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 Ca²⁺ 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) 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 Golgi fractions (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-e-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 cytometry 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 × 10⁵ cells/coverslip) or in suspension (1 × 10⁶ cells/ml) were stimulated to secrete by either sequential incubation with anti-DNP IgE and dinitrophenyl–bovine serum albumin (DNP-BSA) or with the Ca²⁺ 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 CaCl₂, 0.4 mM MgCl₂, 1 mg/ml glucose, 1 mg/ml BSA (Ca²⁺ medium) and the cells were

*Abbreviations used in this paper: DME, Dulbecco’s modified Eagle’s medium; DNP, dinitrophenyl; RBL, rat basophilic leukemia.
incubated for 45 min at 37°C with 0.03 μg/ml DNP-BSA (43 mol of DNP conjugated per mol of BSA) in Ca²⁺ medium. Stimulation of secretion by the Ca²⁺ ionophore A-23187 was performed by incubating the cells for 45 min at 37°C with 2 μg/ml A-23187 in Ca²⁺ medium.

Measurement of Serotonin Secretion in RBL Cells

Release of [³H]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 x 10⁶ cells/ml with 2 μCi/ml of 5,4,12-[³H]-hydroxytryptamine, in tissue culture medium (New England Nuclear, Boston, MA). Cells were then resuspended to 1 x 10⁶ 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. Incubation was stopped by removing the cells by centrifugation for 3 min to 10,000 g at 4°C. The washed pellets, containing mainly 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 N₂ until used.

Immunoprecipitation

Integral membrane proteins from RBL cells solubilized with 1% Triton X-100 were labeled with ¹²⁵I using the lactoperoxidase-g-glucose oxidase method (15), and chromatography on DEAE 52-cellulose (Whatman Inc., Chifton, NJ) as described (25). The purified antibody was labeled with ¹²⁵I to a specific activity of 45 μCi/μg using the lactoperoxidase-g-glucose oxidase method (15). Internalization of the surface-exposed 5GI0 antigen in secreting RBL cells was studied as follows. 2 x 10⁶ 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 x 10⁶ cpm ¹²⁵I-labeled 5GI0, 4 μg/ml normal mouse IgG (Sigma Chemical Co.), and, when required, 5 μg unlabeled 5GI0. The reaction was terminated by centrifuging the cells at 13,000 g for 1 min through a dithylbutylphosphate 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 5GI0 antibody was calculated by subtracting the radioactivity incorporated by cells incubated with unlabeled 5GI0 from that measured in cells incubated in the absence of cold 5GI0. Binding of ¹²⁵I-labeled 5GI0 to a membrane fraction from RBL cells was used as described for intact cells (25). The purified antibody was labeled using the lactoperoxidase-D-glucose oxidase method (15) and chromatography on DEAE 52-cellulose (Whatman Inc., Chifton, NJ) as described (25). The purified antibody was labeled using the lactoperoxidase-D-glucose oxidase method (15).

Results

Characterization of the 5GI0 Antigen in RBL Cells

Antibody 5GI0 specifically immunoprecipitated a single 80-kD protein when incubated with integral membrane proteins from RBL cells, solubilized with Triton X-100, and labeled with ¹²⁵I (Fig. 1).
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. 2e).

Immunofluorescence microscopy studies of these preparations showed that the granules were specifically stained with antibody 5G10 (Fig. 2f). Studies with nonpermeabilized unstimulated RBL cells revealed that very little 5G10 antigen was expressed on the surface (Fig. 2g).

**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. 2h). 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. 3a). Binding of anti-DNP IgE to cells did not change these low basal levels of surface fluorescence (Fig. 3b). However, when cells were stimulated to secrete by incubation with anti-DNP IgE followed by DNP-BSA (Fig. 3c) or with the Ca\(^{2+}\) ionophore A-23187 (Fig. 3d), 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
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. 7A), 78% of the antibody bound at 37°C remained associated with the cells after the acid wash (Fig. 7B). 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. 7C). 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...
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 [125I]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
from secretory granules inserted into the plasma membrane has permitted us to specifically label the membranes on the surface of cells upon stimulation of secretion. The localization of 5GI0 antigen in the membranes from secretory granules has also been established by its expression against an integral membrane protein from liver Golgi vesicles (3, 13, 21), and glycoprotein III (13, 19) have been used to specifically bind ~21-labeled 5GI0 remaining after treatment at low pH. The exposure of the 5GI0 epitope on the surface of secretory granules in secreting RBL cells. This antibody, initially raised to secrete, with either DNP-BSA bound to anti-DNP IgE or the Ca++ ionophore A-23187, produced a clear increase in the levels of 5GI0 antigen on the cell surface. It is noteworthy that the Ca++ ionophore has been always more effective in stimulating the secretion of serotonin and the expression of 5GI0 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++, 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 5GI0 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 5GI0 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 5GI0 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 5GI0 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 5GI0 antigen, after stopping secretion. This experiment shows that the half-time of the 5GI0 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 5GI0 antigen corresponds to that of the other components of the membranes of secretory granules. This will depend on whether the internalization of the 5GI0 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 125I 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.

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