Study of the Transit of an Integral Membrane Protein from Secretory Granules through the Plasma Membrane of Secreting Rat Basophilic Leukemia Cells Using a Specific Monoclonal Antibody

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Abstract. The monoclonal antibody 5G10 reacted specifically with an 80-kD integral membrane protein in rat basophilic leukemia (RBL) cells. Immunofluorescence microscopy studies of RBL cells, fixed and permeabilized, revealed that the 80-kD protein was located in the membrane of cytoplasmic vesicles. The vesicles were identified as secretory granules by their content in immunoreactive serotonin. Expression of the 5G10 antigen on the surface of unstimulated RBL cells was low. However, RBL cells stimulated to secrete with anti-dinitrophenyl IgE followed by dinitrophenyl–bovine serum albumin or with the Ca^{2+} ionophore A-23187 displayed an increased expression of the antigen on their surface. Surface exposure of the 5G10 antigen was maximal at 5 min after stimulation of secretion. Removal of dinitrophenyl–bovine serum albumin from the incubation medium resulted in internalization of 50% of the antigen within 10 min.

Rat basophilic leukemia (RBL)[1] cells display regulated secretion (22). They have high-affinity IgE receptors on their surface (11, 12). When a multivalent antigen is specifically recognized by the surface-bound IgE, an influx of calcium leads the cells to secrete the content of their secretory granules (23). Secreted products include histamine, serotonin, heparin, and a number of proteins (16, 22). Both the mechanism and characteristics of secretion have been intensively investigated by measuring the products released. However, the likely transit of membrane components of secretory granules through the plasma membrane has not been studied mainly due to the lack of specific probes for those components. Here we report the use of a monoclonal antibody, 5G10, obtained from a mouse immunized with integral membrane proteins of Golgi vesicles purified from rat liver, to study the traffic of membranes from secretory granules through the plasma membrane in secreting RBL cells. These studies show that monoclonal antibodies against membrane proteins from secretory vesicles are useful tools for studying the traffic of these membranes during and after secretion.

Materials and Methods

Cell Culture

RBL cells (2H3-AB subline, a gift of Dr. Henry Metzger, NIADDK, National Institutes of Health) were routinely grown at densities of 3–8 × 10^5 cells/ml in glass spinner bottles using 45% Dulbecco's modified Eagle's medium (DME), 45% Ham's F12 medium, and 10% fetal calf serum containing 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin in an atmosphere of 93% air–7% CO_2 at 37°C. Rat mast cells were purified from peritoneal washings as reported (4). For immunofluorescence microscopy studies, cells were grown adherent to glass coverslips for 48 h under the same conditions described above, with the exception of mast cells that were used 2 h after preparation.

Antibodies

The monoclonal antibody, 5G10, was produced by a hybridoma obtained from the fusion of SP2/0 cells with splenic lymphocytes (7) obtained from a mouse injected with a preparation of integral membrane proteins from rat liver Golgi vesicles (8). The mouse monoclonal antibody 8D12, reacting with both the cytoplasmic microtubule-associated protein-1 and a 280-kD nuclear protein (p280), was obtained and characterized in our laboratory (1). The anti-serotonin rat monoclonal antibody YC5/45 (2) was purchased from Accurate Chemical & Scientific Corp., Westbury, NY. Mouse monoclonal anti-dinitrophenyl (anti-DNP) IgE, purified from ascitic fluid of mice bearing the tumor H1 dinitrophenyl-ϵ-26.82 (14) was a gift of Dr. Henry Metzger. Rhodamine- or fluorescein-conjugated goat anti-mouse IgG or rhodamine-conjugated goat anti-rat IgG (Cappel Laboratories, Cochranville, PA) were used as second antibodies in immunofluorescence microscopy and flow cytofluorometry studies. For double-immunofluorescence experiments, fluorescein-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-rat IgG were pre-absorbed on columns of rat IgG-Sepharose and mouse IgG-Sepharose (Cappel Laboratories), respectively, to prevent unwanted cross-reactivity with the first antibodies. For experiments in which RBL cells were incubated with IgE, the second antibodies were absorbed on an IgE–Sepharose column.

Induction of Secretion in RBL Cells

RBL cells growing adherent to glass coverslips (5 × 10^5 cells/coverslip) or in suspension (1 × 10^6 cells/ml) were stimulated to secrete by either sequential incubation with anti-DNP IgE and dinitrophenyl–bovine serum albumin (DNP–BSA) or with the Ca^{2+} ionophore A-23187. Allergen-mediated cell secretion was induced as follows. Cells were washed with DME containing 0.1% BSA (DME–BSA) and incubated for 1 h at 37°C with 10 μg/ml of anti-DNP IgE in DME–BSA. Excess antibody was removed by two washes with 10 mM Hepes, pH 7.4, containing 135 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, 0.4 mM $MgCl_2$, 1 mg/ml glucose, 1 mg/ml BSA (Ca^{2+} medium) and the cells were

[1] *Abbreviations used in this paper:* DME, Dulbecco's modified Eagle's medium; DNP, dinitrophenyl; RBL, rat basophilic leukemia.

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incubated for 45 min at 37°C with 0.03 μg/ml DNP–BSA (43 mol of DNP conjugated per mol of BSA) in Ca^{2+} medium. Stimulation of secretion by the Ca^{2+} ionophore A-23187 was performed by incubating the cells for 45 min at 37°C with 2 μg/ml A-23187 in Ca^{2+} medium.

Measurement of Serotonin Secretion in RBL Cells

Release of [^{3}H]serotonin by RBL cells was measured essentially as described by Taurog et al. (24). Cells were cultured in suspension overnight at a density of 5×10^5 cells/ml with 2 μCi/ml of 5-(1,2-[^{3}H]N)-hydroxytryptamine binoxalate (New England Nuclear, Boston, MA). Cells were then resuspended to 1×10^6 cells/ml and aliquots of 1 ml incubated by triplicate in Eppendorf tubes with either anti-DNP IgE plus DNP–BSA or A-23187 as described above. Secretion was stopped by removing the cells by centrifugation for 3 min to 10,000 *g* at 4°C and 800 μl of the culture medium counted for [^{3}H] using an LS-5801 Beckman scintillation counter (Beckman Instruments, Inc., Palo Alto, CA). Results were expressed as the percentage of the total [^{3}H]serotonin stored in the cells released into the medium. Standard deviation values were always <2% of the calculated mean values.

Immunofluorescence Microscopy Studies

Cells were fixed–permeabilized with cold (−20°C) methanol for 2 min or fixed with 4% *p*-formaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature and then permeabilized with 0.1% saponin in PBS for 15 min at 37°C. After a brief wash with PBS, the cells were processed as follows. For study of the cellular distribution of 5G10, cells were incubated for 1 h at 37°C with 50 μl of a 1:10 solution of ascitic fluid containing 5G10 antibody in PBS. Excess antibody was removed by washing the cells with PBS for 15 min. The washed cells were incubated for 30 min at 37°C with 50 μl of a solution containing 7.5 μg of fluorescein- or rhodamine-conjugated goat anti-mouse IgG and 12.5 μg of normal goat IgG in PBS. Excess second antibody was removed by washing the cells for 15 min and the coverslips were mounted on glass slides using gelvatol (Monsanto Co., St. Louis, MO). The localization of 5G10 antigen in serotonin-containing secretory granules was studied by double-immunofluorescence microscopy using a mixture of 5G10 antibody and the anti-serotonin monoclonal antibody YC5/45 as first antibodies and a mixture of fluorescein-conjugated goat anti-mouse IgG, rhodamine-conjugated goat anti-rat IgG, and normal goat IgG as second antibodies. Studies of nonpermeabilized cells were performed as follows. Cells were washed with ice cold PBS containing 0.02% NaN_3 (PBS-NaN_3) and incubated for 30 min at 4°C with 50 μl of 0.3 mg/ml purified goat IgG in PBS-NaN_3. Excess goat IgG was removed by washing the cells with ice cold PBS-NaN_3 and then the cells were incubated for 2 h at 4°C with 50 μl of a 1:10 dilution of ascitic fluid containing the corresponding monoclonal antibody in PBS-NaN_3. After removing excess antibody by a 15-min wash with PBS-NaN_3, the cells were incubated for 1 h at 4°C with 50 μl of a solution containing 60 μg/ml fluorescein-conjugated goat anti-mouse IgG and 0.3 mg/ml purified goat IgG in PBS-NaN_3. Excess second antibody was removed by washing the cells with ice cold PBS-NaN_3 for 15 min and then the cells were fixed with cold (−20°C) methanol for 2 min, washed briefly with PBS, mounted on glass slides using Gelvatol, and studied using an ICM 405 inverted Zeiss microscope.

Flow Cytofluorometry

Unstimulated and stimulated RBL cells in suspension (1×10^6 cells) were washed twice with ice cold PBS-NaN_3 using low speed centrifugation (400 *g* for 4 min at 4°C) and incubated for 30 min at 4°C with 0.5 ml of 0.3 mg/ml normal goat IgG in PBS-NaN_3. The goat IgG–treated cells were then collected by centrifugation and incubated for 1 h at 4°C with a 1:10 dilution of ascitic fluid containing the corresponding monoclonal antibody prepared in 0.5 ml PBS-NaN_3. Excess antibody was removed by two washes with ice cold PBS-NaN_3 using low speed centrifugation and then the cells were incubated for 1 h at 4°C with 60 μg/ml fluorescein-conjugated goat anti-mouse IgG and 0.3 mg/ml of normal goat IgG in 0.5 ml PBS. After two washes with ice cold PBS-NaN_3, the cells were suspended in 1 ml of the same buffer and the surface fluorescence of 2.5×10^4 cells was analyzed using a fluorescence-activated cell sorter (cytofluorograf 50 H, Ortho Diagnostic Systems Inc., Raritan, NJ). Determinations were done in duplicate and the calculated standard deviations were always <2% of the mean.

Preparation of Integral Membrane Proteins from RBL Cells

RBL cells grown in suspension cultures (5×10^7 cells) were washed twice with ice cold PBS and resuspended in 10 ml of 10 mM Tris-HCl, pH 7.4, 10 μg/ml leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride. After incubation for 15 min on ice, the cells were lysed with a Dounce-type homogenizer (50 strokes, B pestle). The cell lysate was centrifuged at 1,000 *g* for 10 min at 4°C and the resulting supernatant centrifuged again at 100,000 *g* for 1 h at 4°C. The pelleted membranes were washed twice with 10 ml of 0.1 M Na_2CO_3, pH 11.3, for 30 min at 4°C, once with 10 ml 0.1 M Tris-HCl, pH 7.4, and collected by centrifugation at 100,000 *g* for 1 h at 4°C. The washed membranes, containing exclusively integral membrane proteins, were then treated with 1% Triton X-100 in PBS for 15 min at 4°C and the insoluble material was removed by centrifugation at 130,000 *g* for 10 min using a Beckman airfuge. The resulting detergent-solubilized integral membrane proteins were stored frozen under liquid N_2 until used.

Immunoprecipitation

Integral membrane proteins from RBL cells solubilized with 1% Triton X-100 were labeled with ^{125}I using the lactoperoxidase-D-glucose oxidase method (15) (enzymobeads, Bio-Rad Laboratories, Richmond, CA). Immunoprecipitation was carried out by incubating overnight at 4°C the ^{125}I-labeled integral membrane proteins (4×10^7 cpm) with 5 μl of ascitic fluid containing either antibody 8D12 (nonspecific control) or 5G10 in 200 μl of 0.5% Triton X-100, 0.1% BSA, 0.02% NaN_3 in PBS. Samples were then incubated for 2 h at 4°C with 40 μl of Protein A–Sepharose. The immunocomplexes bound to Sepharose were washed five times with 1 ml of ice cold PBS containing 1% Triton X-100, 0.2% sodium desoxycholate, and 0.02% NaN_3 by centrifugation for 5 min at 10,000 *g* at 4°C using an Eppendorf microfuge. They were boiled for 5 min in electrophoresis sample buffer, resolved by SDS PAGE on 12.5% acrylamide gels, and analyzed by exposing the dried gels onto Kodak XAR-5 film.

Studies on the Internalization of 5G10 Antigen in Secreting RBL Cells

Antibody 5G10 was purified from ascitic fluid by ammonium sulfate precipitation, gel filtration on Ultrogel AcA 34 (LKB Instruments, Inc., Gaithersburg, MD) and chromatography on DEAE 52-cellulose (Whatman Inc., Clifton, NJ) as described (25). The purified antibody was labeled with ^{125}I to a specific activity of 45 μCi/μg using the lactoperoxidase-D-glucose oxidase method (15). Internalization of the surface-exposed 5G10 antigen in secreting RBL cells was studied as follows. 2×10^6 cells, stimulated to secrete with anti-DNP IgE and DNP–BSA (see above), were incubated for 1 h at 37°C or 2 h at 4°C in 0.25 ml Ham's F12–0.2% BSA containing 0.03 μg/ml DNP–BSA, 1×10^6 cpm ^{125}I-labeled 5G10, 4 μg/ml normal mouse IgG (Sigma Chemical Co.), and, when required, 5 μg unlabeled 5G10. The reaction was terminated by centrifuging the cells at 13,000 *g* for 1 min through a dibutylphtalate cushion. The surface-bound antibody was removed by washing the cells with 0.25 M acetic acid/0.25 M NaCl for 15 s before centrifugation as described (26). Specific uptake of the 5G10 antibody was calculated by subtracting the radioactivity incorporated by cells incubated with unlabeled 5G10 from that measured in cells incubated in the absence of cold 5G10. Binding of ^{125}I-labeled 5G10 to a membrane fraction from RBL cells was studied as described for intact cells using 50 μg of membrane proteins, except that the reaction was terminated by dilution with ice cold incubation medium and centrifugation for 5 min at 13,000 *g* to count the radioactivity in the pellet.

Results

Characterization of the 5G10 Antigen in RBL Cells

Antibody 5G10 specifically immunoprecipitated a single 80-kD protein when incubated with integral membrane proteins from RBL cells, solubilized with Triton X-100, and labeled with ^{125}I (Fig. 1).

Cellular Distribution of the 5G10 Antigen in Unstimulated RBL and Rat Peritoneal Mast Cells

Immunofluorescence microscopy studies of unstimulated RBL cells, fixed with *p*-formaldehyde and permeabilized with saponin, showed that antibody 5G10 reacted specifically with an antigen present in numerous cytoplasmic vesicles (Fig. 2 *a*). Fixation and permeabilization with cold (−20°C) methanol resulted in swelling of vesicles stained by antibody 5G10, revealing that the antigen was located in the vesicle membrane (Fig. 2 *b*). The vesicles were identified as secretory granules by their content in serotonin, as shown by their staining with

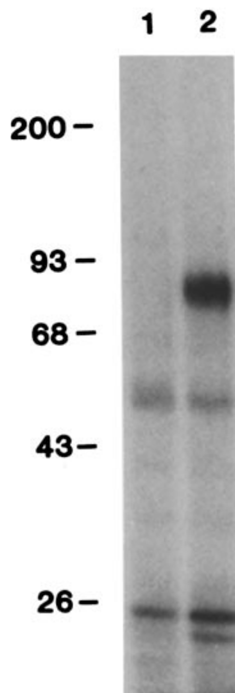

Figure 1. Characterization of the antigen reacting with 5G10 in RBL cells. Triton X-100–solubilized integral membrane proteins from RBL cells, labeled with ^{125}I (5×10^7 cpm), were immunoprecipitated with the monoclonal antibodies 8D12 (lane *1*, control) or 5G10 (lane *2*) as described in Materials and Methods. The immunoprecipitates were analyzed by SDS PAGE on 12.5% acrylamide gels and autoradiography. The positions of molecular mass markers are indicated on the left. The 24-kD species appearing in lane *2* was not reproducibly immunoprecipitated in similar experiments.

both 5G10 antibody and the anti-serotonin monoclonal antibody YC5/45 in double-immunofluorescence experiments (Fig. 2, *c* and *d*). In this experiment and to prevent the extraction of serotonin, the cells were fixed with *p*-formaldehyde before being permeabilized with saponin. Antibody 5G10 also reacted with vesicles contained in the cytoplasm of rat peritoneal mast cells. In these studies we observed that mast cells were lysed when treated with cold methanol and only the secretory granules remained attached to the glass coverslips as shown by phase-contrast microscopy (Fig. 2*e*). Immunofluorescence microscopy studies of these preparations showed that the granules were specifically stained with antibody 5G10 (Fig. 2*f*). Studies with nonpermeabilized unstimulated RBL cells revealed that very little 5G10 antigen was expressed on the surface (Fig. 2*g*).

Immunofluorescence Microscopy Studies of Secreting RBL Cells

During secretion, cells fuse their secretory granules with the plasma membrane. As a result, the luminal side of the secretory granule membrane is exposed on the cell surface. RBL cells stimulated to secrete with the Ca^{2+} ionophore A-23187 displayed an increase in the surface expression of the 5G10 antigen as shown by immunofluorescence microscopy studies of nonpermeabilized cells (Fig. 2*h*). Control experiments using either antibody 6D1 reacting with a Golgi cisternae antigen (Bonifacino, J. S. and I. V. Sandoval, unpublished results) or 8D12, reacting with cytoplasmic microtubule-associated protein-1 and nuclear p280 proteins did not show any increase in surface fluorescence. The increase in the surface expression of 5G10 antigen was detected because of the localization of the epitope recognized by the monoclonal antibody 5G10 in the lumen of secretory granules. Such increase indicated the insertion of the membranes of secretory granules in the plasma membrane during exocytosis. Similar results were obtained when RBL cells primed with anti-DNP IgE were stimulated to secrete with DNP-BSA (not shown).

Quantitation of the Exposure of 5G10 Antigen on the Surface of Secreting RBL Cells by Flow Cytofluorometry and Its Correlation with Serotonin Secretion

The changes in the surface expression of 5G10 antigen in RBL cells during exocytosis were quantitated by flow cytofluorometry. As already observed by immunofluorescence microscopy, unstimulated RBL cells displayed low levels of surface fluorescence with only 6% exhibiting fluorescence intensities greater than 720 U (Fig. 3*a*). Binding of anti-DNP IgE to cells did not change these low basal levels of surface fluorescence (Fig. 3*b*). However, when cells were stimulated to secrete by incubation with anti-DNP IgE followed by DNP–BSA (Fig. 3*c*) or with the Ca^{2+} ionophore A-23187 (Fig. 3*d*), an increase in surface fluorescence was observed, indicating the incorporation of the 5G10 antigen into the plasma membrane. Under these conditions, 43% of the cells stimulated with IgE/DNP–BSA and 56% of the cells stimulated with A-23187 fluoresced over the threshold of 720 U. No change in the level of fluorescence was observed under the same conditions when 5G10 was replaced by the 8D12 (Fig. 3, *e–h*) or 6D1 antibodies (not shown).

When serotonin secretion was measured in parallel in these experiments, a close correlation with the expression of 5G10 antigen on the cell surface was observed (Fig. 4). Unstimulated cells displaying low surface levels of 5G10 antigen secreted small amounts of serotonin. A similar result was obtained with cells incubated with anti-DNP IgE. However, incubation of anti-DNP IgE–treated cells with DNP–BSA resulted in secretion of 20% of the serotonin contained in the cells and increase in the surface expression of 5G10 as described above. Furthermore, cells stimulated with A-23187 expressed more 5G10 antigen on the cell surface and secreted significantly more serotonin than cells stimulated with anti-DNP IgE and DNP–BSA.

The kinetics of the surface expression of 5G10 antigen were studied in RBL cells primed with anti-DNP IgE that were incubated with DNP–BSA for different periods of time. Analysis by flow cytofluorometry revealed that the appearance of 5G10 antigen on the cell surface was a very rapid process, with maximal levels of exposure attained 5 min after addition of DNP–BSA (Fig. 5). This level of surface expression remained unchanged for nearly 45 min before decreasing slowly (Fig. 5).

Internalization of the 5G10 Antigen Exposed on the Cell Surface during Secretion

Removal of DNP–BSA from the incubation medium resulted in a rapid decrease in the expression of 5G10 antigen on the surface of RBL cells (Fig. 5, dashed line). The time-course of this decrease is shown in Fig. 6. From the rate of disappearance, we calculated that the half-life of the 5G10 antigen on the cell surface was ~10 min. Dissociation of cell-bound DNP–BSA with DNP-Lys (9, 20) had no effect on this rate. Removal of 5G10 antigen from the cell surface was completely inhibited at 0°C.

To investigate whether the disappearance of surface 5G10 antigen was due to internalization, we conducted experiments in which RBL cells stimulated with anti-DNP IgE and DNP–BSA were incubated for 2 h at 4°C or for 1 h at 37°C with ^{125}I-labeled 5G10 antibody. After incubation, the cells were

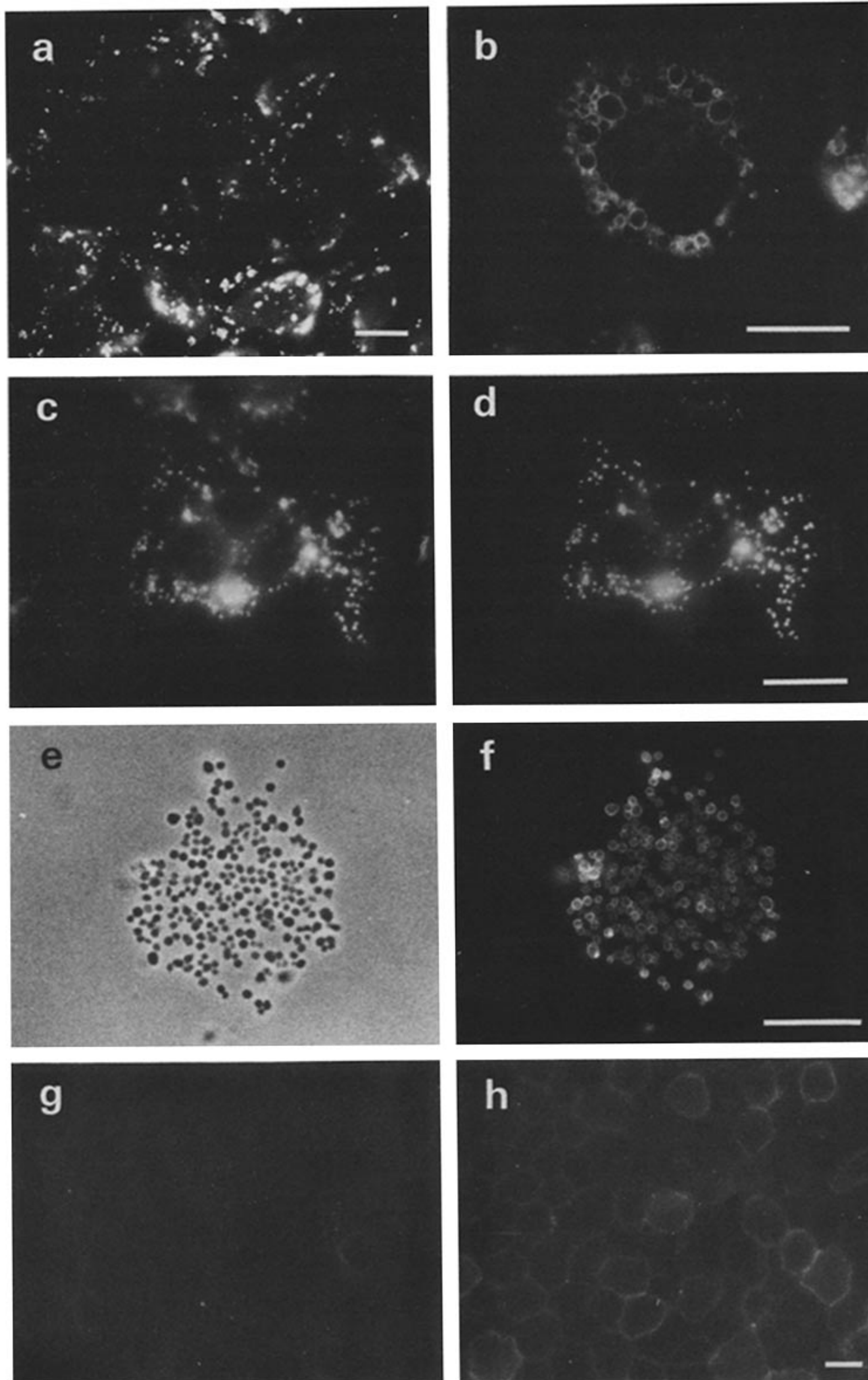

Figure 2. Immunofluorescence microscopy studies of RBL and mast cells. (*a*) RBL cells fixed with 4% *p*-formaldehyde for 15 min at room temperature and permeabilized with 0.1% saponin for 15 min at 37°C were incubated with 5G10 and rhodamine-conjugated goat anti-mouse IgG. (*b*) RBL cell fixed-permeabilized with cold (−20°C) methanol incubated with 5G10 antibody and stained with rhodamine-conjugated goat anti-mouse IgG. (*c* and *d*) RBL cells fixed with 4% *p*-formaldehyde and permeabilized with 0.1% saponin were incubated with 5G10 (*c*) and YC5/45 (*d*) antibodies and stained with fluorescein-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-rat IgG. Observe the co-localization of 5G10 and serotonin in the same cytoplasmic vesicles. (*e* and *f*) Rat peritoneal mast cells fixed-permeabilized with cold (−20°C) methanol, studied by phase-contrast (*e*), and immunostained with 5G10 and rhodamine-conjugated goat anti-mouse IgG (*f*). (*g*) Nonpermeabilized, unstimulated RBL cells incubated with 5G10 antibody and immunostained with fluorescein-conjugated goat anti-mouse IgG. (*h*) Nonpermeabilized RBL cells, stimulated with 2 μg/ml A-23187 for 45 min at 37°C, incubated with 5G10 antibody, and immunostained with fluorescein-conjugated goat anti-mouse IgG. Notice the enhanced surface staining after stimulation of secretion. Bars, 5 μm.

treated with 0.25 M acetic acid/0.25 M NaCl to dissociate the surface-bound antibody. We observed that whereas nearly all of the antibody specifically bound at 4°C could be removed by this treatment (Fig. 7 *A*), 78% of the antibody bound at 37°C remained associated with the cells after the acid wash (Fig. 7 *B*). However, when the labeled antibody was bound at 37°C to a membrane fraction from RBL cells, only 7% remained bound to the membranes after the acid wash (Fig. 7 *C*). These observations indicate that at 4°C, the antibody binds to an antigen that remains exposed on the cell surface whereas at 37°C the bound antibody is readily internalized, becoming resistant to the effect of external pH changes.

Discussion

During the release of secretory products by exocytosis, the membranes from secretory granules are continuously inserted into the plasma membrane. To compensate for the addition

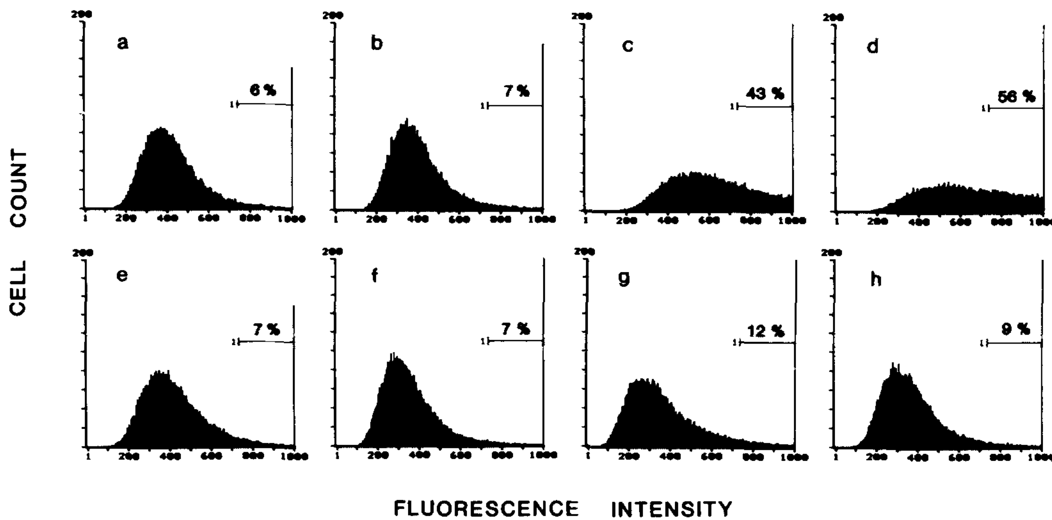

Figure 3. Flow cytofluorometry studies of unstimulated and stimulated RBL cells incubated with 5G10. RBL cells grown in suspension cultures were incubated with no additions (*a* and *e*), with anti-DNP IgE (*b* and *f*), with anti-DNP IgE followed by DNP-BSA (*c* and *g*), or with 2 μg/ml A-23187 (*d* and *h*) as described in the Materials and Methods section. Cells were then immunostained with 5G10 (*a–d*) or 8D12 (*e–h*) followed by fluorescein-conjugated goat anti-mouse IgG. Immunostained cells were analyzed with a fluorescence-activated cell sorter. The percentage of positive cells is indicated on each histogram and represents the proportion of cells having fluorescence intensities higher than 720 arbitrary units.

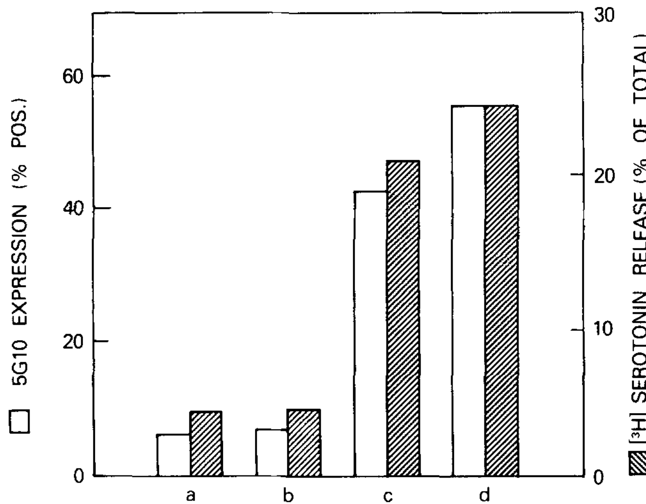

Figure 4. Correlation between serotonin secretion and surface expression of 5G10 antigen during exocytosis. RBL cells at a density of 5 × 10^5 cells/ml were incubated overnight with 2 μCi/ml of 5-(1,2-[^{3}H]N)-hydroxytryptamine binoxalate. Aliquots of 1 ml were separately incubated with no additions (*a*), anti-DNP IgE (*b*), anti-DNP IgE followed by DNP-BSA (*c*), or A-23187 as described in Materials and Methods. The secretion of serotonin (hatched bars) and surface expression of the 5G10 antigen (open bars) were then measured in parallel.

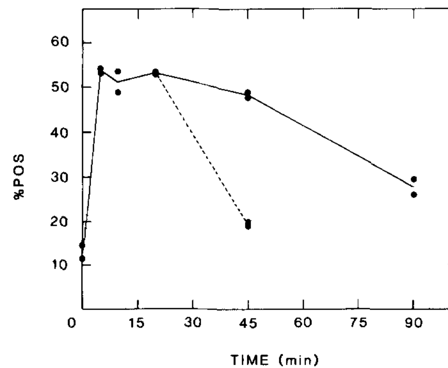

Figure 5. Time-course of appearance of 5G10 antigen on the surface of RBL cells upon stimulation of secretion. RBL cells primed with anti-DNP IgE were incubated with 0.03 μg/ml DNP-BSA for different periods of time at 37°C. Samples were then immunostained with 5G10 and fluorescein-conjugated goat anti-mouse IgG and analyzed by flow cytofluorometry as described in the Materials and Methods section. The percentage of positive cells (*% POS*) was determined as indicated in legend to Fig. 3. Determinations were performed in duplicate. The dashed line shows the effect of removing DNP-BSA from the medium at 20 min and incubating the cells in growth medium for an additional 25 min.

of these membranes and to maintain the specific composition of the plasma membrane, the membranes inserted by exocytosis must be selectively removed by endocytosis for as long as secretion proceeds. Therefore, in secreting cells, the membranes of secretory granules are in continuous movement. The first studies of the traffic of these membranes in secreting cells were performed using tracers, such as cationized ferritin (5, 17) and [^{125}I]iodine (28), which label proteins exposed on the cell surface without differentiating their origin. These studies revealed a major route of membrane traffic between the plasma membrane and new secretory granules, suggesting that the membranes from secretory granules inserted into the plasma membrane by exocytosis are retrieved and used in the formation of new secretory granules. However, the nonspecific labeling of surface proteins with these tracers hindered the study of the insertion of membranes from secretory granules into the plasma membrane and made it difficult to interpret the presence of labeled membrane components in Golgi apparatus (5, 17, 28) and lysosomes (5, 17) after internalization by endocytosis.

A different approach to study the traffic of the membranes from secretory granules has been to use specific antibodies as tracers. Recently, polyclonal antibodies against the membrane

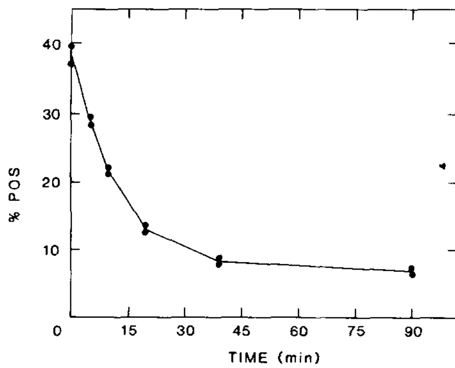

Figure 6. Time-course of disappearance of 5G10 antigen from the surface of RBL cells upon removal of DNP–BSA. RBL cells primed with anti-DNP IgE were incubated with 0.03 μg/ml DNP–BSA for 45 min at 37°C. Cells were then washed twice with ice cold PBS, resuspended in growth medium, and incubated at 37°C for different periods of time. Cells taken at each time point were immunostained with 5G10 and fluorescein-conjugated goat anti-mouse IgG and analyzed by flow cytofluorometry as described in the Materials and Methods section. The percentage of positive cells (*% POS*) was determined as indicated in legend to Fig. 3. Determinations were performed in duplicate.

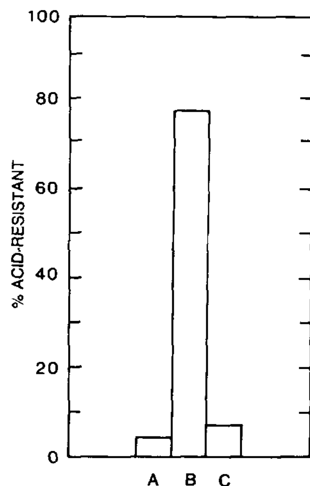

Figure 7. Internalization of ^{125}I-labeled 5G10 in RBL cells. RBL cells were stimulated to secrete by incubation with anti-DNP IgE and DNP-BSA as indicated in the Materials and Methods section. Stimulated cells were then incubated for 2 h at 4°C (*A*) or for 1 h at 37°C (*B*) with ^{125}I-labeled 5G10 (see Materials and Methods). RBL cell membranes were also incubated for 1 h at 37°C with the labeled antibody (*C*). At the end of incubation, part of the samples were treated for 15 s with 0.25 M acetic acid/0.25 M NaCl and the specific binding to control and acid-washed cells was determined. Results are expressed as the percentage of specifically bound ^{125}I-labeled 5G10 remaining after treatment at low pH.

proteins from chromaffin granules, dopamine β-hydroxylase (3, 13, 21), and glycoprotein III (13, 19) have been used to study the movement of these membranes during secretion in chromaffin cells.

In the present study we have used the monoclonal antibody 5G10 to monitor the traffic of membranes from secretory granules in secreting RBL cells. This antibody, initially raised against an integral membrane protein from liver Golgi vesicles specifically reacts with an 80-kD integral membrane protein from RBL cells. The antibody clearly stains the serotonin-containing secretory granules of RBL cells and of mast cells. The localization of 5G10 antigen in the membranes from secretory granules has also been established by its expression on the surface of cells upon stimulation of secretion.

The exposure of the 5G10 epitope on the surface of secreting cells has permitted us to specifically label the membranes from secretory granules inserted into the plasma membrane using the monoclonal antibody 5G10. An important advantage of this approach is that we have been able to monitor the insertion of the membranes from secretory granules into the plasma membrane during secretion by comparing the binding of the antibody to the surfaces of unstimulated and stimulated RBL cells. In these studies, we found that unstimulated RBL cells expressed low but significant levels of 5G10 antigen on their surface. This low expression of 5G10 antigen in the plasma membrane was probably the result of some constitutive secretion as indicated by measurements of serotonin released into the culture medium. Stimulation of the cells to secrete, with either DNP–BSA bound to anti-DNP IgE or the Ca^{2+} ionophore A-23187, produced a clear increase in the levels of 5G10 antigen on the cell surface. It is noteworthy that the Ca^{2+} ionophore has been always more effective in stimulating the secretion of serotonin and the expression of 5G10 antigen on the cell surface than the binding of the allergen–IgE complex to the plasma membrane. This difference probably reflects the greater effectiveness of the ionophore in increasing intracellular levels of Ca^{2+}, although variations in individual cell responses to the binding of DNP–BSA/IgE can not be discarded. Also, it is important to note that the surface expression of the 5G10 antigen was not enhanced by incubating the cells with IgE alone and that it sharply decreased when the DNP–BSA/IgE complex was dissociated with DNP-lysine. All these results show a close correlation between secretion and the expression of 5G10 antigen on the cell surface, indicating that the latter is the result of the insertion of the membranes from secretory granules into the plasma membrane during the release of secretory products by exocytosis.

Study of the cells after stimulation of secretion has revealed that the expression of the 5G10 antigen on the cell surface is a fast process that reaches a maximum within 5 min. It is noteworthy that this value is comparable to that obtained in similar studies of secretion in chromaffin cells (3, 13, 19, 21). We observe that when the cells are continuously stimulated to secrete, the expression of the 5G10 antigen on the cell surface is sustained for ~45 min before decreasing slowly. The sustained expression of the antigen probably results from the continuous insertion of the membranes of secretory granules into the plasma membrane and their rapid removal of endocytosis, as pointed out by the continuous uptake of the surface-bound antibody by these cells. This membrane retrieval is most likely carried out by the secreting cells to maintain the surface and chemical composition of the plasma membrane constant.

We have studied the retrieval of the membranes of secretory granules from the plasma membrane by measuring the decrease in the surface expression of the 5G10 antigen, after stopping secretion. This experiment shows that the half-time of the 5G10 antigen on the plasma membrane is ~10 min, indicating a fast mechanism of retrieval whose rate is comparable to that measured in the study of the traffic of the membranes from chromaffin granules (3, 13). It is not known whether the rate of internalization of the 5G10 antigen corresponds to that of the other components of the membranes of secretory granules. This will depend on whether the internalization of the 5G10 antigen occurs simultaneously with the internalization of those others components. Diffusion in the plasma membrane could result in their internalization with similar or different rates, depending on their diffusion

coefficients, the existence of different retrieving signals, and their pathway of internalization. A low rate of diffusion of the components with respect to the rate of internalization would result in endocytosis of intact membrane patches, and therefore in the simultaneous recovery of all the membrane components. In this case, the rate of internalization of the membranes from secretory granules would correspond to that of the 5G10 antigen.

The mechanism by which secretory cells retrieve the membranes of secretory granules from the plasma membrane is not known. Probably, the components of the granule membranes contain or acquire signals that are recognized by a retrieval system. The number of components carrying a retrieving signal may depend on whether or not the membranes are retrieved intact. Recovery of intact membranes may only require the introduction of such a signal into one or a few components. If a signal is acquired, it is probably through a system residing in the plasma membrane that is able to discriminate the molecules that are to be internalized by endocytosis. The internalization of the membranes from secretory granules does not seem to require any extracellular signal. By contrast, external signals are common in the receptor-mediated internalization of hormones, growth factors, immunoglobulins, and allergens (for reviews see references 10 and 18). Despite this difference the rate of internalization of the 5G10 antigen is similar to those of ligands internalized by receptor-mediated endocytosis (27). Among the ligand–receptor complexes whose rate of internalization is similar to that of the 5G10 antigen, is the allergen–IgE receptor complex, used in this study to stimulate RBL cells to secrete (6). The similar rates of receptor-mediated and receptor-independent endocytosis, as measured in different systems, suggest the existence of a similar mechanism for both forms of endocytosis.

We do not yet know the pathway followed by the membranes of secretory granules retrieved from the plasma membrane. As mentioned before, studies of the pathways of membrane internalization in secreting cells labeled with cationized ferritin and ^{125}I have revealed that proteins from these membranes can be traced in the Golgi apparatus (5, 17, 28) and lysosomes (5, 17). Nevertheless, the nonspecificity of these tracers raises the question of the origin of the membrane labeled in these studies. Antibody 5G10 can be a useful tool to study the pathway of the secretory vesicle membranes retrieved by endocytosis.

We are grateful to Drs. Henry Metzger and Rodolfo Quarto (NIADDK, National Institutes of Health) for helpful discussions and reagents; Mike Walker (NICHD, National Institutes of Health) for his assistance with flow cytofluorometry analysis; and Katy Perry and Wanda Ridley for expert secretarial assistance.

Received for publication 16 July 1985, and in revised form 22 October 1985.

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