascites (Liu *et al.*, 1980). IgE was conjugated to Alexa-555 (Invitrogen) according to the manufacturer's instructions. Anti-arrestin rabbit polyclonal serum that reacts with arrestin2 was provided by Dr. Jeffrey L. Benovic (Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA). Anti-GFP monoclonal antibody was purchased from Chemicon International (Temecula, CA). BD Living Colors full-length A.v. polyclonal antibody was purchased by BD Biosciences (Palo Alto, CA). Anti-clathrin monoclonal antibodies (X22) were from Abcam (Cambridge, MA), and PY-20 and PY-99 anti-phosphotyrosine antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) and Upstate Biotechnology (Lake Placid, NY), respectively. Auro Probe gold particles conjugated with goat anti-mouse IgG and goat anti-rabbit IgG were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, United Kingdom). Anti-fluorescein isothiocyanate (FITC) polyclonal antibodies were a gift from Dr. Larry Sklar (Department of Pathology, University of New Mexico, Albuquerque, NM). Polyclonal rabbit antibodies to the α-subunits of G_s and G_{i1,2} were provided by Drs. Allen Spiegel and Teresa Jones (National Institutes of Health, Bethesda, MD); these antibodies were raised to decapeptides from the C termini of the α-subunit and affinity purified on the corresponding peptide. For G_s, this sequence was RMHLRQYELL; for G_{i1,2}, it was KENLKDCGLF. Monoclonal antibodies to the FcεRI β-subunit were kindly provided by Dr. Juan Rivera (National Institutes of Health).

Cell Line and cDNA

The cDNA encoding the FPR was obtained from a human HL-60 granulocyte library. Plasmid DNA designed to express FPR fused to green fluorescent protein at the C termini was transfected into RBL-2H3 cells by a lipid-based (Effectene; QIAGEN, Valencia, CA) method. Briefly, cells were plated overnight, transfected with 1 μg of plasmid DNA, and then selected for 8–12 d in Geneticin (G-418; Sigma). Surviving cells were pooled, and receptor expression levels were analyzed by flow cytometry after labeling with 10 nM N-fNleLFNleYK. Receptor numbers were quantitated using Simply Cellular calibration beads (Bangs Laboratories, Fisk, IN). Stably transfected

RBL^{GFP-FPR} cells were maintained at 37°C in 5% CO₂ in MEM-Earle's medium with 15% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 1 mg/ml G-418.

Receptor Internalization

To examine the internalization of formyl peptide receptors in response to ligand stimulation, RBL^{GFP-FPR} cells were suspended in serum-free MEM and allowed to incubate at 37°C for 10 min. Cells were then stimulated with 1 μM fMLF for selected times, followed by plunging into ice-cold MEM and incubation on ice for 15 min to stop internalization. Cells were washed three times in cold Hanks' buffered saline solution. Receptors remaining on the cell surface were fluorescence labeled using 10 nM 6-pep-FITC and analyzed on a FACScan flow cytometer (BD Biosciences). Only viable cells were included in the assay as determined by gating on forward and side scatter. Nonspecific binding was determined in the presence of 1 μM fMLF. Receptor internalization was expressed relative to the total number of cell surface receptors on untreated cells.

TEM and Immunogold Labeling

Membrane sheets were prepared by a modification of the method of Sanan and Anderson (1991) as described previously (Wilson *et al.*, 2000). In brief, cells were plated on 15-mm round, clean glass coverslips and cultured overnight. After defined periods of incubation at 37°C with or without stimuli, cells on the coverslips were fixed in 0.5% paraformaldehyde for 7 min at room temperature (RT). Coverslips were then immersed in ice-cold HEPES buffer (25 mM HEPES, pH 7, 25 mM KCl, and 2.5 mM Mg-acetate) and inverted onto dry Formvar and poly-L-lysine-coated nickel electron microscopy grids. Pressure was applied to the coverslip for 20 s by bearing down with a cork. The coverslips were lifted, leaving sections of the upper cell surface adherent to the poly-L-lysine-coated grid. Membranes were further fixed in 2% paraformaldehyde for 10 min at 4°C. The results of previous single particle tracking and fluorescence recovery after photobleaching studies (Wilson *et al.*, 2006) established that this fixation is sufficient to immobilize membrane proteins. After washing, membrane proteins were labeled from the inside by incubating sequentially with primary antibodies and gold-conjugated secondary reagents. Membrane sheets were postfixed with 2% glutaraldehyde for 10 min at RT, processed for TEM analysis, and examined using a Hitachi H7500 transmission electron microscope. Sheets were selected for analysis at low magnification based on their favorable appearance (no rips or tears), and images were taken at high magnification with no further selection. A minimum of 10 images were taken in at least two separate experiments for each experimental condition.

For TEM imaging of ultrathin sections, monolayer cultures of RBL^{GFP-FPR} cells were incubated for defined time periods with 10 nM FITC-fMLF and dinitrophenol (DNP)-bovine serum albumin (BSA) gold (10 nm). Cells were fixed with 0.5% paraformaldehyde for 7 min, followed by incubation with polyclonal anti-FITC antibodies and 5-nm gold secondary antibodies. To establish the distribution of resting receptors, cells were fixed with paraformaldehyde before addition of ligand-gold reagents. Cells were postfixed in 2% glutaraldehyde, osmicated in 2% osmium tetroxide, and stained en bloc with 2% uranyl acetate. Propylene oxide was used to lift cell monolayers from plastic, and monolayers were spun into a pellet and embedded in Epon. Ultrathin sections were placed on copper grids, stained with uranyl acetate and lead citrate, and images were acquired using a Hitachi 600 transmission electron microscope (Pfeiffer *et al.*, 1985).

Mapping and Analyzing Gold Particle Distributions

Electron microscopy (EM) negatives were digitized using an ArtixScan1100 scanner (Microtek, Carson, CA). ImageJ (National Institutes of Health) was the platform for image analysis. A customized plugin was built to count and find the coordinates of 5- and 10-nm particles automatically (Zhang *et al.*, 2005). For statistical measure of clustering, coordinates of gold particles from at least 10 independent sheets from two separate experiments were analyzed using the Hopkins spatial statistic (Jain and Dubes, 1988; Wilson *et al.*, 2004; Zhang *et al.*, 2005). The Ripley's K bivariate function was used to define spatial relationships of two different sizes of gold, again based on distributions measured on multiple sheets (Haase, 1995; Wilson *et al.*, 2004; Zhang *et al.*, 2005). When experimental values fall outside the confidence envelope in this test, the deviation from complete spatial randomness is statistically significant. Data also were evaluated by a coclustering algorithm that scores particles within a defined distance (typically 20 nm) as clustered (Zhang, unpublished data).

Live Cell Imaging

RBL^{GFP-FPR} cells were plated overnight on 25-mm coverslips and incubated for 2 h on the day of the experiment with 2 μg/ml Alexa-555-conjugated (DNP-specific) IgE. After a medium change, coverslips were mounted in a temperature-controlled holder (Warner Instruments, Hamden, CT) on the stage of an inverted Zeiss LSM510 confocal microscope. Cells were illuminated with alternating excitation wavelengths of 488 (for GFP) and 543 (for Alexa-555) by line (Δx, y = 110 nm; Δz = 1 μm). Fluorescence emissions for GFP and Alexa-555 were captured using 505–530 and 560–615 band pass filters, respectively. Images were acquired at 3-s intervals for 10–30 s, followed by addition of 1 μM fMLF and 1 μg/ml

DNP-BSA. Clustering and internalization of receptors were tracked for 5–10 min after addition of ligands. Data were compiled and analyzed using Zeiss LSM software (Carl Zeiss, Thornwood, NY).

Immunoprecipitation and Western Blotting

Cells were treated for specified intervals with 1 $\mu\text{g}/\text{ml}$ DNP-BSA to cross-link anti-DNP IgE-primed Fc ϵ RI or with 10 nM FMLF, followed by rapid chilling. Cells were suspended in ice-cold lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, and a cocktail of phosphatase inhibitors [Pierce Biotechnology]) and lysates were clarified by microcentrifugation. Supernatants were rocked for 1 h at 4°C with monoclonal antibodies to clathrin conjugated to protein A/G-Sepharose. Proteins bound to wash beads were eluted by boiling in 2 $\times$ Laemmli buffer and separated by SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoretic transfer to nitrocellulose, blots were probed with horseradish peroxidase-conjugated, anti-phosphotyrosine antibodies, immersed in chemiluminescence substrate solution, and exposed to film.

Inositol-(3,4,5)-trisphosphate (IP₃) Production

Cells were washed once and suspended in Hanks'-BSA medium at 20×10^6 cells/ml. Aliquots (400 μl) were activated with either 100 ng/ml DNP-BSA or 100 nM FMLF. Reactions were stopped by the addition of 16% trichloroacetic acid. Inositol 1,4,5-trisphosphate levels were determined using the competitive binding assay of Deanin *et al.* (1991).

Secretion

RBL_{GFP-FPR} cells were plated into individual wells of a 24-well plate and primed overnight with 1 $\mu\text{g}/\text{ml}$ DNP-specific IgE. After washing and replacement with fresh medium, agonists were dispensed as described into duplicate wells, leaving wells without agonists for untreated controls and totals. Plates were incubated at 37°C for 20 min, followed by rapid chilling on ice and

addition of ice-cold phosphate-buffered saline. Supernatants from each well were transferred to tubes for colorimetric assay of β -hexosaminidase released by degranulation. Secretion was expressed as a percentage of total, obtained by detergent lysis of replicate wells.

Computer Simulations

Simulations were performed using Signaling Pathway Simulator, a three-dimensional, discrete-time, agent-based simulator developed by G. Hsieh, W. Shu, and J. Edwards at the University of New Mexico (unpublished data). The program is implemented in C++ and has been tested under Linux. Particle behavior is assigned by an expandable function table. The geometry of the model includes a patch of cell membrane bounded on each side by an extracellular domain and the cell cytoplasm. The cell surface area in the simulation is represented by a two-dimensional Cartesian plane with continuous values, so that the location of the particles can be determined precisely. Particle diffusion is modeled as Brownian motion approach and has been validated by Popov and Agmon's algorithm (Popov and Agmon, 2001). Particles are not permitted to overlap during simulation. The size of the simulated space is 2 μm^2 and is $\sim 1/1000$ of the actual cell membrane; time steps are typically 100 ms. Individual receptors diffuse at a rate of 0.077 $\mu\text{m}^2/\text{s}$. Transient clustering is simulated by use of a slow down rule, where nearby receptors (of the same species) slow by a factor of 5 and resume the faster diffusion rate whenever their random movement separates them from other receptors.

RESULTS

Figure 1 shows the distribution of the formyl peptide receptor on native plasma membrane sheets prepared from rest-

Figure 1. Membrane distributions of the FPR before (A) and after (B–D) stimulation with a low concentration of FMLF. In resting membranes (A), 5-nm immunogold labeling GFP-tagged FPR is found in small clusters (black circles). Membranes in B, C, and D were prepared from cells treated at 37°C for 1 min (B), 5 min (C), or 8 min (D) with 10 nM FMLF. Circles and arrows highlight FPR clusters, which increase in size during this treatment. Coated pits in A and C (inset) are identified by arrows. (E–H) The Hopkins statistic shows that the FPR is significantly clustered at all time points (>10 micrographs analyzed per condition). Bars (A–D), 100 nm.

Figure 2. (A–C) Membrane distributions of the FPR over a time course of exposure to a high concentration of fMLF. Membranes were prepared from cells treated at 37°C for 30 s (A), 1 min (B), or 5 min (C) with 1 μ M fMLF and labeled with 5-nm gold for GFP-tagged FPR. Arrows point to FPR clusters. (D and E) The Hopkins test for clustering at 1 and 5 min of treatment at 1 μ M fMLF (>10 micrographs analyzed per condition). (F) Flow cytometry-based measurements of FPR internalization after stimulation with low (10 nM) and high (1 μ M) concentrations of fMLF. Bars (A–C), 100 nm.

ing or fMLF-stimulated RBL^{FPR-GFP} cells. FPR-GFP fusion proteins were immunolocalized on the cytoplasmic face of paraformaldehyde-fixed membrane sheets by incubating with polyclonal antibodies to GFP, followed by anti-rabbit secondary antibodies conjugated to 5-nm colloidal gold particles. This procedure marks both unligated and ligated chimeric receptors. After labeling, sheets were postfixed in glutaraldehyde and imaged by transmission electron microscopy. As shown, the FPR is found in singlets and small clusters on the membranes of resting cells (Figure 1A) and in cells treated for up to 1 min with a low concentration of fMLF (10 nM) (Figure 1B). Over 5 min (Figure 1C) and 8 min (Figure 1D) of stimulation, the FPR clusters become progressively larger and often contain dozens of gold particles. As noted previously for aggregated Fc ϵ RI (Wilson *et al.*, 2000), FPR clusters are typically found in “dark” membrane. Particularly notable in the case of the FPR, this membrane often seems to be protruding out from the sheet (Figure 1C, top right; also Figure 2B). This observation is consistent with the concentration of FPR in membrane invaginations seen by ultrathin section (see Figure 7). It should be noted that, because of the bivalent nature of antibody probes, the number of gold particles per cluster is likely to underrepresent the number of receptors in each cluster. The inset in Figure 1C illustrates the lack of gold label within a clathrin-coated pit, which is easily distinguished from the FPR-containing protrusion by its honeycomb appearance. Analysis of multiple micrographs established that label marking FPR was very rarely seen within clathrin-coated pits in fMLF-treated cells.

As a statistical measure of clustering, we applied the Hopkins test (Wilson *et al.*, 2004; Zhang *et al.*, 2005). In this test, particle distributions are compared with the expected distribution for the same number of random points, represented by a black line centered around 0.5. Histograms in Figure 1, E and F, shows a shift to the right of center, indicating that the small clusters of FPR seen in resting membranes and in membranes after 1-min exposure to 10 nM fMLF are statistically different from random. Histograms in Figure 1, G and H, progressively shift to the right, providing statistical confirmation that FPR clustering increases significantly following 5 and 8 min of continuous exposure to 10 nM fMLF.

Results in Figure 2 show that treatment with high concentration of fMLF accelerates FPR clustering as well as receptor internalization. Within 30 s of 1 μ M fMLF treatment (Figure 2A), FPR are found in large clusters. Figure 2B shows immunogold-labeled membrane prepared after 1 min of stimulation. This image again captures clusters of FPR within membrane invaginations that lack the regular shape and honeycomb structure of traditional clathrin-coated pits and are also larger and more irregular than the caveolae that are common features of nonhematopoietic cells (Sanan and Anderson, 1991). We speculate that these represent preendocytic structures specialized for nonclathrin-mediated internalization. Consistent with this hypothesis, there is a marked reduction in FPR gold label within 5 min of stimulus with 1 μ M fMLF and a correspondingly smaller size of the remaining clusters (Figure 2C). This observation is confirmed by a shift to the left in the Hopkins test, when particle

Figure 3. Colocalization of FPR with G_i (A) and arrestin2 (C) after stimulation with 10 nM FMLF. (B) Lack of colocalization between the FPR and G_s . Membranes were prepared from cells treated at 37°C for 1 min (A and B) or 8 min (C). In all three images, GFP-tagged FPR are marked with small (5-nm gold particles) and doubly labeled for G proteins or arrestin by using large (10-nm) gold particles. Plots D–F report results of Ripley's bivariate coclustering analysis for the FPR with G_i (D) or G_s (E) at 1-min stimulation and the FPR with arrestin2 (F) at 8-min stimulation. Asterisks in D and F indicate significant coclustering (>10 micrographs analyzed per condition). Bars (A–C), 100 nm.

distributions after 5-min treatment are compared with 1-min treatment (Figure 2, D and E). Data in Figure 2F report rates of FPR internalization, as measured by flow cytometry. These data confirm that the higher concentration of FMLF leads to more rapid and complete internalization of FPR.

Figure 3 tracks the recruitment of positive and negative signaling elements in the FPR signaling pathway to FPR clusters. As shown in Figure 3A, immunogold marking the α -subunit of G_i (10-nm gold) coclusters with FPR (5-nm gold) at early times after stimulation with 10 nM FMLF (30 s). The statistical significance of FPR- G_i coclustering is confirmed by the Ripley's covariant test (Figure 3D), where experimental values for $L(t) - t$ (solid black line) fall outside the red dotted lines marking the expected values for unrelated pairs of points or clusters. In contrast, immunogold label for the α -subunit of G_s does not colocalize with the FPR at any time point (see image in Figure 3B and results of Ripley's test in Figure 3E). Results in Figure 3, C and F, show that arrestin2 (10-nm gold) colocalizes strongly with FPR clusters (5-nm gold) after longer periods (8 min) of stimulation with 10 nM FMLF. This leads to the conclusion that, although large clusters of FPR persist on the membrane after prolonged exposure to agonist, the clustered receptors are

largely down-regulated by association with arrestin2. Most importantly, these experiments establish that FPR clusters have the characteristics of bona fide signaling domains based upon their sequential recruitment of G_i and arrestin.

Because mast cells and basophils express multiple classes of receptors, it is important to evaluate the potential for cross-talk between the G_i -coupled FPR and the tyrosine kinase-coupled IgE receptor Fc ϵ RI. Our previous work (Hall *et al.*, 1997) showed that simultaneous treatment of RBL^{FPR} cells with both ligands resulted in synergistic effects on secretory and calcium responses. Similar results were obtained in RBL^{FPR-GFP} cells (Figure 4A), confirming that the chimeric FPR-GFP receptors behave the same as the wild-type receptors. The synergistic effects on secretion are explained in part by the large increase in IP_3 production (Figure 4B) that is observed when cells are stimulated with a combination of FMLF and IgE-polyvalent antigen. Importantly, when both stimuli are applied, secretion and IP_3 production are more than the sum of the responses induced by either individual species of receptor.

As a possible mechanism of FPR-Fc ϵ RI cross-talk, we evaluated the possibility that the two different receptors might colocalize in the same signaling patch. Figure 5 shows

Figure 4. Secretion and IP₃ production are potentiated by simultaneous stimulation of FPR and FcεRI. (A) RBL^{GFP-FPR} cells were stimulated at 37°C with a range of IgE-specific antigen (0.001, 0.01, 0.1 and 1.0 µg/ml DNP-BSA), either 10 or 100 nM fMLP, or a combination of either 10 or 100 nM fMLP over the whole range of DNP-BSA. Secretion of granule contents was based upon a colorimetric assay for β-hexosaminidase. Each experiment was performed in duplicate and β-hexosaminidase release was expressed as a percentage of total. (B) Cells were stimulated with 100 nM fMLP, 100 ng/ml DNP-BSA, or a combination and incubated for 0.5 or 5 min at 37°C. Levels of IP₃ in lysed samples were measured by competitive binding assay with tritiated IP₃.

representative receptor distributions on membrane sheets that were prepared from cells treated with a combination of 10 nM fMLF as monovalent ligand for FPR and 1 µg/ml DNP-BSA as a polyvalent ligand for IgE-primed FcεRI. Sheets were double-labeled with 5-nm immunogold particles to tag the FPR and with 10-nm immunogold particles to tag the FcεRI β-subunit. Results of coclustering analysis, using Ripley's K test on multiple sheets, showed that only 11% of membrane sheets prepared from resting cells pass the Ripley's test for coclustering. The occasional coclustering of resting FPR and FcεRI (data not shown) is probably not significant, because computer simulations suggest this is a function of the density of the two receptors expressed on the cell surface (Figure 5C and Supplemental Figure). Levels of endogenous FcεRI in RBL-2H3 cells are ~200,000 per cell, whereas transfected FPR averages ~100,000 per cell. When stochastic simulations were performed that recapitulate clustering of individual receptor species at these values, two of 15 (13%) of the simulated images falsely passed the Ripley's test for coclustering.

Importantly, after 1 min of dual stimulation, 53% of the membrane sheets pass the Ripley's K test for coclustering of the two receptors. A representative Ripley's K plot for this data set is shown in Figure 5C. These results are illustrated in Figure 5, A and B, and they are summarized in the table

embedded in Figure 5C. By 8 min of stimulus, there is a marked reduction in FcεRI on the cell surface (Figure 5D). As expected based upon the results in Figure 1, large FPR clusters persist on the plasma membrane at this late time point. Thus, these results suggest that the two receptors significantly colocalize during signaling but that internalization of the FPR is less efficient (particularly at this low dose of ligand).

Live cell imaging was used to explore the internalization routes for the two receptors under dual stimulation conditions. RBL^{FPR-GFP} cells were incubated for 2 h with Alexa-555-conjugated DNP-specific IgE to provide a red fluorescent tag for FcεRI-IgE complexes. Cells grown on coverslips were mounted on a temperature-controlled stage and imaged by confocal microscopy for up to 10 min at 35°C after the simultaneous addition of 1 µM fMLF and 1 µg/ml DNP-BSA (Figure 6 and Supplemental Movie). Here, the higher dose of fMLF was chosen to maximize FPR-GFP internalization and to improve the odds of detecting internalized vesicles containing both receptors. For comparison, experiments were also conducted at room temperature, a condition that slows but does not prevent internalization (data not shown). Results clearly document endocytic vesicles that contain both species of receptors as well as many vesicles that seem to contain only one species. This is illustrated in Figure 6C, where the merged images of FPR (green, Figure 6A) and FcεRI (red, Figure 6B) show spots that contain only FPR (green arrows) or only FcεRI (red arrow). This pattern was seen in all cells observed, although internalized mobile vesicles containing a single species of receptor cargo were consistently most abundant. In addition, at later times it became clear that internalization of the FcεRI was more complete than that of the FPR, which retains at least 25% of total receptors on the cell surface even after stimulation with higher concentrations of ligand (Figure 2). This is illustrated in Figure 6, D–F. Five minutes of stimulus results in marked clearance of red fluorescent IgE-bound receptors from the cell surface and concentration of the FcεRI in endocytic structures (Figure 6E). In contrast, a significant amount of GFP-FPR persists on the plasma membrane at this same time point (Figure 6D); this observation agrees well with the EM results in Figures 1–3 and 7.

At least two distinct internalization mechanisms seem to operate in mast cells to mediate uptake of receptors. The rare appearance of FPR label in coated pits is consistent with previous work where FPR internalization failed to be inhibited by dominant-negative forms of clathrin and dynamin (Gilbert *et al.*, 2001). The major pathway for endocytosis of cross-linked FcεRI is the coated pit (Pfeiffer *et al.*, 1985), although recent work suggests that the FcεRI can also efficiently use a nonclathrin-mediated pathway (Fattakhova *et al.*, 2006). We reasoned that traditional ultrathin transmission electron microscopy methods might provide insight into the morphological features of the membrane surrounding receptor clusters and possibly capture receptors in non-clathrin endocytic structures. Figure 7 shows images from TEM experiments where RBL^{GFP-FPR} cells were tracked by double labeling from the outside for FPR (5-nm gold) and FcεRI (10-nm gold). To label the FPR, we used FITC-conjugated fMLF, followed by fixation and incubation with anti-FITC gold reagents. IgE-primed FcεRI were labeled and stimulated with DNP-conjugated gold. Resting cells were prefixed with paraformaldehyde, followed by incubation with gold reagents. Figure 7A shows the edge of a resting cell with gold label for both receptors. The two sizes of gold are segregated on the membrane, with a small cluster of

Figure 5. Transient colocalization of FPR with Fc ϵ RI when both receptors are stimulated simultaneously. Membranes were prepared from DNP-specific IgE-primed cells that were treated at 37°C for 1 min (A and B) or 8 min (D) with 10 nM fMLF and 1 μ g/ml DNP-BSA. In all three images, GFP-tagged FPR are labeled with small (5-nm) gold particles and Fc ϵ RI are labeled with large (10-nm) gold particles. Receptor clusters and coclusters are marked with arrows. The plot in C reports results of Ripley's bivariate coclustering analysis for the FPR and Fc ϵ RI at 1-min stimulation with both activating reagents; the asterisk indicates significant coclustering. A summary of coclustering analysis (>9 micrographs per condition) is also provided in (C). Bars, 100 nm.

resting FPR in a membrane invagination (arrow). Unligated FPR was also found on flat areas of membrane and microvilli, although it was common to observe it in membrane invaginations. As expected from previous TEM and scanning EM experiments (Pfeiffer *et al.*, 1985; Seagrave *et al.*, 1991), the small clusters of resting Fc ϵ RI were well distributed.

Cells in Figure 7, B and C, were incubated with FITC-fMLF and DNP-gold for 2 min before fixation and completion of the labeling protocol for the FPR. These images of membrane invaginations containing both sizes of gold (Figure 7, B and C, arrows) provide further evidence that the FPR and Fc ϵ RI may occupy the same membrane domain at early time points after addition of the two agonists. Because there is only a hint of a coat, these deeply cupped membrane invaginations may represent preendocytic structures of the nonclathrin-mediated pathway. For comparison, vesicles with a more typical appearance of a clathrin coat are shown in Figure 7E; these particular vesicles contain only large gold particles marking the Fc ϵ RI.

Further evidence that the lightly coated invaginations may represent specialized membrane domains for nonclathrin-mediated endocytosis comes from observations at later times after FPR stimulation. Figure 7D illustrates a membrane labeled for the FPR after 5 min of stimulus. Here, FPR

is found clustered in a flat region of membrane and in two deeply cupped membrane invaginations (Figure 7D, arrows). Note that, although the left-hand invagination in Figure 7D seems to be a closed vesicle, the labeling procedure only reaches ligand-bound FPR still exposed to the extracellular buffer. Similar clusters of FPR within membrane invaginations are seen in Figure 7, F and G, also after 5 min of stimulus. Importantly, all the membrane invaginations containing ligated FPR have a similar, "wispy" coat. In contrast to FPR, the DNP-gold is both ligand and gold marker for the Fc ϵ RI and so can be "chased" into the cells in this experiment. This is demonstrated in Figure 7, F and G, where gold-conjugated ligands marking internalized Fc ϵ RI have reached late endosomal compartments or multivesicular bodies (bold arrows), accompanied by loss of their clathrin coats.

Brodsky and colleagues reported previously that TCR internalization was accompanied by tyrosine phosphorylation of clathrin (Crozier *et al.*, 2004). Therefore, we examined the state of clathrin phosphorylation after stimulation of either the Fc ϵ RI or the FPR. To generate the results in Figure 7H, cells were incubated at 37°C with or without 10 nM fMLF or 1 μ g/ml DNP-BSA for specified time periods. Clathrin was then immunoprecipitated from detergent cell lysates, followed by SDS-PAGE and Western blotting with anti-PY99/PY20 antibodies

Figure 6. Partial colocalization and internalization of GFP-FPR and Alexa-555 IgE-primed Fc ϵ RI in live cells. (A–C) Confocal images of cells stimulated for ~1 min with a combination of 1 μ M fMLF and 1 μ g/ml DNP-BSA, where A shows GFP fluorescence, B shows IgE fluorescence, and C shows the merged image. Yellow arrows in C point to spots containing both green FPR and red Fc ϵ RI, whereas green arrows point to spots containing only green fluorescence for the GFP-FPR chimera, and red arrows point to spots containing only red fluorescence IgE. (D–F) Confocal images of a 1- μ m optical slice near the bottom of a cell, taken at 5 min after addition of both ligands. As noted above, GFP and IgE fluorescence are in D–E and the merged image is in F. These images illustrate the marked loss of Fc ϵ RI from the membrane, whereas a significant amount of FPR persists at the membrane.

to detect phosphotyrosine. Only antigen stimulation of Fc ϵ RI (top), and not agonist stimulation of the FPR (bottom), induced a significant increase over the low basal state of clathrin tyrosine phosphorylation.

DISCUSSION

Most receptors are transmembrane proteins made up of one or more membrane-spanning subunits. For some receptors, for example, the ErbB family of growth factor receptors, extracellular ligand binding activity and intracellular catalytic activity are built into a single transmembrane protein. Other receptors, including the Fc ϵ RI and FPR studied here, lack intrinsic catalytic activity but couple after ligand binding to tyrosine kinases (Fc ϵ RI) or G proteins (FPR) that in turn initiate signaling. There is strong evidence for interactions between signaling pathways that integrate signals for cell survival, differentiation, proliferation, and other activities. As noted, costimulating Fc ϵ RI and FPR leads to calcium and secretory responses in mast cells that are larger, more rapid and longer lived than those induced by stimulating either receptor alone (Hall *et al.*, 1997; Lee *et al.*, 1997; also see Figure 4). Similarly, signals from either G protein-coupled receptors and cytokine receptors affect the strength, duration, and character of signals from ErbB receptors in breast cancer cells and from insulin receptors in diabetes (Hynes *et al.*, 2001; Hupfeld *et al.*, 2003; Chakravarti *et al.*, 2005; Morisco *et al.*, 2005). Thus, it is important not only to understand mechanisms that regulate the efficiency, specificity, and duration of signaling through specific receptors but also to understand the interactions between different receptors

and their signaling pathways that modulate signal strength and the nature of the physiological response.

Our particular interest has been in the role of membrane topography in controlling signaling. Previous studies showed that cross-linked Fc ϵ RI redistribute during signaling to distinct membrane domains that also recruit the tyrosine kinase Syk and a large number (but not all; Wilson *et al.*, 2001) of the proteins implicated in remodeling membrane phospholipids, propagating signals (for example to calcium mobilization and Ras/mitogen-activated protein kinase pathway activation) and also in terminating signaling (generally through the activation of lipid and protein phosphatases). These membrane regions often seem darker than bulk membrane in TEM studies. Studies by x-ray spectral microscopy showed they have an elevated carbon content (Wilson *et al.*, 2004), whereas atomic force microscopy revealed that they have more height extending into the cytoplasm in comparison with bulk membrane (Frankel *et al.*, 2006). Together, these data suggest that the dark domains contain a higher concentration of protein and associated lipids and/or cholesterol than adjacent areas of membrane. They also may represent areas of significant membrane curvature. Here, we asked whether ligand–FPR complexes also redistribute to distinct domains during signaling and whether they are accompanied by proteins implicated in signal propagation and desensitization. Importantly for understanding cross-talk between receptors, we also asked whether FPR and Fc ϵ RI cocluster during signaling and/or cointernalize during signal termination.

Our results show that FPR indeed clusters during signaling. Furthermore, proteins implicated in signal propagation and signal termination cocluster with the receptors. This

Figure 7. Ultrathin sections examined by TEM show that FPR and FcεRI can occupy the same preendocytic structures within 2-min addition of both ligands (FITC-fMLF and DNP-gold). After stimulus, cells in B–G were fixed with paraformaldehyde and incubated with anti-FITC gold to label the FPR. Cells in A were prefixed and then incubated with ligand–gold reagents to establish that FPR (5-nm gold) and FcεRI (10-nm gold) are not colocalized under resting conditions. Images in B and C show the presence of both sizes of gold (arrows) in deeply cupped membrane invaginations. Bold arrow in C shows large gold marking FcεRI in an endosome. Small gold particles in D, F, and G (arrows) mark FPR–ligand–gold probe complexes within lightly coated invaginations on the plasma membrane. Bold arrows in C, E, F, and G point to internalized FcεRI. (H) Clathrin was immunoprecipitated from RBL^{FPR-GFP} cells treated for specified times with 1 μg/ml DNP-BSA to cross-link IgE-primed FcεRI (top) or with 10 nM fMLF (bottom). Proteins in the immunoprecipitates were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose. Blots were probed with anti-phosphotyrosine antibodies. Gels were routinely stripped and reprobed with anti-clathrin as a loading control (data not shown). Bar (left), 100 nm.

coclustering occurs sequentially. The signal-initiating protein complex G_i associates with receptor immediately after ligand binding. At later times, G_i is replaced by the signal-terminating protein arrestin. This sequential recruitment is similar to the progressive loss of Lyn from FcεRI signaling patches and the subsequent recruitment of Syk (Wilson *et al.*, 2000). As expected, the rate and extent of FPR clustering and the rates of receptor loss from the surface vary with ligand concentration. The mechanism of FPR clustering is not yet understood. Unlike the FcεRI, external cross-linking does not play a role, because the ligand is monovalent. Current models to explain such clustering invoke membrane microdomains that provide favorable locations for receptor either because of their specialized lipid composition or because they represent regions of close protein–protein interaction (Magee *et al.*, 2005). Consistent with this kind of model, we observed that FPR concentrate in membrane invaginations in both resting and activated cells.

FcεRI and FPR cluster distributions are independent in resting cells (Figure 5A) and in cells where only one receptor is stimulated (data not shown). We base this conclusion on stochastic simulations of receptor clusters at this density (100,000 for the transfected FPR and 200,000 for the endogenous FcεRI) that predict intermixing of up to 20% (Supple-

mental Figure). At these concentrations of receptors in the membrane, even the usually reliable Ripley's test for coclustering will occasionally give a false positive result (Figure 5C). Importantly, costimulation of both FcεRI and FPR leads to their rapid and statistically significant colocalization. We speculate that cross-talk occurs when the two species of receptors occupy the same signaling patch early after stimulation. The mechanism is unknown, but it may, for example, occur by a more rapid and extensive synthesis of phosphatidylinositol-(3,4,5) trisphosphate and of IP_3 (Figure 4B) when multiple isoforms of PI3K and PLC are concentrated in a single membrane domain via their association with coclustered receptors.

Evidence has been mounting that nonclathrin-mediated internalization mechanisms are important alternatives for the uptake of receptors and water-soluble particles in the extracellular milieu (for review, see Kirkham and Parton, 2005). In the system studied here, we track the internalization of two distinct classes of receptors known to use both clathrin-dependent and -independent pathways. Using live cell imaging (Figure 6), we were able to document instances where both stimulated receptors entered the cell in the same endocytic vesicle. However, in general, we observed that the FcεRI internalized more completely and more rapidly than

the FPR, even at maximal concentrations of fMLF. This can be readily observed in two movies available as Supplemental Material. Both FPR and FcεRI can occupy lightly coated membrane invaginations at early times after dual stimulus, but only the FPR persists on the cell membrane in these invaginations after prolonged exposure to agonists (Figure 7, F and G). We speculate that these membrane invaginations may be the hematopoietic cell equivalent of caveolae that are implicated in both cell signaling and internalization (Razini *et al.*, 2002; Hommelgard *et al.*, 2005).

Continued work will address the mechanisms by which the cell accomplishes the dramatic postsignaling movements of receptors into both common and separate pathways of internalization. Work from the Brodsky laboratory has suggested that there is strong adaptive pressure to maximize immunoreceptor internalization. The BCR, for example, demonstrates inherent adaptability to use both clathrin and raft-dependent pathways (Stoddart *et al.*, 2005). Brodsky's group was also the first to correlate clathrin tyrosine phosphorylation with endocytosis of the TCR (Crotzer *et al.*, 2004). Preliminary results presented here raise the possibility that the more frequent observation of FcεRI in an obvious clathrin "honeycomb" caged vesicle on membrane sheets is associated with its ability (and not the FPR's ability) to stimulate clathrin phosphorylation. We have speculated that the lightly coated membrane invaginations that label for both receptors early after addition of dual agonists but that contain only FPR at later times may be either 1) preendocytic structures specialized for clathrin-independent internalization or 2) caveolae-like signaling domains. We must at least consider a third explanation for the light coat on the invaginations containing FPR at late times: they could be phosphorylation-deficient, incomplete assemblies of the more classical adaptin-clathrin coat.

In summary, receptor clustering for signaling and internalization seems to be a widespread phenomenon induced by both multivalent and monovalent ligands and characterizing both G protein-coupled and tyrosine kinase-coupled receptors. The proximity of two active receptors and their associated signaling partners in the same signaling domains provides a potential mechanism for localized cross-talk leading to changes in the rate, duration, and extent of the signaling response. In the FPR and FcεRI, the potential for continued cross-talk seems to be limited by their different rates of internalization and by the arrestin-mediated desensitization of FPR, some of which can remain for prolonged periods on the plasma membrane. The particular receptors studied here are implicated in causing the symptoms of allergy and asthma (FcεRI) and in early host defense against infection (FPR). Their cross-talk may be relevant to understanding and preventing the worsening of asthma that often accompanies a bacterial or viral infection.

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