Endocytosis of Activated Receptors and Clathrin-coated Pit Formation: Deciphering the Chicken or Egg Relationship

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Abstract. The fundamental mechanisms by which receptors once targeted for endocytosis are found in coated pits is an important yet unresolved question. Specifically, are activated receptors simply trapped on encountering preexisting coated pits, subsequently being rapidly internalized? Or do the receptors themselves, by active recruitment, gather soluble coat and cytosolic components and initiate the rapid assembly of new coated pits that then mediate their internalization? To explore this question, we studied the relationship between activation of IgE-bound high affinity Fc receptors (FcεRI) and coated pit formation. Because these receptors are rapidly internalized via clathrin-coated pits only when cross-linked by the binding of multivalent antigens, we were able to separate activation from internalization by using an immobilized antigen. The FcεRIs, initially uniformly distributed over the cell surface, relocalized and aggregated on the antigen-exposed membrane. The process was specific for the antigen, and temperature- and time-dependent. This stimulation initiated a cascade of cellular responses typical of FcεRI signaling including membrane ruffling, cytoskeletal rearrangements, and, in the presence of Ca^{2+}, exocytosis. Despite these responses, no change in coated pit disposition or in the distribution of clathrin and assembly protein AP2 was detected, as monitored by immunoblotting and by quantitative (vertical sectioning) confocal microscopy analysis of immunofluorescently stained cells. Specifically, there was no decrease in the density of clathrin-coated pits in regions of the cell membrane not in contact with the antigen, and there was no apparent increase in clathrin-coated pits in proximity to stimulated FcεRI receptors as would have been expected if the receptors were inducing formation of new pits by active recruitment. These results indicate that de novo formation of clathrin-coated pits is not a prerequisite for rapid internalization or a direct response to stimulation of FcεRI receptors. Therefore, increases in coated pits reported to occur in response to activation of some signaling receptors must be consequences of the signal transduction processes, rather than strictly serving the purpose of the internalization of the receptors.

RECEPTOR-MEDIATED endocytosis provides a pathway by which cells interact with and ensure responsiveness to their environment. Through this process, cells can internalize required nutrient transport proteins (e.g., transferrin, low density lipoprotein) and receive extracellular signals (such as growth factors, hormones, and antigens). To the extent that endocytosis is also responsible for removing signaling receptors from the plasma membrane, it may also play a critical role in the signal transduction process itself (e.g., by desensitization [69*a*, 74]).

Localization of cell surface receptors in clathrin-coated pits is the characteristic hallmark of receptor-mediated endocytosis (for review see 68, 70). After a lifetime of ~1 min, ligands appear in detached vesicles within the cell (2). Nutritive receptors generally are constitutively internalized. In contrast, rapid and efficient internalization of signaling receptors (e.g., EGF receptor) generally occurs only after ligand binding and receptor activation. As coated pits mediate the initial step in this process, the mechanisms responsible for regulating their dynamics at the plasma membrane are likely to be critical, possibly rate-determining factors in the endocytosis process. Control of the coated pit to coated vesicle transition, in which dynamin has been implicated (16, 69), likely represents one aspect of regulation. Another important potential site of modulation is the formation of the coated pit itself. It is this process that we focus on here.

The major structural components of coated pits at the plasma membrane are clathrin and the assembly or adap-

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tor protein (AP)2[1] (for review see 32, 57). In addition to binding and assembling clathrin into discrete coat structures, there is evidence that AP2 may interact directly with specific motifs on the cytoplasmic domain of some cell surface receptors, thus serving as an adaptor (47, 51, 52, 62). Interestingly, several studies have shown that addition of certain ligands (e.g., EGF [14], NGF [13], carbachol [21], insulin [15]) or certain membrane events (e.g., fertilization of sea urchin eggs [20]) lead to rapid increases in coated pits, or to increases in membrane-bound forms of coat components suggestive of coated pit assembly. These stimulus-induced increases in coated pits seemed to well accommodate the adaptor hypothesis noted above. A direct link between receptors and assembly promoting APs provided a plausible, although unproven, connection between receptor-induced formation of coated pits and rapid receptor internalization. Some previous work addressing such a causal relationship has generally been indirect and subject to limitations (for review see 3, 60).

This receptor-coated pit relationship has been directly tested in the present work that focuses on the early steps that lead to rapid internalization of activated receptors, namely the association of stimulated receptors with clathrin-coated pits. We attempted to answer the metaphoric "chicken or egg" question about the genesis of coated pits. Specifically, does rapid internalization of activated receptors result from receptor-induced recruitment of protein components to form new coated pits (i.e., the egg)? Or do activated receptors have to migrate to preexisting coated pits (i.e., the chicken)?

To approach these questions, we used an immobilized ligand surface to generate a lawn of specific, activated receptors. Takemura et al. had performed related experiments with mouse peritoneal macrophages immobilized on an immune-complex surface (65). While increases in plasma membrane–coated pits were observed, a multitude of other extensive membrane changes also occurred. As these workers later showed (5), these included complete vesiculation of the Golgi that accounted for the disappearance of Golgi clathrin, and the appearance of a novel vacuolar compartment at the basal surface that contained both Golgi markers and numerous coated pits and vesicles. These myriad changes were likely a consequence of the experimental conditions that would be expected to activate the functionally heterogeneous Fcγ receptors (potentially RI, RII, and RIII) expressed by macrophages (55). This and the complexity of the membrane changes precluded an unequivocal interpretation of the nature of the linkage between receptor activation and coated pit changes at the plasma membrane.

RBL-2H3 cells, a continuous rat mast cell line homologue, provide an alternative experimental system for investigating the role of receptors in clathrin-coated pit nucleation. These cells are particularly useful in this context for several reasons. Under our experimental conditions only high affinity Fc receptors for monomeric IgE (FcεRI) are activated by immobilized multivalent antigens (while the low affinity FcεRII are not). In addition, there is extensive knowledge of both the signaling events and the receptor behavior that follow FcεRI stimulation (for review see 7, 9, 40). In resting cells, the FcεRI is found distributed over the entire cell surface. However, upon antigen-induced crosslinking, the receptors become localized into clathrin-coated pits and are rapidly cleared from the cell surface via clathrin-coated vesicles with a half-time of <8 min, (54, 56, 61, 64). In addition to the rapid redistribution and internalization of receptors, aggregation of FcεRI receptors also initiates a cascade of biochemical events that include activation of several tyrosine (10, 17) and serine/threonine kinases (12, 48, 59, 66), as well as of phospholipases C (50), A2 (28), and D (27, 33). This leads to elevation of diglycerides and mobilization of Ca^{2+} (6, 43) and culminates in cell degranulation (54).

Exposure of IgE-primed RBL-2H3 cells to immobilized antigen induced full cell activation as indicated by a variety of biochemical and morphological criteria (e.g., FcεRI aggregation, cell spreading, membrane ruffling, and exocytosis) without affecting the integrity of the Golgi. Despite these cellular responses, no changes were observed in the distribution of clathrin and AP2 in the cell in toto.

These observations indicate that rapid formation of new clathrin-coated pits is neither a direct response to stimulation of FcεRI receptors nor an absolute prerequisite for their rapid internalization. To the extent that these results can be expected to apply to other receptors whose rapid internalization is also activation dependent, endocytosis is likely to result primarily from receptors moving to preexisting coated pits. A corollary of our findings is that increases in coated pits in response to agonists must be important consequences of the signal transduction process, rather than strictly serving the purpose of the internalization of the receptors.

Materials and Methods

Reagents

Reagents were obtained from the following sources: *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, DNP-specific mouse monoclonal IgE, and DNP-lysine were from Sigma Chemical Co. (St. Louis, MO); the antigen, DNP_{40}-BSA, was from Calbiochem-Novabiochem Corp. (La Jolla, CA); Fluorescein-5-isothiocyanate "isomer 1" was from Molecular Probes, Inc. (Eugene, OR); and the enhanced chemiluminescence (ECL) detection kit was from Amersham Corp. (Arlington Heights, IL). Other chemicals were reagent grade or better.

Antibodies

Clathrin was detected using either R5 (rabbit polyclonal against clathrin heavy and light chains), X22, or TD-1 (mAbs against clathrin heavy chain or mAbs to the COOH-terminal domain). AP2 was detected using AP.6 (mAbs against the ear region of the α chain) or R2 (rabbit polyclonal antibodies raised using the β chain residues 511–536). X22, TD-1, and AP.6 were gifts from Dr. F.M. Brodsky (Department of Pharmacology, University of California at San Francisco, San Francisco, CA). All of these antibodies have been previously characterized (8). Antibody against the β chain of the FcεRI was kindly supplied by Dr. David A. Holowka (Department of Chemistry, Cornell University, Ithaca, NY). DNP-specific mouse monoclonal IgE was labeled with fluorescein-5-isothiocyanate "isomer 1" using a published procedure (39) with a fluorescein (FL)/mIgE ratio of 100:1. Other antibodies were from the following sources: mAb against mannosidase II, p135, from BAbCO (Berkeley, CA); FL-conjugated or rhodamine-lissamine–conjugated affinity-purified donkey or goat

1. *Abbreviations used in this paper*: AP, clathrin assembly protein; FcεRI, high affinity Fc receptor for monomeric IgE; FL, fluorescein; FL-IgE, FL-conjugated DNP-specific IgE.

anti-rabbit or -mouse polyclonal antibodies were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Cell Culture and Measurement of Secretion

Experiments were performed with RBL-2H3(m1) cells kindly supplied by Dr. Michael A. Beaven (National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD) (11, 30). Cultures (0.55×10^6 cells/ml) were incubated overnight or for 2 h with DNP-specific IgE (0.5 μg/ml or 5 μg/ml, respectively), in complete growth medium (48). Experiments were performed in buffer C: a glucose-saline, Pipes-buffered medium, pH 7.2, that contained 0.1% BSA and 1 mM Ca^{2+}; calcium was omitted and replaced by 0.1 mM EGTA for calcium-free experiments (34).

Release of hexosaminidase, a granule marker, was measured by monitoring *p*-nitrophenol released upon hydrolysis of *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (48). Cells cultured overnight in the presence of DNP-specific IgE were trypsinized, washed, and resuspended in buffer C. They were then added to BSA- or DNP_{40}-BSA–coated surfaces (see next section) (3×10^5 cell per 250 μl of volume) and incubated at 37°C with or without a 1-h preincubation at 0°C. Different time points were taken. Absorbance readings were made at 405 nm, and release was expressed as percentage of total hexosaminidase content in the cells, the latter determined by solubilization of unstimulated cells in 0.1% Triton X-100.

Preparation of DNP_{40}-BSA–coated Surfaces

Glass coverslips (No. 1 thickness, 12-mm round or 22-mm square), were coated with DNP_{40}-BSA by a modification of the procedure of Michl et al. (41). Briefly, after being coated with poly-L-lysine (Sigma Chemical Co.) and exposed to 2.5% glutaraldehyde (EM grade; Polyscience, Niles, IL), coverslips were incubated for 30 min in the dark with a 1 mg/ml (unless otherwise indicated) solution of DNP_{40}-BSA in PBS, followed by a 5-min incubation with 10 mg/ml $NaBH_4$ solution in 1:1 methanol/PBS to quench the unreacted glutaraldehyde groups. The coverslips were then rinsed with PBS and stored in the dark overnight with 0.2 M glycine in 10 mM sodium phosphate buffer, pH 7.2. Control coverslips were prepared using BSA instead of DNP_{40}-BSA. This same procedure was used to coat petri dishes when a larger number of cells had to be processed (e.g., preparation of cell lysates for immunoblotting, measurement of hexosaminidase). To determine the effect of different immobilized antigen concentrations on the secretory response, petri dishes were prepared using DNP_{40}-BSA solutions ranging from 0.01–2.0 mg/ml, all made up to a final 2 mg/ml using BSA.

Cell Stimulation by Immobilized Antigen

RBL-2H3m1 cells were primed with anti-DNP mIgE. Control cells were either left unstimulated or exposed to BSA-coated coverslips. Frustrated cells were obtained by incubation on immobilized DNP_{40}-BSA. Normally, for microscopy study cells were plated on coverslips at 3×10^4 cells per cm^2. Unless otherwise stated, the exposure of the cells to the immobilized antigen was performed at 4°C for 1 h and was followed by an incubation at 37°C for varied periods of time (1 min–2 h). During the time interval of the stimulation, the cells remained viable as indicated by their ability to exclude trypan blue.

Transferrin Internalization

The ability of unstimulated and frustrated cells to internalize transferrin was measured by following the endocytosis of FL-labeled transferrin (a gift from Dr. F.R. Maxfield, Columbia University, New York). Briefly, unstimulated cells and cells that had been exposed to immobilized DNP_{40}-BSA at 37°C for 25 min (see previous section) were incubated for an additional 20 min in the presence of 5 μg/μl FL-labeled transferrin. Cells were washed three times with PBS, fixed, and mounted on glass slides.

Immunofluorescence and Confocal Microscopy

Coverslips were rinsed three times with PBS, fixed for 10 min in 3.7% formaldehyde, permeabilized 10 min in 0.05% Triton X-100, and blocked for 10 min in 4% normal goat serum in 0.05% Triton X-100, all in solutions prepared in PBS. Cells were incubated with primary antibody for 30 min at 37°C. Clathrin was detected using R5 (1:100) or X22 (60 μg/ml), and AP2 (62 μg/ml) using AP.6. The Golgi apparatus was visualized by using mAb anti-mannosidase II, p135, a Golgi resident protein. The primary antisera were detected with a rhodamine-lissamine- or FL-conjugated donkey anti-rabbit or donkey anti-mouse IgG.

Fluorescence images were observed and photographed on a microscope (Axiovert 405M; Carl Zeiss, Inc., Oberkochen, Germany), using a ×63 or ×100 oil immersion lens (Plan-Neo; Carl Zeiss, Inc.). Confocal analysis was performed on a laser scanning confocal microscope (MRC-600; Bio-Rad Laboratories, Hemmelholsteadt, UK) attached to a microscope (Axiovert 100; Carl Zeiss, Inc.) with ×63 1.40 NA oil immersion lens (Plan-Apo; Carl Zeiss, Inc.). Vertical (x–z) sections were obtained with pinhole Vernier setting of 2.

Quantitation of Coated Pit Density

Quantitation of coated pit density was estimated by comparing pixel density due to AP2 localization on the apical and basal surface of cells imaged by confocal x–z scans. We chose to use AP2 as coated pit marker rather than clathrin because of the lower background and absence of Golgi staining; however, AP2 localization always coincided with clathrin in frustrated and unstimulated cells (see Fig. 6). Images were selected whose pixel intensity fell entirely within the linear photomultiplier output range, e.g., 1 to 254. Using image analysis software (Bio-Rad Laboratories), background was estimated by sampling an area outside the cell silhouette and an equal area within the cell and averaging the pixel intensity values. An area was then drawn that contoured the cell apical and the ventral membrane. The integrated pixel intensity within that area, corrected by the integrated intensity for the same region using the background value obtained as described above, was divided by the size of the area sampled. The pixel density obtained was used to compare AP2 density on the apical and ventral membrane of each cell and was expressed as a percentage of the total. Data are expressed as mean value (±SD) for the indicated number of samples.

While there are limitations to quantitation using laser scanning confocal microscopy, our analysis avoided these complications as it was restricted to a comparison of cell surface profiles observed within a single field in an individual specimen. Quantitation of coated pit distribution in a variety of cell types, including RBL, has yielded values of ~75 coated pits per mm of plasma membrane (38, 42). Our x–z images (e.g., Fig. 7) indicated ~10–15 coated pits per 25 μm of apical or ventral irradiated membrane surface, or 400–600 coated pits per mm. Correction for the larger size of the confocal laser beam diameter (0.45 μm) (53) compared with the thickness of an ultrathin section (0.07 μm) yielded values of 62 to 93 coated pits per mm, in excellent agreement with published results noted above. This suggests that the method may be of general use for evaluation of cell surface signals.

Differential Interference Contrast Microscopy

Cells were fixed for 10 min in 3.7% formaldehyde, rinsed three times in PBS, mounted, and analyzed on a Zeiss Axiovert 405M microscope, using a Zeiss Plan-Neo ×100 oil immersion lens.

Scanning Electron Microscopy

The scanning EM analysis was performed at the SEM facility (Department of Rheumatology, Thomas Jefferson University, Philadelphia, PA). After the appropriate treatment, samples that had been prepared on 12-mm round coverslips as described above were rinsed in 0.1 M cacodylate buffer, pH 7.4, and fixed in 2.5% glutaraldehyde. After incubation in 1% OsO_4, specimens were ethanol dehydrated and critical-point dried. Coverslips were then mounted onto aluminum slabs, sputter coated with gold, and viewed using a scanning electron microscope (35-C; JEOL USA, Peabody, MA).

Lysis, Gel Electrophoresis, and Immunoblotting

To study the distribution of clathrin and AP2 between unassembled and assembled forms, we used a procedure modified from Goud et al. (25). RBL cells, 2×10^6 cells per sample, unstimulated or exposed to immobilized antigen for various periods of time, were scraped in 150 μl of buffer A (100 mM sodium 2-(*N*-morpholino) ethane-sulfonate, Na-MES, pH 6.5, 0.5% Triton X-100, 1 mM EGTA, 0.5 mM $MgCl_2$, 2.5 mM *p*-nitrophenylphosphate, 1 mM Na_3VO_4, 10 μg/ml each of leupeptin, chymostatin, and antipain, and 0.5% aprotinin) and briefly homogenized in a 1.5-ml microcentrifuge tube. After 10 min on ice, samples were spun at 245,000 *g* (TL-100; Beckman Instruments, Fullerton, CA). The unassembled coated vesicle proteins were recovered in the supernatants. The assembled coat proteins

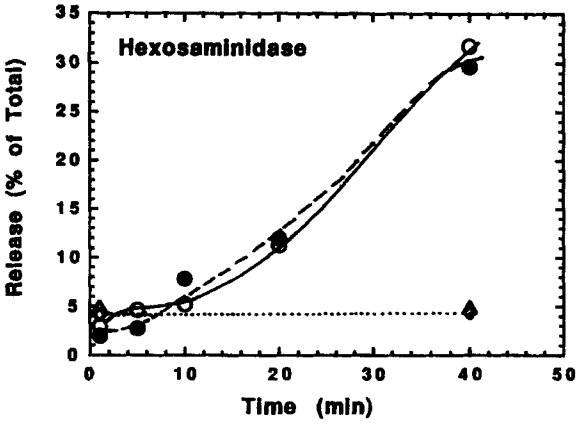

Figure 1. Exposure to immobilized antigen induces a secretory response in RBL-2H3 cells. Incubation of IgE-primed RBL-2H3 cells at 37°C on immobilized DNP_{40}-BSA, preceded (●) or not (○) by 1-h preincubation on ice, induced exocytosis as measured by the release into the medium of hexosaminidase, a granule marker. IgE-primed cells incubated on plates coated with BSA alone (△) or uncoated (◇) gave no release.

present in the pellets were solubilized in buffer B (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 2.5 mM *p*-nitrophenylphosphate, 1 mM Na_3VO_4, 10 μg/ml each of leupeptin, chymostatin, and antipain, and 0.5% aprotinin) and, after 10 min on ice and another high speed centrifugation, recovered in the supernatants. Proteins were solubilized in Laemmli buffer, boiled, separated by electrophoresis on 7.5% SDS–polyacrylamide gels (Bio-Rad Laboratories) and transferred onto nitrocellulose membranes. Clathrin and AP2 were detected using mAb TD-1 and R2, respectively. HRP-conjugated secondary antibodies were visualized with the ECL detection system.

Results

Immobilized Antigen Induces a Secretory Response: Time Course and Effect of Preincubation

The degree of FcεRI activation can be evaluated by the extent of the secretory response after exposure to an inducing antigen (36). We therefore exposed RBL-2H3 cells to immobilized antigen and followed the release into the medium of hexosaminidase, a granule marker. As can be seen in Fig. 1, immobilized antigen induced a secretory response in IgE-primed RBL-2H3 cells, indicating active receptor signaling. Secretion occurred with similar kinetics, whether or not the incubation at 37°C had been preceded by 1-h preincubation on ice. The secretory response was specific for the antigen and did not occur in IgE-primed cells incubated on uncoated plates or on plates coated with BSA alone.

A biphasic relationship exists between the number of receptors aggregated and the secretory response (35–37, 61). After an initial phase in which greater receptor aggregation yields increasing exocytosis, a second phase follows in which further aggregation inactivates receptors and reduces secretion. To avoid the possibility that the degree of receptor cross-linking induced by the immobilized DNP_{40}-BSA had reached an inhibitory level, we prepared coated surfaces using different DNP_{40}-BSA concentrations. Time course measurement of secretion on these surfaces con-

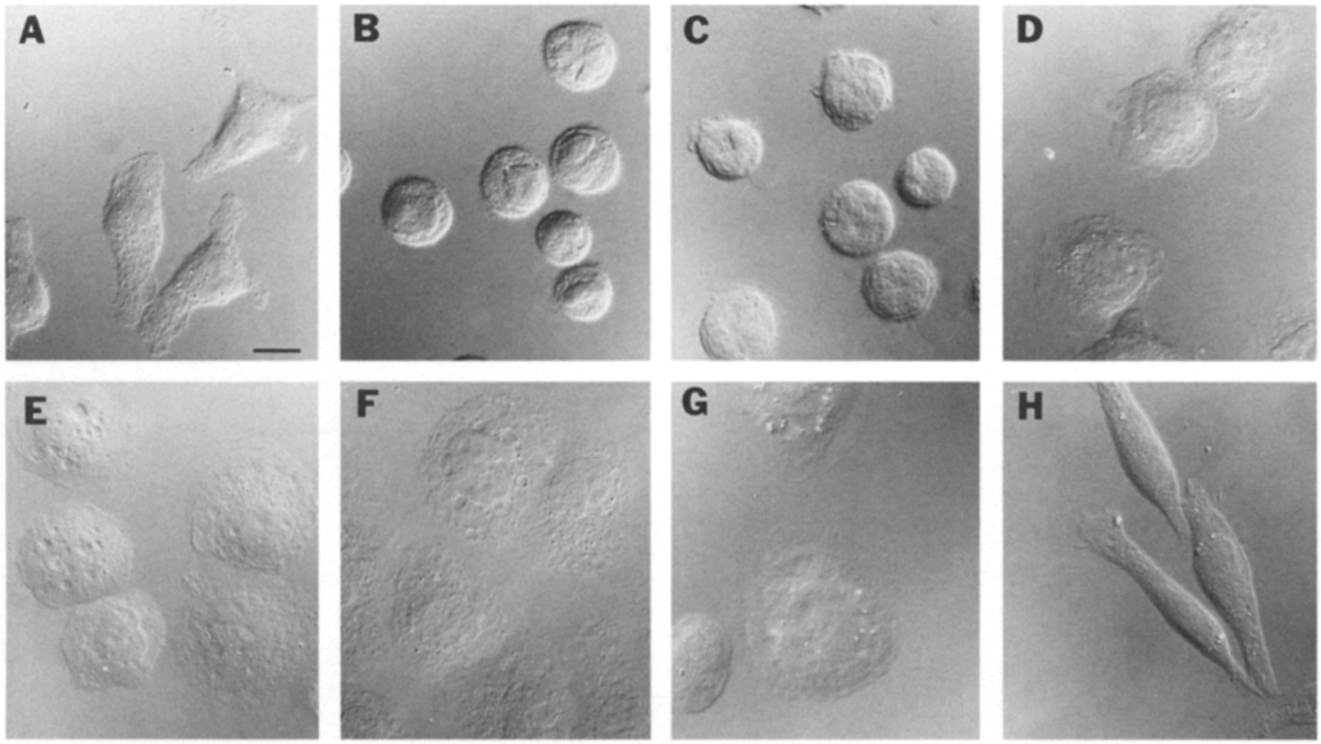

Figure 2. Transmitted light micrographs showing cell spreading on a DNP_{40}-BSA–coated surface. Anti-DNP IgE-sensitized RBL-2H3 cells were plated on glass coverslips coated with BSA (*A*), or DNP_{40}-BSA (*B–G*) at 0°C. After 1 h cells were either fixed immediately (*B*), or shifted to 37°C for 5 min (*C*), 10 min (*D*), 15 min (*E*), 30 min (*F*), or 60 min (*G*) and then fixed. In *H* cells were grown on a regular glass coverslip overnight. Bar, 10 μm.

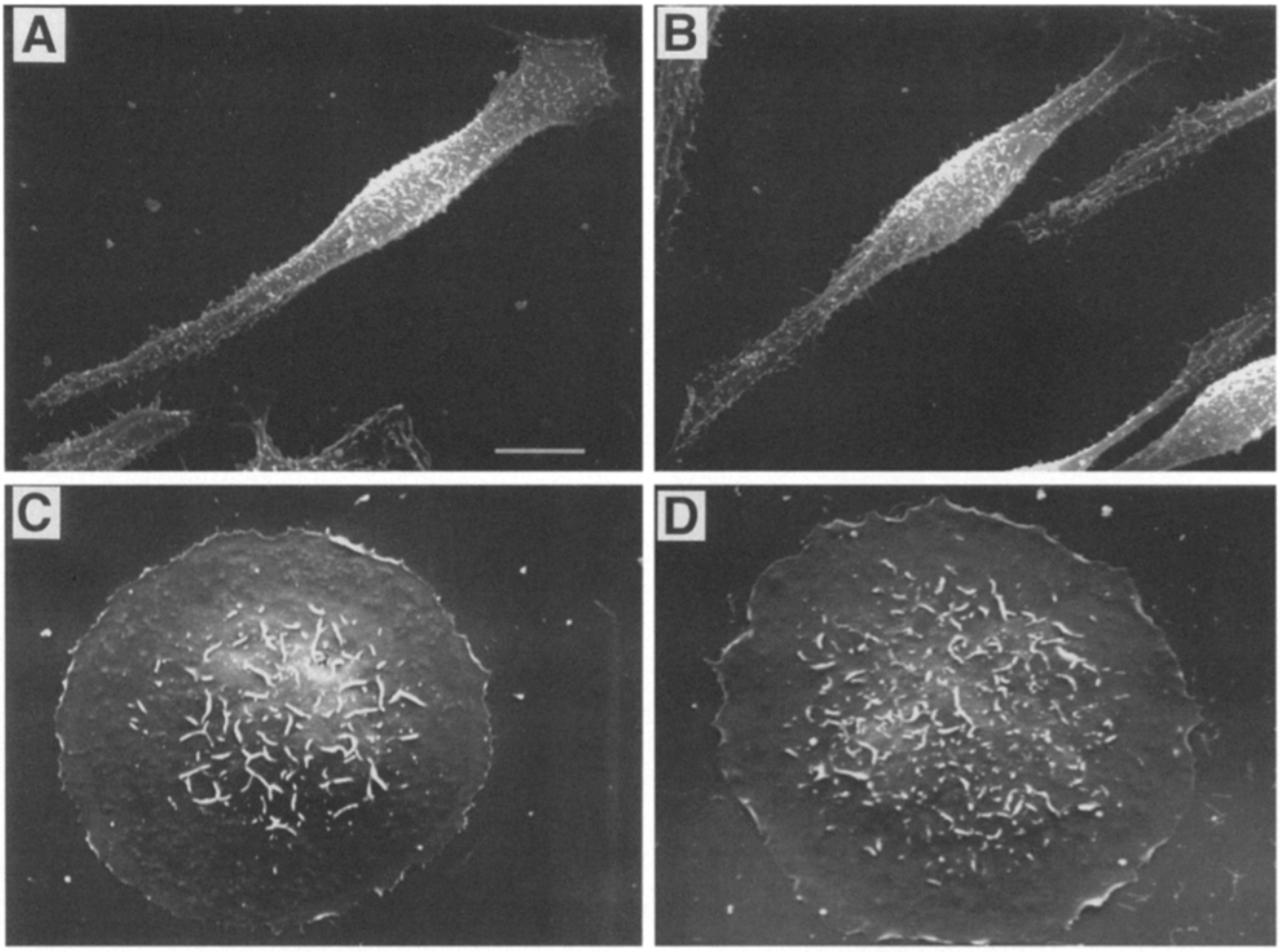

Figure 3. Exposure of DNP-sensitized cells to immobilized DNP_{40}-BSA causes extensive morphological changes including spreading and membrane ruffling. Scanning electron micrographs of RBL-2H3 cells left unstimulated (*A*, and *B*), or exposed to coverslips coated with DNP_{40}-BSA for 1 h at 0°C followed by 30 min at 37°C (*C* and *D*). Bar, 10 μm.

firmed that 1 mg/ml DNP_{40}-BSA was indeed in the stimulatory range (data not shown).

Spreading and Membrane Ruffling in Cells Exposed to Immobilized Antigen

Upon FcεRI cross-linking, mast cells spread and their surfaces, in resting conditions covered by microvilli, become ruffled as a result of cytoskeletal rearrangement (4, 54, 56). To assess if exposure to immobilized antigen could induce such changes and to determine the time frame in which they occurred, we observed RBL-2H3 by transmitted differential interference contrast light microscopy. Anti-DNP IgE-sensitized RBL-2H3 cells were plated on glass coverslips coated with BSA (Fig. 2 *A*), or DNP_{40}-BSA (Fig. 2, *B–G*) at 0°C for 1 h and then either fixed immediately (Fig. 2 *B*), or after incubation at 37°C (Fig. 2, *C–G*). We observed dramatic changes in the appearance of the cells, with extensive spreading and flattening yielding the appearance we define as a "frustrated" morphology, more fully characterized below. This response was specific for DNP_{40}-BSA and did not occur in cells plated on BSA alone (compare Fig. 2 *G* with *A*). It was temperature dependent, as it occurred only after raising the temperature from 0° to 37°C (compare Fig. 2 *B* with *C–D*). Finally, activation was also time dependent as it reached a peak between 10 (Fig. 2 *D*) and 15 min (Fig. 2 *E*) that was maintained for 60 min (Fig. 2 *G*) to 2 h (not shown). The appearance of cells grown on uncoated glass coverslips overnight is shown in (Fig. 2 *H*).

These antigen-induced morphological changes could also be visualized by scanning EM (Fig. 3). This technique better revealed the differences in cell surface topography, in particular the appearance of numerous ruffles, between unstimulated cells (Fig. 3, *A* and *B*) and cells exposed to immobilized DNP_{40}-BSA for 1 h at 0°C followed by 30 min at 37°C (Fig. 3, *C* and *D*).

Redistribution of the IgE Receptors toward the Substratum in Cells Exposed to Immobilized DNP_{40}-BSA

To gain information about the timing and extent of the FcεRI relocalization, samples were prepared at different times and analyzed by confocal microscopy (Fig. 4). FcεRI receptors were directly labeled by incubating cells with

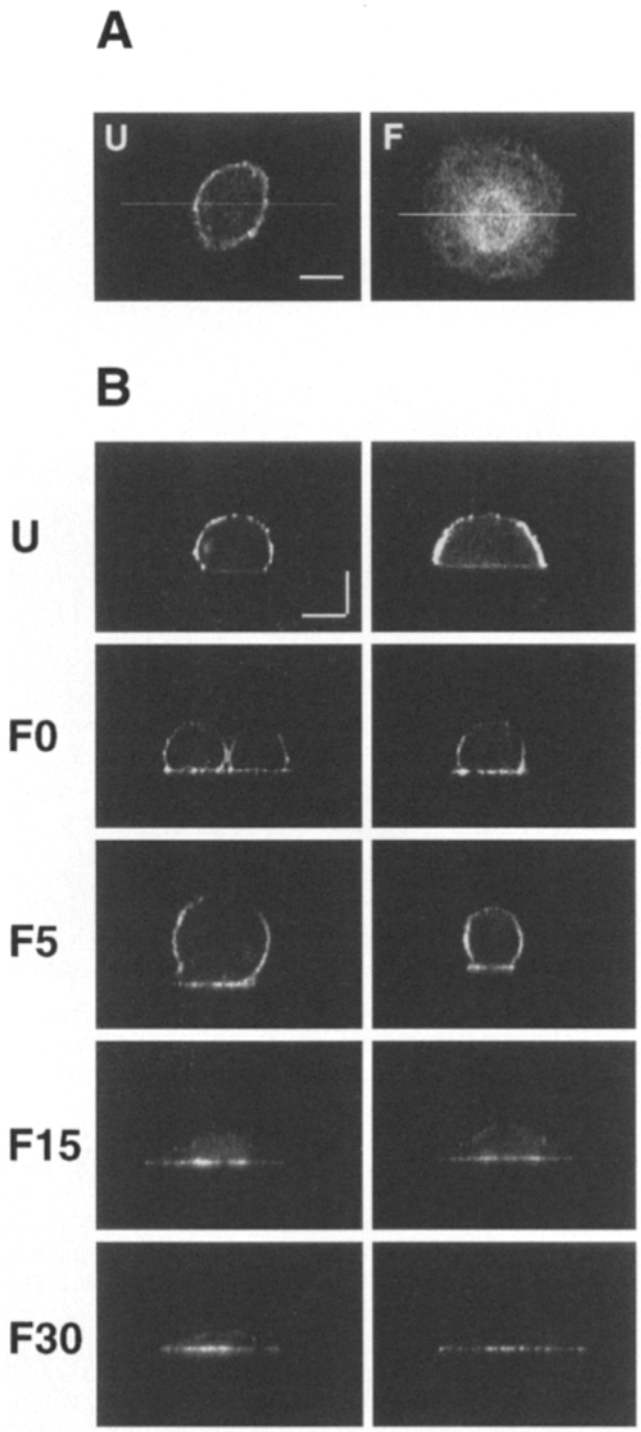

Figure 4. Exposure of RBL-2H3 cells to immobilized DNP_{40}-BSA induces a gradual relocalization of the IgE receptor from the apical to the ventral surface. (*A*) Confocal micrographs, taken with an equatorial focal plane just above the substrate surface, of the distribution of FL-IgE–labeled receptors on unstimulated cells (*U*) or cells exposed to DNP_{40}-BSA–coated surfaces for 30 min at 37°C (*F*). The lines bisecting the images are examples of the position of the *x–z* plane used in *B*. (*B*) Confocal vertical *x–z* images (cross sections) of two examples of unstimulated cells (*U*), and of cells exposed to DNP_{40}-BSA–coated surfaces for 1 h at 0°C (*F0*) and then warmed up to 37°C for 5 min (*F5*), 15 min (*F15*), or 30 min (*F30*). Bar, 10 μm.

FL-conjugated DNP-specific IgE (FL-IgE). Cells were then exposed to control BSA-coated surfaces, termed "unstimulated," or to DNP_{40}-BSA–coated surfaces, termed "frustrated" (Fig. 4 *A*; *U* and *F*, respectively), for 1 h at 0°C followed by incubation at 37°C for different times. As revealed by conventional *x-y* images, in the absence of the antigen (Fig. 4 *A*; *U*), the IgE–receptor complexes were distributed uniformly on the plasma membrane, revealing a ring in images taken with the focal plane near the center of the cell. However, after 30 min on the DNP_{40}-BSA–coated surface (Fig. 4 *A*; *F*), the fluorescent signal had formed a complex pattern at the site of contact with the antigen.

Because individual images like those in Fig. 4 *A* do not completely describe the distribution of surface markers, as mental integration of a series of through-cell focal planes is required, FcεRI receptor distribution was monitored using a focal plane perpendicular to the substrate (Fig. 4 *B*, *x–z* cross sections). Upon exposure to immobilized DNP_{40}-BSA, a gradual relocalization of the IgE receptors from the apical membrane to the ventral membrane that was in contact with the antigen was evident. The FcεRI redistribution was a temperature-dependent process and did not occur during 1-h preincubation at 0°C (Fig. 4 *B*; *F0*). Once cells were shifted to 37°C, though, redistribution took place over a period of 15 min and was complete by 30 min (Fig. 5 *B*; *F5–F30*). The absence of intracellular FL-IgE indicated that IgE-bound receptors were not endocytosed. Similar results were obtained when FcεRI receptors were localized by indirect immunofluorescence using anti-FcεRI β-chain antibody (data not shown). Together, these results provided direct evidence that the receptors had been engaged and activated, but not endocytosed, thus resulting in a frustrated phenotype.

Internalization of Transferrin in Cells Exposed to Immobilized Antigen

Because in frustrated cells endocytosis of FcεRI receptors was inhibited, we asked whether endocytosis of other receptors was still occurring. We chose to follow the uptake of FL-labeled transferrin, a clathrin-mediated process (1, 67). Unstimulated cells and cells exposed to immobilized

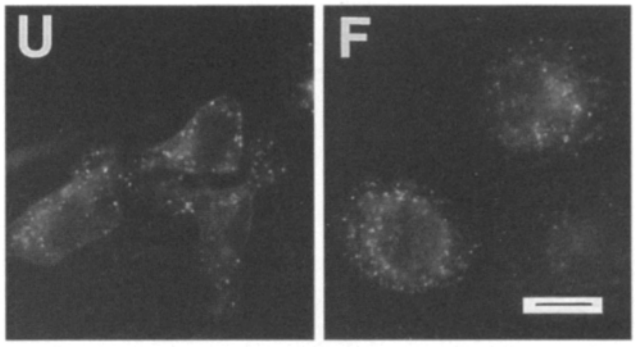

Figure 5. RBL-2H3 cells stimulated by immobilized DNP_{40}-BSA are still capable of internalizing transferrin. Cells unstimulated (*U*) or frustrated on a DNP_{40}-BSA surface for 25 min (*F*) were incubated with fluorescently labeled transferrin for 20 min at 37°C. After washing in PBS, cells were fixed and observed. Bar, 10 μm.

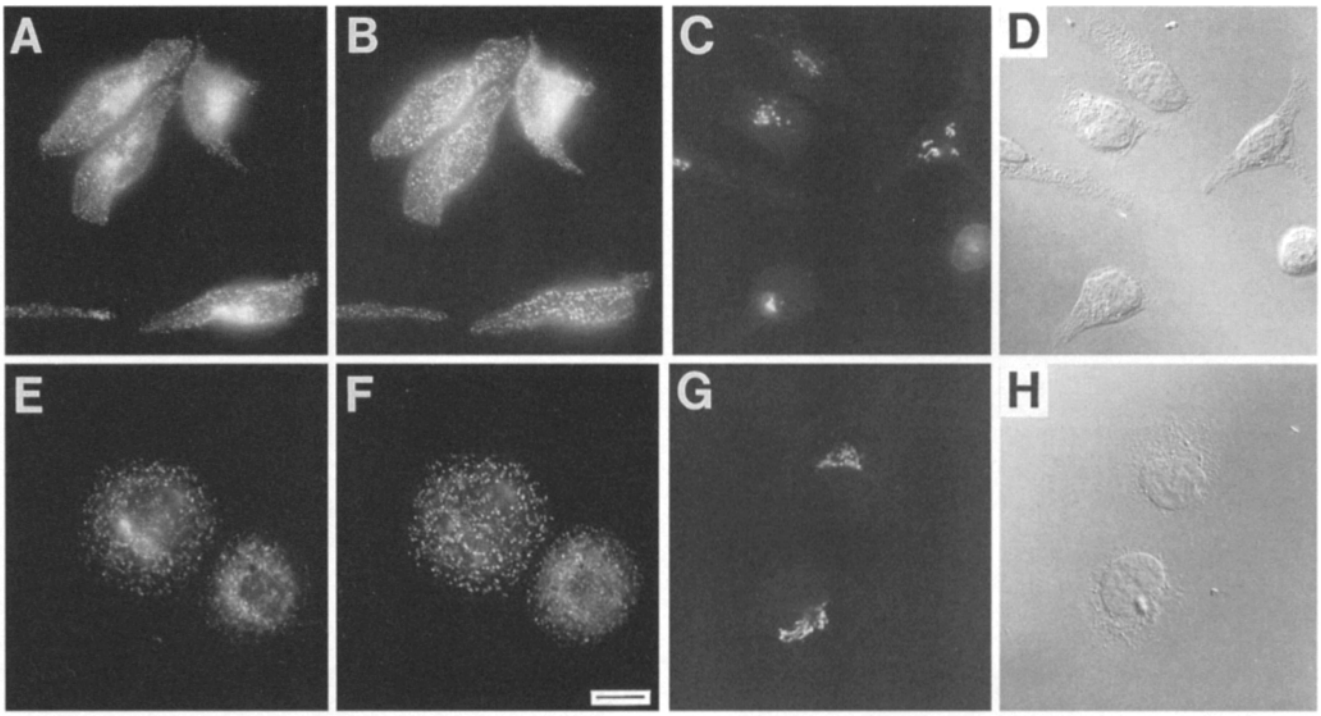

Figure 6. Distribution of clathrin, AP2, and p135 mannosidase II (Golgi marker) in unstimulated and immobilized antigen exposed RBL-2H3 cells. RBL-2H3 cells primed with anti-DNP IgE were exposed to an uncoated or DNP_{40}-BSA–coated coverslip at 4°C for 1 h, followed by 30 min at 37°C. Clathrin (*A* and *E*), AP2 (*B* and *F*), and mannosidase II (*C* and *G*) were then detected in unstimulated (*A–C*) and frustrated (*E–G*) cells. *D* and *H* show the differential interference contrast images of *C* and *G*, respectively. Bar, 10 μm.

DNP_{40}-BSA for 25 min were incubated in the presence of FL-transferrin for an additional 20 min before being prepared for fluorescence microscopy. As shown in Fig. 5, endocytosis of transferrin occurred in both unstimulated (Fig. 5 *U*) and frustrated cells (Fig. 5 *F*).

Stimulation of Cells by Immobilized Antigen Does not Affect the Clathrin and AP2 Distribution or the Integrity of the Golgi

One model of ligand-induced endocytosis suggests that activated receptors recruit coat components triggering the formation of new clathrin-coated pits. We therefore investigated the distributions of clathrin and AP2 in cells exposed to immobilized antigen and compared them with those of unstimulated cells using double labeling indirect immunofluorescence (see Materials and Methods).

As in fibroblasts and other cell types (75), in unstimulated RBL-2H3 cells, staining for clathrin produced a punctate pattern extending to the periphery of cell, as well as in a perinuclear region identified as *trans*-Golgi (Fig. 6 *A*) by comparison with anti-mannosidase II, a Golgi protein marker (Fig. 6 *C*). In the same cells, AP2 staining was confined to the plasma membrane (Fig. 6 *B*) and was essentially superimposable with that of clathrin. Remarkably, in cells frustrated at 37°C, except for the change in cell shape, we did not observe appreciable differences in the intensity, density, or distribution of either clathrin or AP2 for up to 90 min. For example, Fig. 6 shows the distribution of clathrin (*E*) and AP2 (*F*) in cells that had been frustrated for 30 min. Examination of the cells by conventional microscopy revealed no appreciable differences in the overall distribution and intensity of clathrin (Fig. 6 *E*) and AP2 (Fig. 6 *F*), although the cell shape had changed considerably. In particular, it should be noted that Golgi was still present (Fig. 6 *G*), and Golgi-associated clathrin was still prominent (Fig. 6 *E*). Note also that the distribution of aggregated receptors on the ventral membrane of frustrated cells (Fig. 4 *A*; *F*) showed a pattern extremely different from that of clathrin (Fig. 6 *E*) or AP2 (Fig. 6 *F*), indicating the absence of colocalization of coats and receptors.

Fig. 7 shows several examples of *x–z* confocal scans of the distribution of clathrin (*upper panels*) and AP2 (*lower panels*) in RBL-2H3 cells unstimulated (*U*) or exposed for 30 min to immobilized antigen (*F30*). It is more convincing with this approach that unstimulated and frustrated cells showed the same distribution of clathrin and AP2. Moreover, there was no detectable increase in AP2 and/or clathrin on the ventral membrane of frustrated cells, bearing activated receptors, as compared with the apical membrane, as we would have expected if the receptors were inducing formation of new pits by active recruitment. These observations were extended by determining the sum of AP2-integrated pixel intensities in confocal images to obtain a quantitative estimate of coated pit distribution on apical and ventral surfaces of individual cells (see Materials and Methods). This analysis showed that the ratio of coated pit density was unchanged between unstimulated and frustrated cells (Table I).

Essentially the same results were obtained even when the assay conditions were varied. For example, we did not

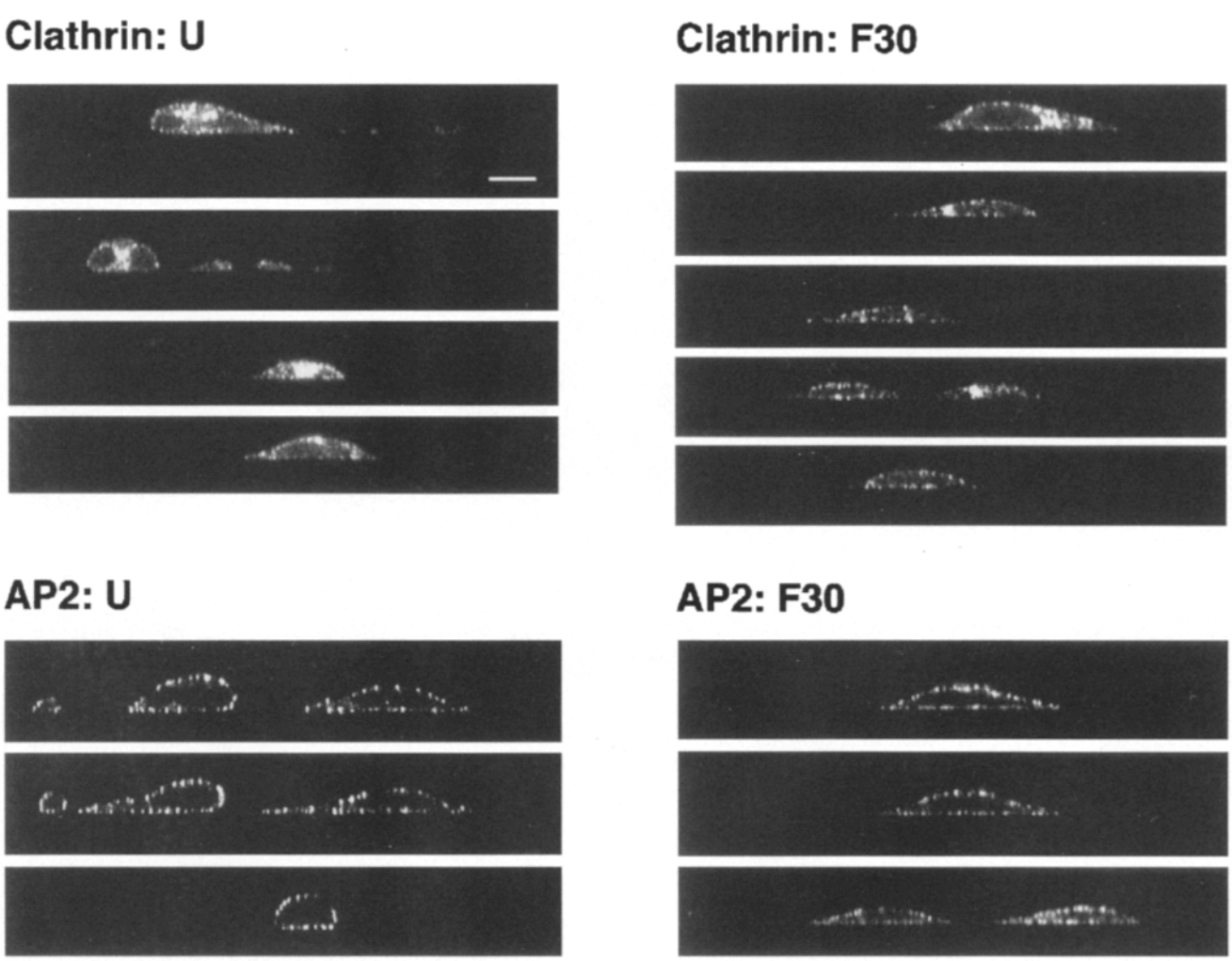

Figure 7. Punctate clathrin and AP2 distributions are unchanged in cells stimulated by immobilized DNP_{40}-BSA. Confocal microscopy analysis showing the distribution of clathrin (*upper panels*) and AP2 (*lower panels*) in RBL-2H3 cells on uncoated (*U*) or immobilized antigen–coated surfaces (*F30*) for 30 min. Bar, 10 μm.

observe any difference in the clathrin and AP2 staining pattern when cells were stimulated without a 0°C preincubation step. This was also true when cells were frustrated in the absence of extracellular calcium. The latter condition, by inhibiting the burst of exocytosis after activation, allowed us to separately evaluate receptor-induced endocytosis without concomitant membrane recycling. It should be noted that although most of the data presented were obtained with 30-min frustrated cells, we carried out experiments at intervals from 1 to 120 min with similar results.

Finally, the frustrated condition was readily reversible by addition of soluble DNP_{40}-BSA, which maintained FcεRI cross-linking and activation but allowed endocytosis (data not shown). When cells that had been subjected to immobilized antigen for 30 min were incubated with soluble antigen, a rapid return (>1 to 8 min) to the rounded morphology was observed (corresponding to change from Fig. 4; *F30* to *F5* or *F0*). Importantly, at no time was an increase in clathrin-coated pits observed.

Table I. Coated Pit Distribution in Unstimulated and Immobilized Antigen–stimulated RBL-2H3 Cells

	Coated pits			
	Apical	Ventral	SD	(*n*)
	%	%		
Unstimulated	50.9	49.1	±3.3	9
Frustrated	51.6	48.4	±3.1	25

Coated pit density was quantitated by analyzing pixel intensity on AP2 images (*x-z* scan) of unstimulated cells (*Unstimulated*) and of cells exposed to immobilized antigen for 30 min (*Frustrated*). Distribution of clathrin-coated pits (as pixel per μm^2) on the apical and ventral membrane is expressed as percentage of the total. Analysis was performed as described in Materials and Methods; data are presented as mean ± SD, where (*n*) equals number of cells examined.

Unchanged Distribution of Clathrin-coated Pit Components between Soluble and Assembled Pools in Stimulated Cells

In the cell, clathrin and AP2 exist in soluble and assembled forms (25). The morphological observations described above are expected to be best at revealing the state of assembled clathrin coat structures within the cell. To complement and extend these findings, we compared the proportion of unassembled and assembled clathrin and

AP2 in frustrated and unstimulated cells by immunoblotting techniques.

IgE-sensitized RBL-2H3 cells were exposed to plates coated with BSA (Fig. 8; *Un*) or DNP_{40}-BSA (*0′*, *15′*, *30′*) at 0°C for 1 h before being incubated at 37°C for 0 (*0′*), 15 (*15′*), and 30 (*Un*, *30′*) min. At various times during the 37°C incubation, samples were lysed (see Materials and Methods), and soluble and assembled clathrin and AP were analyzed by immunoblotting.

The results indicate that ~20% of clathrin and 50% of β (which may include both AP2 β and AP1 β′ subunits) were present as assembled, sedimentable forms (Fig. 8). Analysis of frustrated cells revealed no pronounced changes in the proportions of these coated pit proteins. Furthermore, in all cases a substantial pool of soluble, disassembled coated pit proteins was observed. With time, a significant amount of clipping of the β subunit was observed, likely a consequence of postlysis degradation by proteases released upon receptor activation.

Discussion

That coated pits mediate the endocytic uptake of many receptors from the plasma membrane of mammalian cells is a well-established concept (24). According to the current paradigm of receptor-mediated endocytosis, detached and internalized coated vesicles with receptor cargo rapidly lose their coat proteins. Subsequently, these coat proteins in soluble form recycle back to the membrane to form new coated pits. Kinetic studies suggest that in many cells the lifetime of a coated pit at the plasma membrane is about a minute, and that ~1,000 coated pits detach from the plasma membrane each minute (70).

While the overall pathway is clear, the fundamental mechanisms by which activated receptors once targeted for endocytosis are found in coated pits is an important yet unresolved question, with broad implications for our understanding of how cells interact with their environment. Specifically, are activated receptors simply trapped on encountering preexisting coated pits, subsequently being rapidly internalized? Or do the receptors themselves, by active recruitment, gather the necessary soluble coat and cytosolic components and initiate the rapid assembly of new coated pits that then mediate their internalization? In view of the central importance of this issue, we directly addressed this proverbial "chicken and egg" question using an experimental system that would allow us to gather information specifically about the earliest steps of receptor-coated pit interaction.

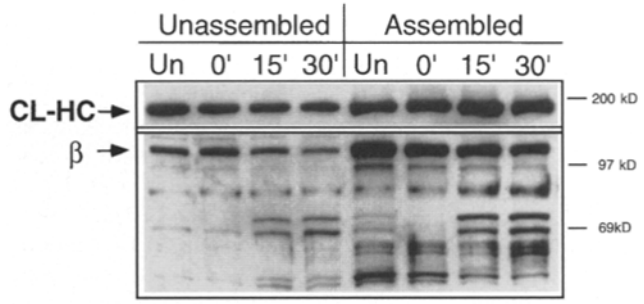

Figure 8. Unassembled pools of clathrin and AP persist in frustrated cells. Immunoblots showing repartition between unassembled and assembled clathrin and AP during frustrated endocytosis. Anti-DNP IgE-primed RBL-2H3 cells were exposed to BSA-(*Un*) or DNP_{40}-BSA–(*F*) coated plates at 0°C for 1 h before being transferred to a 37°C water bath. At 0 (*0′*), 15 (*15′*), and 30 (*30′*) min during the 37°C incubation, samples were processed to identify unassembled and assembled pools of clathrin (*CL-HC*) and AP (*β*) as described in Materials and Methods. Note that proteins derived from 2×10^4 cells were used per lane for the analysis of Unassembled proteins and from 10^5 cells per lane for the analysis of Assembled proteins.

RBL-2H3 cells, a continuous rat mast cell line, express multichain FcεRI receptors that, in the absence of antigen, are present almost exclusively on the cell surface. Addition of multivalent antigen (here BSA with multiple covalently attached DNP residues) induces cross-linking of receptor-bound IgE (here anti-DNP) and rapid internalization of the complexes via clathrin-coated pits with a half-time of <8 min (54, 56, 61, 64). We used a system in which IgE receptors were stimulated while their endocytosis was blocked by exposing cells primed with antigen-specific IgE to surface-immobilized antigen. In a sense, this experimental system is a physiological version of the surface plasmon resonance technique used to study protein–protein interactions (45), with the cytoplasmic face of the plasma membrane providing an in vivo biosensor surface.

The rationale behind our approach was that if activated receptors directly act as coated pit component recruiters, activation of receptors on the ventral surface combined with inhibition of their internalization should have elicited one or more of the following: (*a*) an increase in the number of coated pits on the ventral plasma membrane surface; (*b*) colocalization of the activated receptors with these new coated pits; and (*c*) recruitment of soluble coated pit components to the ventral frustrated surface from the apical plasma membrane and, possibly, the Golgi region.

From the experimental point of view, the system has the important benefits of avoiding the use of either pharmacological agents or overexpression of receptors, both of which can have unanticipated and undetected effects. Under the experimental conditions used here, the RBL cells continued to take up transferrin, suggesting that overall membrane transport had not been blocked. Unlike related experiments in macrophages (5, 65), the organization of the Golgi in the stimulated RBL cells was intact as assessed by the behavior of both integral (mannosidase II) and peripheral (clathrin) membrane proteins. Additionally, frustration in these cells could be rapidly reversed by addition of soluble antigen. Finally, this system has relevance to mast cell physiology and pathology. Mast cells can encounter multivalent antigens (e.g., pollen particles) far too large (50–200 μm) to be internalized (18), and such interactions are believed to play a role in secondary allergic responses such as asthma and rheumatoid arthritis (26, 72).

Immobilized antigen induced the full array of changes characteristic of normal receptor activation including alteration in cell shape and membrane morphology, reflecting actin-mediated cytoskeletal rearrangements, and degranulation (4, 54). IgE receptors migrated from a dispersed distribution and were found aggregated on the membrane in contact with the immobilized antigen. As there are ~4×10^5 FcεRI receptors per cell (36), it is im-

portant to note that their relocalization resulted in an effective membrane density on the ventral surface equivalent to almost 10^6 receptors per cell.

Despite the generation of this high density lawn of activated receptors on the adherent surface, neither clathrin nor AP2 staining coincided with Fcε receptors even when stimulation was monitored for up to 2 h. Nor did we find significant changes during this period in the overall morphological distribution of coated pit proteins in frustrated cells when compared to unstimulated cells, or in the quantitative distribution of coated pits on the ventral and apical surface profiles of the cells before and after frustration (Table I). Finally, no evidence for an increase in coated pits was detected on release from frustration with soluble antigen.

These observations were supported by immunochemical analysis that showed the continued presence of a large soluble pool of unassembled coat proteins in frustrated cells, excluding the possibility that these proteins were quantitatively unavailable for recruitment by activated IgE receptors. In addition, the ratio of membrane-bound and soluble clathrin and APs, presumably corresponding to assembled and unassembled pools, was not significantly changed by the treatment. As the soluble pool comprises 50–80% of the total, a 200–400% increase in coated pits on the activated surface should have been attainable, yet changes of 5% or less were observed.

A conclusion emerging from this study is that for FcεRI receptors the "chicken" model of receptor-mediated endocytosis is accurate: rapid internalization of activated receptors is not a consequence of recruitment of components to form de novo coated pits, but rather results from receptor movement to preexisting coated pits. We believe that, while restricted to FcεRI receptors within RBL cells, our observations are of general relevance. Preliminary experiments with two other receptors, transferrin and mannose-terminated glycoproteins, have provided no evidence for increases in coated pit formation when receptors were concentrated on one surface of the cell (Santini, F., and J.H. Keen, unpublished observations). Collectively, these results also argue that clathrin-coated pits are essentially immobile in the plane of the plasma membrane.

In addition, our findings are quite consistent with some earlier kinetic analysis on the internalization of nutritive, nonsignaling receptors. Henis and co-workers (19, 31) examined the behavior of constitutively internalized asialoglycoprotein receptors and influenza HA mutated to rapidly internalized forms. Mobility of these receptors on the cell surface was consistent with random movements and occasional encounters of receptors with coated pits, culminating in immobilization and internalization. Similar conclusions were drawn by Paccaud et al. (49) in studies on complement receptor type 1 mutations. Finally, biophysical studies using single particle tracking of LDL and α_2-macroglobulin ligands and receptors on cell surfaces are consistent with random diffusional movements within short restricted regions, with internalization suggested to result from encounter with preexisting pits (22, 58). On the other hand, fibroblasts overexpressing transferrin receptor were initially reported to exhibit an increased number of functional clathrin-coated pits at the plasma membrane (29). However, subsequent work showed that only anomalous flat clathrin lattices had increased, and that there was no correlation between the number of receptors expressed and the number of coated pits (44), again suggesting that coated pit number is not directly related to the number of these nonsignaling receptors on the cell surface. In this context, the failure of activated receptors to recruit AP2, despite the evidence that AP2 can form stable interactions with some receptors (23, 47, 62, 63), suggests that the AP2–receptor complex forms in vivo only as result of receptor movement to sites of AP2 localization, i.e., as receptors move to coated pits. The recent observation that deletion of the high affinity EGF receptor binding site for AP2 (63) had no effect on the rate of EGF receptor endocytosis (46) raises further questions about the role of the interaction in the initial events of endocytosis.

Yet, rapid increases in coated pit number after activation of certain membrane signaling systems have been demonstrated convincingly in several studies, as noted in the Introduction. Although these observations pointed to nucleation of new coated pits by activated receptors, careful kinetic analysis of endocytic rates in at least one case actually suggested a lack of correlation between ligand concentration and receptor internalization (73). Lack of proportionality between activated receptor number and increases in coated pits is also suggested by several other examples. The most direct comparison of our work with FcεRI can be made with earlier findings with EGF, as both receptors are internalized at comparable rates. In contrast to our findings with RBL-2H3 cells that contain 400,000 FcεRI receptors (or the equivalent of almost 10^6 receptors per cell on the stimulated surface), only 40,000 EGF receptors dispersed on either PC12 cells or neurons mediated a rapid and sustained three- to fourfold increase in coated pits. An even more extreme case is the rapid appearance of extensive coated pits on previously uncoated plasma membrane after fertilization of a sea urchin egg by a single sperm (20). Additionally, changes in coated pit distribution have been implicated in the initial stages of cell attachment to substrates (71, 76).

Our results demonstrate that de novo coated pit formation is neither a requirement for rapid receptor internalization nor an obligate consequence of receptor activation. A corollary of our and other results is that the formation of new clathrin-coated pits is a downstream result of signaling by certain agonists, and that multiple mechanisms regulate coated pit dynamics, though they may not be coupled to receptor internalization rates. New coated pits could form in response to certain effector(s) (e.g., G-proteins) or product(s) (e.g., phosphoinositides) that modulate the equilibrium between unassembled and assembled coat components directly, or by creating new nucleation sites. It will be important to dissect this process to identify the agents, likely to be common to several signaling systems, that trigger the specific mobilization of coated pit components.

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References

1. Ajioka, R.S., and J. Kaplan. 1986. Intracellular pools of transferrin receptors result from constitutive internalization of unoccupied receptors. *Proc. Natl. Acad. Sci. USA.* 83:6445–6449.
2. Ajioka, R.S., and J. Kaplan. 1987. Characterization of endocytic compartments using the horseradish peroxidase–diaminobenzidine density shift technique. *J. Cell Biol.* 104:77–85.
3. Anderson, R.G.W. 1993. Dissecting clathrin-coated pits. *Trends Cell Biol.* 3:177–179.
4. Apgar, J.R. 1991. Regulation of the antigen-induced F-actin response in rat basophilic leukemia cells by protein kinase C. *J. Cell Biol.* 112:1157–1163.
5. Bainton, D.F., R. Takemura, P.E. Stenberg, and Z. Werb. 1989. Rapid fragmentation and reorganization of Golgi membranes during frustrated phagocytosis of immobile immune complexes by macrophages. *Am. J. Pathol.* 134:15–26.
6. Beaven, M.A., and J.R. Cunha-Melo. 1988. Membrane phosphoinositide-activated signals in mast cells and basophils. *Progr. Allergy.* 42:123–184.
7. Beaven, M.A., and H. Metzger. 1993. Signal transduction by Fc receptors: the Fcε case. *Immunol. Today.* 14:222–226.
8. Beck, K.A., M. Chang, F.M. Brodsky, and J.H. Keen. 1992. Clathrin assembly protein AP-2 induces aggregation of membrane vesicles: a possible role for AP-2 in endosome formation. *J. Cell Biol.* 119:787–796.
9. Benhamou, M., and R.P. Siraganian. 1992. Protein-tyrosine phosphorylation: an essential component of FcεRI signaling. *Immunol. Today.* 13: 195–197.
10. Benhamou, M., V. Stephan, K.C. Robbins, and R.P. Siraganian. 1992. High-affinity IgE receptor-mediated stimulation of rat basophilic leukemia (RBL-2H3) cells induces early and late protein-tyrosine phosphorylations. *J. Biol. Chem.* 267:7310–7314.
11. Choi, O.H., J.H. Lee, T. Kassessinoff, J.R. Cunha-Melo, S.V.P. Jones, and M.A. Beaven. 1993. Carbachol and antigen mobilize calcium by similar mechanisms in a transfected mast cell line (RBL-2H3 cells) that express m1 muscarinic receptors. *J. Immunol.* 151:5586–5595.
12. Choi, O.H., R.S. Adelstein, and M.A. Beaven. 1994. Secretion from rat basophilic RBL-2H3 cells is associated with phosphorylation of myosin light chains by myosin light chain kinase as well as phosphorylation by protein kinase C. *J. Biol. Chem.* 269:536–541.
13. Connolly, J.L., S.A. Green, and L.A. Greene. 1981. Pit formation and rapid changes in surface morphology of sympathetic neurons in response to nerve growth factor. *J. Cell Biol.* 90:176–180.
14. Connolly, J.L., S.A. Green, and L.A. Greene. 1984. Comparison of rapid changes in surface morphology and coated pit formation of PC12 cells in response to nerve growth factor, epidermal growth factor, and dibutyryl cyclic AMP. *J. Cell Biol.* 98:457–465.
15. Corvera, S. 1990. Insulin stimulates the assembly of cytosolic clathrin onto adipocyte plasma membranes. *J. Biol. Chem.* 265:2413–2416.
16. Damke, H., T. Baba, D.E. Warnock, and S.L. Schmid. 1994. Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. *J. Cell Biol.* 127:915–934.
17. Eiseman, E., and J.B. Bolen. 1992. Engagement of the high-affinity IgE receptor activates src protein-related tyrosine kinases. *Nature (Lond.).* 355: 78–80.
18. Fadal, R. 1993. IgE-mediated hypersensitivity reactions. *Otolaryngol.-Head Neck Surg.* 109:565–578.
19. Fire, E., D.E. Zwart, M.G. Roth, and Y.I. Henis. 1991. Evidence from lateral mobility studies for dynamic interactions of a mutant influenza hemagglutinin with coated pits. *J. Cell Biol.* 155:1585–1594.
20. Fisher, G.W., and L.I. Rebhun. 1983. Sea urchin egg cortical granule exocytosis is followed by a burst of membrane retrieval via uptake into coated vesicles. *Dev. Biol.* 99:456–472.
21. Gelsow, M.J., and J. Childs. 1985. Cholinergic stimulation of chromaffin cells induces rapid coating of the plasma membrane. *Euro. J. Cell Biol.* 38:51–56.
22. Ghosh, R.N., and W.W. Webb. 1994. Automated detection and tracking of individual and clustered cell surface low density lipoprotein receptor molecules. *Biophys. J.* 66:1301–1318.
23. Gilboa, L., R. Ben-Levy, Y. Yarden, and Y.I. Henis. 1995. Roles for a cytoplasmic tyrosine and tyrosine kinase activity in the interactions of neu receptors with coated pits. *J. Biol. Chem.* 270:7061–7067.
24. Goldstein, J.L., R.G. Anderson, and M.S. Brown. 1979. Coated pits, coated vesicles, and receptor-mediated endocytosis. *Nature (Lond.).* 279:679–685.
25. Goud, B., C. Huet, and D. Louvard. 1985. Assembled and unassembled pools of clathrin: a quantitative study using an enzyme immunoassay. *J. Cell Biol.* 100:521–527.
26. Gruber, B.L. 1991. Immunoglobulin E, mast cells, endogenous antigens, and arthritis. *Rheum. Dis. Clin. North Am.* 17:333–342.
27. Gruchalla, R.S., T.T. Dinh, and D.A. Kennerly. 1990. An indirect pathway of receptor-mediated 1,2-diacylglycerol formation in mast cells. I. IgE receptor-mediated activation of phospholipase D. *J. Immunol.* 144:2334–2342.
28. Hirasawa, N., F. Santini, and M.A. Beaven. 1995. Activation of the mitogen-activated protein kinase/cytosolic phospholipase A2 pathway in a rat mast cell line: indication of different pathways for release of arachidonic acid and secretory granules. *J. Immunol.* 154:5391–5402.
29. Iacopetta, B.J., S. Rothenberger, and L.C. Kühn. 1988. A role for the cytoplasmic domain in transferrin receptor sorting and coated pit formation during endocytosis. *Cell.* 54:485–489.
30. Jones, S.V.P., O.H. Choi, and M.A. Beaven. 1991. Carbachol induces secretion in a mast cell line (RBL-2H3) transfected with the m1 muscarinic receptor gene. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 289:47–50.
31. Katzir, Z., N. Nardi, I. Geffen, C. Fuhrer, and Y.I. Henis. 1994. Dynamic interactions of the asialoglycoprotein receptor subunits with coated pits: enhanced interactions of H2 following association with H1. *J. Biol. Chem.* 269:21578–21575.
32. Keen, J.H. 1990. Clathrin and associated assembly and disassembly proteins. *Annu. Rev. Biochem.* 59:415–438.
33. Lin, P.Y., G.A. Wiggan, and A.M. Gilfillan. 1991. Activation of phospholipase D in a rat mast (RBL-2H3) cell line: a possible unifying mechanism for IgE-dependent degranulation and arachidonic acid metabolite release. *J. Immunol.* 146:1609–1616.
34. Ludowyke, R.I., I. Peleg, M.A. Beaven, and R.S. Adelstein. 1989. Antigen-induced secretion of histamine and the phosphorylation of myosin by protein kinase C in rat basophilic leukemia cells. *J. Biol. Chem.* 264: 12492–12501.
35. Maeyama, K., R.J. Hohman, H. Metzger, and M.A. Beaven. 1986. Quantitative relationships between aggregation of IgE receptors, generation of intracellular signals and histamine secretion in rat basophilic leukemia (2H3) cells: enhanced responses with heavy water. *J. Biol. Chem.* 261: 2583–2592.
36. Maeyama, K., R.J. Hohman, H. All, J.R. Cunha-Melo, and M.A. Beaven. 1988. Assessment of IgE-receptor function through measurement of hydrolysis of membrane inositol phospholipids: new insights on the phenomena of biphasic antigen concentration-response curves and desensitization. *J. Immunol.* 140:3919–3927.
37. Magro, A.M., and A. Alexander. 1974. Histamine release: In vitro studies of the inhibitory region of the dose-response curve. *J. Immunol.* 112: 1762–1765.
38. Mao, S.-Y., J.R. Pfeiffer, J.M. Oliver, and H. Metzger. 1993. Effects of subunit mutation on the localization of coated pits and internalization of cross-linked IgE-receptor complexes. *J. Immunol.* 151:2760–2774.
39. Menon, A.K., D. Holowka, and B. Baird. 1984. Small oligomers of immunoglobulin E (IgE) cause large-scale clustering of IgE receptors on the surface of rat basophilic leukemia cells. *J. Cell Biol.* 98:577–583.
40. Metzger, H. 1992. Transmembrane signaling: the joy of aggregation. *J. Immunol.* 149:1477–1487.
41. Michl, J., M.M. Pieczonka, J.C. Unkeless, and S.C. Silverstein. 1979. Effects of immobilized immune complexes on Fc- and complement-receptor function in resident and thioglycollate-elicited mouse peritoneal macrophages. *J. Exp. Med.* 150:607–621.
42. Miettinen, H.M. J.K. Rose, and I. Mellman. 1989. Fc receptor isoforms exhibit distinct abilities for coated pit localization as a result of cytoplasmic domain heterogeneity. *Cell.* 58:317–327.
43. Millard, P.J., T. Ryan, W.W. Webb, and C. Fewtrell. 1989. Immunoglobulin E receptor cross-linking induces oscillations in intracellular free ionized calcium in individual tumor mast cells. *J. Biol. Chem.* 264:19730–19739.
44. Miller, K., M. Shipman, I.S. Trowbridge, and C.R. Hopkins. 1991. Transferrin receptors promote the formation of clathrin lattices. *Cell.* 65:621–632.
45. Nesterov, A., R.C. Kurten, and G.N. Gill. 1995. Association of epidermal growth factor receptors with coated pit adaptins via a tyrosine phosphorylation-regulated mechanism. *J. Biol. Chem.* 270:6320–6327.
46. Nesterov, A., H.S. Wiley, and G.N. Gill. 1995. Ligand-induced endocytosis of epidermal growth factor receptors that are defective in binding adaptor proteins. *Proc. Natl. Acad. Sci. USA.* 92:8719–8723.
47. Ohno, H., J. Steward, M.-C. Fournier, H. Bosshart, I. Rhee, S. Miyatake, T. Saito, A. Gallusser, T. Kirchhausen, and J.S. Bonifacino. 1995. Interaction of tyrosine-based sorting signals with clathrin-associated proteins. *Science (Wash. DC).* 269:1872–1875.
48. Ozawa, K., Z. Szallasi, M.G. Kazanietz, P.M. Blumberg, H. Mischak, J.F. Mushinski, and M.A. Beaven. 1993. Ca^{2+}-Dependent and Ca^{2+}-independent isozymes of protein kinase C mediate exocytosis in antigen-stimulated rat basophilic RBL-2H3 cells: reconstitution of secretory responses with Ca^{2+} and purified isozymes in washed permeabilized cells. *J. Biol. Chem.* 268:1749–1756.
49. Paccaud, J.-P., W. Reith, B. Johansson, K.-E. Magnusson, B. Mach, and J.-L. Carpentier. 1993. Clathrin-coated pit-mediated receptor internalization: role of internalization signals and receptor mobility. *J. Biol. Chem.* 268:23191–23196.
50. Park, D.J., H.K. Min, and S.G. Rhee. 1991. IgE-induced tyrosine phosphorylation of phospholipase C-γ1 in rat basophilic leukemia cells. *J. Biol. Chem.* 266:24237–24240.
51. Pearse, B.M. 1988. Receptors compete for adaptors found in plasma membrane coated pits. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:3331–3336.
52. Pearse, B.M.F., and M.S. Robinson. 1990. Clathrin, adaptors, and sorting. *Annu. Rev. Cell Biol.* 6:151–171.

53. Petersen, N.O., P.L. Höddelius, P.W. Wiseman, O. Seger, and K.-E. Magnusson. 1993. Quantitation of membrane receptor distributions by image correlation spectroscopy: concept and application. *Biophys. J.* 65:1135–1146.
54. Pfeiffer, J.R., J.C. Seagrave, B.H. Davis, G.G. Deanin, and J.M. Oliver. 1985. Membrane and cytoskeletal changes associated with IgE-mediated serotonin release from rat basophilic leukemia cells. *J. Cell Biol.* 101: 2145–2155.
55. Ravetch, J.V. 1994. Fc Receptors: rubor redux. *Cell.* 78:553–560.
56. Robertson, D., D. Holowka, and B. Baird. 1986. Cross-linking of immunoglobulin E-receptor complexes induces their interaction with the cytoskeleton of rat basophilic leukemia cells. *J. Immunol.* 136:4565–4572.
57. Robinson, M.S. 1994. The role of clathrin, adaptors and dynamin in endocytosis. *Curr. Opin. Cell Biol.* 6:538–544.
58. Sako, Y., and A. Kusumi. 1994. Compartmentalized structure of the plasma membrane for receptor movements as revealed by a nanometer-level motion analysis. *J. Cell Biol.* 125:1251–1264.
59. Santini, F., and M.A. Beaven. 1993. Tyrosine phosphylation of a mitogen activated protein (MAP) kinase-like protein occurs at a late step in exocytosis: studies with tyrosine phosphatase inhibitors and various secretagogues in RBL-2H3 cells. *J. Biol. Chem.* 268:22716–22722.
60. Schmid, S.L. 1992. The mechanism of receptor-mediated endocytosis: more questions than answers. *BioEssays.* 14:589–596.
61. Seagrave, J., J.R. Pfeiffer, C. Wofsy, and J.M. Oliver. 1991. Relationship of IgE receptor topography to secretion in RBL-2H3 mast cells. *J. Cell. Physiol.* 148:139–151.
62. Sorkin, A., and G. Carpenter. 1993. Interaction of activated EGF receptors with coated pit adaptins. *Science (Wash. DC).* 261:612–615.
63. Sorkin, A., T. McKinsey, W. Shih, T. Kirchhausen, and G. Carpenter. 1995. Stoichiometric interaction of the epidermal growth factor receptor with the clathrin-associated protein complex AP-2. *J. Biol. Chem.* 270:619–625.
64. Stump, R.F., J.R. Pfeiffer, M.C. Schneebeck, J.C. Seagrave, and J.M. Oliver. 1989. Mapping gold-labeled receptors on cell surfaces by backscattered electron imaging and digital analysis: studies on the IgE receptor of mast cells. *Am. J. Anat.* 185:128–141.
65. Takemura, R., P.E. Stenberg, D.F. Bainton, and Z. Werb. 1986. Rapid redistribution of clathrin onto macrophage plasma membranes in response to Fc receptor-ligand interaction during frustrated phagocytosis. *J. Cell Biol.* 102:55–69.
66. Teshima, R., K. Suzuki, H. Ikebuchi, and T. Terao. 1989. Enhancement of the phosphorylation of membrane bound myosin light chain by antigen stimulation in rat basophilic leukemia cells. *Mol. Immunol.* 26:641–648.
67. Trowbridge, I.S. 1991. Endocytosis and signals for internalization. *Curr. Opin. Cell Biol.* 3:634–641.
68. Trowbridge, I.S., J.F. Collawn, and C.R. Hopkins. 1993. Signal-dependent membrane protein trafficking in the endocytic pathway. *Annu. Rev. Cell Biol.* 9:129–161.
69. van der Bliek, A.M., T.E. Redelmeier, H. Damke, E.J. Tisdale, E.M. Meyerowitz, and S.L. Schmid. 1993. Mutations in human dynamin block an intermediate stage in coated vesicle formation. *J. Cell Biol.* 122:553–563.
69a. von Zastrow, M., and B.K. Kobilka. 1994. Antagonist-dependent and -independent steps in the mechanism of adrenergic receptor internalization. *J. Biol. Chem.* 269:18448–18452.
70. Watts, C., and M. Marsh. 1992. Endocytosis: what goes in and how? *J. Cell Sci.* 103:1–8.
71. Weigel, P.H., and J.A. Oka. 1991. Coated pits and asialoglycoprotein receptors redistribute to the substratum during hepatocyte adhesion to galactoside surfaces. *Biochem. Biophys. Res. Commun.* 180:1304–1311.
72. Wieslander, E., P. Andersson, M. Linden, B. Axelsson, L. Kallstrom, R. Brattsand, and I. Paulsson. 1985. Importance of particulate antigen for the induction of dual bronchial reaction in guinea-pigs. *Agents Actions.* 16:37–38.
73. Wiley, H.S., and D.D. Cunningham. 1982. The endocytotic rate constant. A cellular parameter for quantitating receptor-mediated endocytosis. *J. Biol. Chem.* 257:4222–4229.
74. Wiley, H.S., J.J. Herbst, B.J. Walsh, D.A. Lauffenburger, M.G. Rosenfeld, and G.N. Gill. 1991. The role of tyrosine kinase activity in endocytosis, compartmentation, and down-regulation of the epidermal growth factor receptor. *J. Biol. Chem.* 266:11083–11094.
75. Willingham, M.C., J.H. Keen, and I.H. Pastan. 1981. Ultrastructural immunocytochemical localization of clathrin in cultured fibroblasts. *Exp. Cell Res.* 132:329–338.
76. Woods, A., C.G. Smith, D.A. Rees, and G. Wilson. 1983. Stages in specialization of fibroblast adhesion and deposition of extracellular matrix. *Eur. J. Cell Biol.* 32:108–116.