Internalization and recycling to serotonin-containing granules of the 80K integral membrane protein exposed on the surface of secreting rat basophilic leukaemia cells

JUAN S. BONIFACINO, LYDIA YUAN and IGNACIO V. SANDOVAL*

Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA

*Present address for correspondence: Instituto de Biología Molecular Facultad de Ciencias, Universidad Autónoma de Madrid, Canto Blanco, 28049 Madrid, Spain

Summary

The 80K (80x10^3 M_r) integral membrane protein, first described in the secretory granules of rat basophilic leukaemia (RBL) cells, is also localized to lysosomes in these cells. The protein displays the same distribution in natural killer lymphocytes (RNK-7), wherein it codistributes with cytolysin in secretory granules. In contrast, the protein is absent from the endocrine and exocrine secretory granules of rat pancreatic acinar and pituitary cells, respectively, where it is confined to lysosomes. The protein colocalizes with lysosomal integral membrane proteins in all the cells studied, indicating that it is largely restricted to secretory granules with lysosomal properties (LSG) and lysosomes. The protein expressed on the surface of secreting RBL cells is internalized by endocytosis via coated pits, and found in coated vesicles, endosomes, multivesicular bodies and Golgi system, before being recycled to LSG and partly delivered to lysosomes. The recycled protein is re-expressed on the surface of cells stimulated to secrete a second time.

Key words: 80K integral membrane protein, RBL cells, serotonin.

Introduction

In a recent study on the process of exocytosis in the rat basophilic leukaemia-derived (RBL) cell line, we used a monoclonal antibody (5G10) that reacts with an 80K (K = 10^3 M_r) protein component of the membrane of the serotonin-containing granules (p80), to examine the insertion of these membranes into the plasma membrane and their subsequent removal (Bonifacino et al. 1986). We demonstrated that stimulation of serotonin secretion with the Ca^{2+} ionophore A23187, or with IgE and a specific allergen, DNP-BSA (dinitrophenol-bovine serum albumin), caused a rapid increase in the surface levels of p80. Upon removal of the stimuli, the amount of p80 exposed on the cell surface decreased quickly to basal levels. This decrease was due to internalization.

In the present study, we have compared the localization of p80 in cells containing secretory granules with lysosomal properties (LSG) (RBL cells, rat natural killer lymphocytes), and in cells displaying endocrine (rat pituitary cells) or exocrine (rat pancreatic acinar cells) secretory granules. We also studied the distribution of p80 on the plasma membrane of secreting RBL1 cells, characterized the morphology of the pathway of internalization, and shown the recycling of the protein to LSG.

Materials and methods

Cell culture

RBL (rat basophilic leukaemia) (2H3-AB subline), NRK (normal rat kidney) PtK2 (potoroo kidney) cells were grown in R medium (45% Dulbecco's modified Eagle's medium, 45% Ham's F12 medium, 10% foetal calf serum, 2mM-L-glutamine, 50 units ml^{-1} penicillin, 50 µg ml^{-1} streptomycin) in an atmosphere of 93% air-7% CO_2 at 37°C. Rat pancreatic acinar cells and rat anterior pituitary cells were prepared by trypsinization of the corresponding tissues. RNK-7 cells, prepared as described (Ward & Reynolds, 1983), were kindly provided by Dr Pierre Henkart (NCI, NIH). Hybridoma clones were grown in Dulbecco's modified Eagle's medium containing 1% Nutridoma (Boehringer).

Antibodies

The mouse monoclonal antibodies used in these studies were the following: antibody 5G10, reacting with an 80K integral membrane protein present in serotonin-containing granules of RBL cells (Bonifacino et al. 1986); antibodies against the lysosomal integral membrane proteins (LIMPs) I, II and III (Barriocanal et al. 1986); antibody 8D12, reacting with the cytosolic microtubule-associated protein MAP1 and a 280K nuclear protein component of the mitotic spindle (Bonifacino et al. 1985); antibody 15C8, that reacts with the luminal domain...
of a 130K integral membrane protein confined to the cis and medial cisternae of the Golgi system (Yuan et al. 1987). The rat anti-serotonin monoclonal antibody YCS/45 (Consolazione et al. 1981) was purchased from Accurate Chemical and Scientific Corp., Westbury, NY. The mouse monoclonal IgE, specific for anti-dinitrophenyl groups (Liu et al. 1980), was a gift from Dr Henry Metager, NIADDK, NIH. The anti-cytosin antibody was a gift from Dr Pierre Henkart, NCI, NIH. The characteristics of the human anti-Golgi antibody have been reported (Gaspar et al. 1988). Rhodamine- or fluorescein-conjugated second antibodies were from Cooper Biomedical, Inc., Malvern, PA. Second antibodies used in double immunofluorescence microscopy studies were made species-specific by absorption with the corresponding mouse, rat, rabbit or human IgG coupled to Sepharose (Cooper Biomedicals). All second antibodies were preabsorbed with mouse IgG-Sepharose. Rabbit anti-rat antibody purified by affinity chromatography on IgG-Sepharose columns and absorbed with human and mouse serum was from Zymed. The 15 nm colloidal-gold-conjugated goat anti-mouse IgG and the 5 nm colloidal-gold-conjugated protein A were from Boehringer-Mannheim and E-Y Laboratories, respectively.

Effect of pH on the dissociation of 125I-labelled 5G10 antibody from RBL cell membranes

RBL cell membranes (1 mg protein ml⁻¹), treated with 0·1 M-Na₂CO₃, pH 11·3 (Bonifacino et al. 1986), were incubated for 2 h at room temperature with 2x10⁶ cts min⁻¹ ml⁻¹ 125I-labelled 5G10 antibody (9x10⁶ Ci mol⁻¹) in PBS (137 mM-NaCl, 3 mM-KCl, 1·8 mM-KH₂PO₄, 8 mM-Na₂HPO₄, pH 7·5) containing 0·1% bovine serum albumin (BSA) and 0·25 mg ml⁻¹ normal mouse IgG, in the absence or presence of 20 µl ml⁻¹ of ascites containing antibody 5G10. After incubation, the membranes were washed twice with ice-cold AP buffer (0·1 % BSA/PBS) and harvested by centrifugation. The pellets were resuspended to 0·1 of the original volume in PBS with a Dounce homogenizer type B using 10 strokes, and 50-μl samples were pipetted over 1 ml portions of buffers with different pH values; 50 mM-sodium acetate or sodium phosphate buffer containing 0·1 M-NaCl was used for the pH ranges of 2·5 and 5·5-7, respectively. After incubation for 10 min at 37°C, the membranes were collected by centrifugation and bound 125I was determined with a gamma counter. Specific binding of antibody to the membranes was calculated by subtracting the values obtained with excess cold 5G10 from values obtained without it. Results were expressed as the percentage of specific binding measured at each pH with respect to the specific binding measured at pH 7.

Degradation of internalized 125I-labelled 5G10 antibody by stimulated cells

RBL cells growing in suspension were resuspended in 6 ml HA medium ( Dulbecco's modified Eagle's medium, 10 mm-Hepes, 0·1 % BSA, 2 mM-glutamine) to a density of 2x10⁶ cells ml⁻¹, stimulated with DNP-BSA and anti-DNP IgE (see above) and incubated for 30 min at 37°C with 125I-labelled 5G10 antibody (2x10⁶ cts min⁻¹ ml⁻¹, 9x10⁶ Ci mol⁻¹), in the absence or presence of 20 µl unlabelled antibody 5G10, in 6 ml HA medium containing 0·03 µg ml⁻¹ DNP-BSA. After incubation, the cells were washed twice with R medium by centrifugation for 5 min at 1000 revs min⁻¹, resuspended in 6 ml of the same medium and incubated for different periods of time at 37°C. Antibody 5G10 specifically internalized and retained by cells (5G10i) was measured by centrifuging 0·2 ml samples of the cell suspension through a 0·15 ml dibutylphosphate cushion. The 125I measured in pellets of cells incubated in the presence of excess 5G10 antibody was subtracted from the 125I counted in pellets of cells incubated in its absence. Degradation of 5G10i to small peptides was studied by measuring the fraction of 5G10i precipitable by trichloroacetic acid. For this purpose 0·5 ml of the cell suspension was mixed with 0·5 ml ice-cold 20% trichloroacetic acid, incubated for 10 min on ice and the precipitated protein was collected by centrifugation and counted for radioactivity. Degradation of 125I-labelled 5G10 antibody to large peptides was studied as follows: 2x10⁶ cells containing 5G10i were incubated for 0 and 6 h in antibody-free R medium, solubilized in 1 ml of ice-cold 1 % Trition X-100, 0·1 % BSA, 0·02 % NaN₃ in PBS for 10 min and centrifuged for 10 min at 4°C in a Beckman airfuge at 30150 min⁻¹ (1 lbf = 6·9 kPa). The supernatant was passed over a 2·5 cm x 45 cm Ultrogel ACA-34 column equilibrated and eluted with solubilization buffer. The fractions eluted were counted for 125I and the radioactivity profile was examined for the presence of antibody fragments in the fractions eluted after the free antibody.

Immunofluorescence microscopy studies

Co-localization of p80 and cytosome in RKN-7 cells. RKN-7 cells were fixed in suspension with 2% paraformaldehyde/PBS for 15 min at room temperature. After washing twice with PBS, 15 µl of the cell suspension was applied to glass coverslips and allowed to dry at room temperature. Cells were permeabilized with cold (−20°C) methanol for 2 min, washed twice with PBS and incubated for 1 h at 37°C with a mixture of antibodies 5G10 (1:50 (v/v) dilution in PBS medium) and rabbit anti-cytosome (50 µg ml⁻¹ of IgG fraction in PBS). Cells were washed for 15 min with PBS and incubated with a mixture of 0·15 mg ml⁻¹ fluorescein-conjugated goat anti-mouse IgG, 0·15 mg ml⁻¹ rhodamine-conjugated goat anti-rabbit IgG and 0·25 mg ml⁻¹ normal goat IgG.

Co-localization of internalized p80 and HRP. Stimulated RBL cells or NRK cells were incubated from 10 min to 2 h at 37°C with a 1:50 (v/v) dilution of antibody 5G10 and 20 mg ml⁻¹ of rhodamine-conjugated horseradish peroxidase (HRP) in HA medium. After incubation, the cells were washed for 30 min at 4°C with PBS, fixed for 15 min with 2% paraformaldehyde/PBS, permeabilized for 15 min at room temperature with 0·1 % saponin/PBS and incubated for 30 min at 37°C with a mixture of 0·15 mg ml⁻¹ fluorescein-conjugated goat anti-mouse IgG and 0·25 mg ml⁻¹ normal goat IgG.

Co-localization of internalized p80 and serotonin. Stimulated cells were incubated for 10 min or 2 h, at 37°C with a 1:50 (v/v) dilution of antibody 5G10 in HA medium and, after removing excess antibody, cultured in R medium for different periods of time at 37°C. At each time point, cells were fixed for 15 min at room temperature with 2% paraformaldehyde in PBS and permeabilized for 15 min at room temperature with 0·1 % saponin in PBS. The permeabilized cells were incubated for 1 h at 37°C with a 1:200 (v/v) dilution of the anti-serotonin antibody YCS/45 in ASP buffer (0·1 % BSA, 0·1 % saponin, PBS). After washing for 15 min with PBS, the cells were incubated for 30 min at 37°C with a mixture of 0·15 mg ml⁻¹ rhodamine-conjugated goat anti-rat IgG, 0·15 mg ml⁻¹ fluorescein-conjugated goat anti-mouse IgG and 0·25 mg ml⁻¹ normal goat IgG in PBS. In all cases, after incubation with the second antibody, the cells were washed for 15 min at room temperature with PBS and the coverslips were mounted on glass slides using Fluormount G (Southern Biotechnology Associates, Birmingham, AL).
50 µl of a 1:25 (v/v) dilution of antibody 5G10 in HA medium. After removing excess antibody by washes with R medium, the cells were incubated for an additional 3 h at 37°C in antibody-free R medium. At the end of the incubation, the cells were washed briefly with HA medium and incubated for 15 min at 37°C with the same medium with or without 4 µg ml⁻¹ of the Ca²⁺ ionophore A23187. The cells were washed for 10 min at 4°C with PBS, incubated for 1 h at 4°C with 0-15 mg ml⁻¹ rhodamine-conjugated goat anti-mouse IgG; 0-25 mg ml⁻¹ normal goat IgG/PBS, washed again for 15 min at 4°C in PBS, fixed for 2 min with −20°C methanol, washed briefly with PBS and mounted on glass slides. To quantify the fluorescence intensity, 10 randomly chosen fields of each sample examined at ×200 magnification were photographed for 4 or 8 s, using Kodak Tri-X Pan film, and negatives corresponding to identical exposures were analysed by two-dimensional densitometry using a Photoscan System P-1000 HS densitometer. The absorbance values recorded were quantified with a Digital PDP 11/34 computer using the program R-Image (Goochee et al. 1980). Values obtained were corrected for the number of cells in the field and photographed using phase-contrast.

**Correlation between secretion of [³H]serotonin and ¹²⁵I-labelled HRP, and surface expression of internalized p80 in stimulated RBL cells**

RBL cells, treated as described in the previous section, were incubated with 2 µCi ml⁻¹ [³H]serotonin or 1X10⁸ cts min⁻¹ ml⁻¹ ¹²⁵I-labelled HRP for 2 h in the presence of either antibody 5G10 or antibody 15C8. After a 3 h incubation of the cells in antibody-free R medium and 15 min stimulation with A23187 (4 µg ml⁻¹), the release of radioactive serotonin and HRP into the medium was measured, and the surface expression of internalized 5G10 and 15C8 antigens was quantified as described above. Secreted serotonin and HRP were expressed as percentages of the total contained in the cells before stimulation of secretion.

**Electron microscopy**

Immunoelectron microscopy studies were performed by a modification of the pre-embedding procedures described previously (Brown & Farquhar, 1984; Tougaard et al. 1980). To study the surface localization of p80, allergen-stimulated RBL cells were incubated for 1 h at 4°C with 1:50 dilution of antibody 5G10 in PBS, washed for 15 min at 4°C with the same buffer, fixed for 15 min at room temperature with 0-1 % glutaraldehyde, 2 % paraformaldehyde, PBS and treated for 10 min at the same temperature with 0-5 mg ml⁻¹ NaBH₄ in PBS to quench free aldehyde groups. In some experiments, stimulated cells were fixed before incubation for 1 h at 37°C with antibody 5G10. The ultrastructural pathway of internalization was studied by incubating stimulated cells for 2 min at 37°C with a 1:50 dilution of antibody 5G10 in HA medium and then with antibody-free medium for different periods of time at 37°C. Cells were fixed as described above, permeabilized for 15 min at room temperature with 0-1 % saponin in PBS, and then incubated for 1 h at 37°C with either antibody 5G10 or the anti-serotonin antibody YCS/45 diluted 1:50 and 1:200 in ASP buffer, respectively. After three washes for 10 min at room temperature with PBS, the cells were incubated for 1 h at 37°C with 30 µg ml⁻¹ of HRP-conjugated F(ab')₂ goat anti-mouse IgG or F(ab')₂ rabbit anti-rat IgG in ASP buffer containing 0-25 mg ml⁻¹ normal goat or rabbit IgG, respectively. Following incubation with the second antibody, the cells were washed three times for 15 min with PBS, fixed for 30 min at room temperature with 1-5 % glutaraldehyde, 0-1 % sodium cacodylate buffer, pH 7-4, washed overnight with cacodylate buffer containing 7-5 % sucrose and finally for 20 min with 50 nm Tris–HCl buffer, pH 7-4, containing 7-5 % sucrose. The peroxidase reaction (Graham & Karnovsky, 1966) was performed by incubating the cells for 1-15 min with 0-2 % 3,3'-diaminobenzidine tetrahydrochloride and 0-01 % H₂O₂ in 0-1 m Tris–HCl buffer, pH 7-4.

Study of the recycling of internalized 5G10 antigen to LSG (lysosome-like secretory granules) was performed using non-stimulated RBL cells secreting small amounts of serotonin and displaying low but significant levels of p80 on their surface (Bonifacino et al. 1986). The cells were cooled at 4°C, incubated for 30 min with antibody 5G10 and rinsed several times with cold AP buffer. After, the cells were incubated at 4°C with 15 nm colloidal-gold-conjugated goat anti-mouse IgG (Boehringer-Mannheim) diluted 1:5 in the same buffer, washed three times with AP and incubated 4 h at 37°C in medium. Then, the cells were fixed with 0-1 % paraformaldehyde, 37 µm-sodium phosphate buffer, pH 7-5 (McLean & Nokane, 1974), at 4°C for 1 h, and at 37°C for another hour, before permeabilization with 0-15 % saponin in AP buffer for 15 min at room temperature. After a 30 min wash with PBS at room temperature, the cells were incubated for 1 h at the same temperature with the monoclonal anti-serotonin antibody YCS/45 diluted 1:50 in AP buffer. The cells were washed again for 30 min with AP buffer and incubated for 1 h at room temperature with 100 µg ml⁻¹ of specific rabbit anti-rat IgG (Zymed), washed with AP buffer and incubated for 1-5 h with 5 nm gold, Texas Red-conjugated protein A (E-Y Laboratories). Control cells incubated without rabbit anti-rat IgG did not display any reaction with protein A as shown by immunofluorescence microscopy in the Texas Red channel. After extensive washing with AP buffer the cells were fixed again with 1-5 % glutaraldehyde in 0-1 m-sodium cacodylate buffer, pH 7-4.

All cells incubated with peroxidase- or gold-conjugated antibodies were washed three times for 10 min with cacodylate buffer and postfixed for 45 min at 4°C with 1 % OsO₄, 1 % potassium ferrocyanide, 0-1 m-sodium cacodylate, pH 7-4. The osmicated cells were dehydrated in graded ethanol, detached from the plastic support with propylene oxide and embedded in Epon 812. Thin sections (800 Å) were prepared using a Sorval MT2-B microtome, stained with lead citrate and examined using a Philips 400 electron microscope.

**Sources of other reagents**

DNP–BSA containing 43 nmol DNP per mol of BSA was a gift from Dr Henry Metzger, NIADDK, NIH. HRP (type VI, inactivated), saponin, 3,3'-diaminobenzidine tetrahydrochloride, paraphenylenediamine dihydrochloride and A23187 were from Sigma Chemical Co., St Louis, MO. Paraformaldehyde was from Aldrich Chemical Co., Milwaukee, WI. Glutaraldehyde was from Ladd Research Industries, Inc., Burlington, VT. Rhodamine-conjugated HRP was from Coper Biomedical Inc., Malvern, PA. 5-(1,2-d[¹H]N)-hydroxytryptamine binoxalate ([¹H]serotonin) was from New England Nuclear, Boston, MA.

**Results**

**Localization of p80 in membranes of lysosomes and lysosomal-like secretory granules**

The localization of p80 was studied in a variety of rat cell types displaying exclusively lysosomes (NRK cells), or lysosomes and secretory granules (RBL, natural killer lymphocytes, pancreatic acinar cells, pituitary cells). In
Fig. 1. Presence of p80 in lysosomes and secretory granules containing lysosomal enzymes, (A,B). Immunofluorescence microscopy of NRK cells incubated for 45 min at 37°C with 50 mg/ml-1 rhodamine–HRP in free RM, fixed with 2% paraformaldehyde, permeabilized with 0·1% saponin, and immunofluorescence-conjugated goat anti-mouse IgG. A. Fluorescein channel; B, rhodamine channel. A few vesicles are not displayed in both channels (arrowheads). Bar, 10 μm. C,D. Distribution of p80 in NRK cells (C) and rat pancreatic acinar cells (D) as studied by electron microscopy with antibody 5G10 using the pre-embedding immunoperoxidase technique. Large secondary lysosomes (l); zymogen granules (g); nucleus (n). Bars, 0·5 μm. E,F. Co-localization of p80 (E, fluorescein channel) and serotonin in RBL cells (F, rhodamine channel). A few vesicles contain only p80 (arrowheads). Bar, 10 μm. G,H. Co-localization of p80 (G, fluorescein channel) and cytolysin (H, rhodamine channel) in cytotoxic T lymphocytes, line RNK-7. Numerous vesicles contain p80 and cytolysin, and a few display either one of them (arrowheads). Bar, 10 μm.

NRK fibroblasts, the p80 localized to vesicles that contained lysosomal membrane proteins, as shown by the staining with the anti-LIMP I, II and III antibodies (Barriocanal et al. 1986) (data not shown), and HRP internalized by endocytosis 45 min before (Fig. 1A,B) (Marsh et al. 1986; Rodman et al. 1986). By both criteria the vesicles appear to be lysosomes. This localization was confirmed by electron microscopy (Fig. 1C). In exocrine pancreatic acinar cells p80 was found in lysosomes but not in exocrine secretory granules (Fig. 1D). The same pattern of protein distribution was observed in endocrine pituitary cells (data not shown). However, in secretory rat basophilic leukaemia cells, p80 was found in secretory granules, identified by the presence of serotonin (Fig. 1E,F), and in vesicles containing no serotonin (Fig. 1E,F) that were identified as lysosomes by immunofluorescence and immunoelectron microscopy using anti-LIMP antibodies (data not shown). In natural killer cells (line RNK-7), the protein was localized to cytotoxic granules, identified by the presence of cytolysin (Henkart, 1985) (Fig. 1G,H) and in vesicles containing no cytolysin (Fig. 1G,H) that were stained with anti-LIMP antibodies and displayed the morphology characteristic of lysosomes as shown by immunofluorescence and immunoelectron microscopy (data not shown). These cells also showed a significant number of small vesicles containing cytolysin but displaying no p80. With respect to the different distribution of p80 between different secretory granules it is worth noting that p80 was expressed in secretory granules with lysosomal properties (LSG), as shown by the content of basophils and granular lymphocytes, line RNK-7. Numerous vessels contain p80 and cytolysin, and a few display either one of them (arrowheads). Bar, 10 μm.

Sensitivity of the p80–antibody complex to dissociation by acidic pH

It was essential, for using the monoclonal antibody 5G10 as a probe to study the pathway of internalization and recycling of p80 to LSG, that it remained firmly bound to the antigen at the acidic pH values existing in the putative organelles involved in these processes. To test the stability of the p80–antibody complex at different pH values, 125I-labelled 5G10 antibody was bound to sodium carbonate-treated membranes from RBL cells and the membranes were incubated at 37°C with buffers adjusted to pH values ranging from 2 to 7. The results of this experiment showed that the antibody was not dissociated from the membranes at pH 5 or at higher pH values. Only

Fig. 2. Degradation of internalized 125I-labelled 5G10 antibody by stimulated RBL cells. Stimulated RBL cells were incubated for 30 min at 37°C with 125I-labelled 5G10 antibody in the presence or absence of excess unlabelled antibody 5G10 as described in Materials and methods. Excess antibody was removed by centrifugation and the cells were incubated in R medium for different periods of time at 37°C. A. Antibody specifically internalized retained by the cells (5G10i) was determined by centrifugation of the cells through a dibutylphthalate cushion (••••). Degradation of internalized 125I-labelled 5G10 antibody into small peptides was studied by measuring trichloroacetic acid-soluble products (O O). B. Degradation of internalized 125I-labelled 5G10 antibody into large peptides was studied by comparing the gel chromatography pattern of Triton X-100-solubilized extracts obtained from cells incubated in antibody-free medium for 0 and 6 h. Markers: V0, void volume; IgG, normal goat IgG; Tf, transferrin; Vn, salt volume.

Recycling of serotonin-containing granules
40% of the antibody was dissociated at pH 4.5. Since organelles potentially involved in the internalization and recycling of p80, such as coated vesicles, endosomes, multivesicular bodies, Golgi system and secretory granules, have an internal pH higher than 5 (Forgac et al. 1983; Glickman et al. 1983; Johnson & Scarpa, 1976;
Degradation of internalized SGIO antibody in RBL cells.

Fig. 3. Immunofluorescence microscopy studies on the acetic acid soluble products after 4 and 6h, respectively. A. Fluorescein channel; B, rhodamine channel. HRP internalized by fluid-phase pinocytosis is used as an endosome marker. C,D. Stimulated RBL cells incubated for 10 min at 37°C with antibody SG10, fixed with 2% paraformaldehyde, permeabilized with 0-1 % saponin and incubated for 30 min at 37°C with fluorescein-conjugated goat anti-mouse IgG. A. Fluorescein channel; B, rhodamine channel. HRP internalized by fluid-phase pinocytosis is used as an endosome marker. C,D. Stimulated RBL cells incubated for 10 min at 37°C with antibody SG10, fixed with 2% paraformaldehyde and permeabilized with 0-1 % saponin. Serotonin was reacted with antibody YCS/45 and stained with rhodamine-conjugated goat anti-rat IgG, whereas internalized antibody SG10 was stained with fluorescein-conjugated goat anti-mouse IgG. C. Fluorescein channel; D, rhodamine channel. E-G. Stimulated cells, incubated for 10 min with antibody SG10 and after 30 min in antibody-free medium fixed and then permeabilized with cold (−20°C) methanol, and incubated for 1h at 37°C without (E) or with (F-G) a human anti-Golgi autoantibody. p80 was stained with rhodamine-conjugated goat anti-mouse IgG (E,F), whereas a fluorescein-conjugated goat anti-human antibody was used to stain the Golgi antigen (G). H-L. Stimulated RBL cells incubated for 2h at 37°C with antibody SG10 and rhodamine-conjugated HRP, and then for 3h in R medium. Cells were fixed with 2% paraformaldehyde, permeabilized with 0-1 % saponin and incubated for 30 min at 37°C with fluorescein-conjugated goat anti-mouse IgG. H. Fluorescein channel; I, rhodamine channel. J-L. Stimulated RBL cells incubated for 2h with antibody SG10 and then for 3h in regular medium. Cells were fixed with either 2% paraformaldehyde and permeabilized with 0-1 % saponin (J,K) or fixed and permeabilized with −20°C methanol (L). Internalized antibody SG10 was detected with fluorescein-conjugated goat anti-rat IgG and serotonin reacted with antibody YCS/45 and stained with rhodamine-conjugated goat anti-rat IgG. J.L. Fluorescein channel; K, rhodamine channel. L. Cell boundaries are marked with arrowheads. Bars, 7-5 µm.

Tycko & Maxfield, 1982; Van Dyke et al. 1984; Ohkuma & Poole, 1978), the SG10 antibody was expected to remain largely bound to p80 after internalization.

Degradation of internalized SG10 antibody in RBL cells

To exclude the possibility that the internalized SG10 antibody was degraded to produce a fragment that could follow an intracellular pathway different from the antigen, we studied the proteolytic degradation of 125I-labelled SG10 antibody internalized by stimulated RBL cells to small (Fig. 2A) and large peptides (Fig. 2B). Degradation to small peptides was limited, since only 10 and 15% of the radioactivity was recovered as trichloracetic acid soluble products after 4 and 6h, respectively (Fig. 2A). Furthermore, no significant degradation to large peptides was found, as shown by the absence of 125I-labelled peptides with molecular weights smaller than the antibody. By comparing the amounts of radioactivity corresponding to the solubilized antigen–antibody complex (peak at fraction 28 in Fig. 2B), we concluded that approximately 70% of the antibody remained attached to the antigen after 6h of internalization (Fig. 2). In spite of the fact that any antibody can potentially alter the intracellular pathway of internalized proteins, we concluded from its slow degradation and tight binding to the antigen that antibody SG10 was a reliable probe for study of the pathway of internalization of surface-expressed p80 antigen.

Internalization and recycling of surface-expressed p80 studied by immunofluorescence microscopy

In a previous study (Bonifacino et al. 1986) we showed that upon stimulation of secretion increased levels of p80 were expressed on the surface of RBL cells. To study the internalization of surface p80 by immunofluorescence microscopy, stimulated RBL cells were incubated for 10 min or for 2h at 37°C with antibody SG10, and then they were either fixed immediately or incubated for various periods of time at 37°C in antibody-free medium before fixation. We observed that cells studied after a 10 min uptake period displayed staining of cytoplasmic vesicles (Fig. 3A,C). Study of the internalization of the antibody in the presence of the fluid-phase pinocytosis marker HRP labelled with rhodamine showed that most of the vesicles displaying the internalized antibody also contained HRP (Fig. 3, compare A and B). Since HRP is known to be concentrated in endosomes shortly after internalization (Marsh et al. 1986; Rodman et al. 1986), this result suggested that endosomes were involved in the internalization pathway of the p80 expressed on the cell surface. It is noteworthy that at this early time of internalization (10 min), the degree of co-localization of internalized antibody SG10 and serotonin (a marker of secretory granules) were negligible (Fig. 3, compare C and D). This result indicated that endocytic vesicles did not undergo rapid fusion with secretory granules, as was shown in normal guinea-pig basophilis (Dvorak et al. 1972). Incubation of the cells for 30 min in antibody-free medium resulted in a progressive concentration of the internalized antibody in the juxtanuclear area containing the Golgi system (Fig. 3E,F,G). Between 1 and 3h after internalization, the antibody was localized in vesicles randomly dispersed throughout the cytoplasm (Fig. 3H,J). After prolonged incubations (2h uptake followed by a 3h chase in antibody-free medium), the internalized antibody displayed only partial co-localization with HRP (Fig. 4, compare H and I) and, in contrast with the correspondence observed at 10min (Fig. 3, compare A and B), many vesicles containing antibody SG10 did not display HRP. Since HRP is mainly delivered to secondary lysosomes (Marsh et al. 1986), this observation could suggest that a substantial amount of the internalized antibody was being delivered to organelles other than lysosomes. Nevertheless, the possibility that HRP was being degraded rapidly in lysosomes could not be discarded. To characterize further the vesicles containing SG10 antibody we also studied the co-distribution of the antibody with serotonin, a marker of LSG in these cells. It was observed that a significant number of cytoplasmic vesicles containing the internalized antibody also contained serotonin (Fig. 3, compare J and K). The number of cytoplasmic vesicles that contained both markers versus the total containing either one or both of them was between 50 and 80%.

Recycling of serotonin-containing granules
Fig. 4. Re-expression of internalized p80-5G10 antibody complex on the surface of cells stimulated to secrete for a second time. IgE/DNP-BSA-stimulated RBL cells were incubated for 2 h at 37°C with antibody 5G10 and then for 3 h at 37°C in R medium, before incubation for 15 min at 37°C without (A,B) or with 4 µg ml⁻¹ A23187 (C,D). The p80-antibody complex re-expressed on the cell surface was detected with rhodamine-conjugated goat anti-mouse IgG. A,C. Phase-contrast; B,D, rhodamine channel. Bars, 7.5 µm.

Fig. 5. Quantification of the re-expression of p80-5G10 antibody on the surface of cells stimulated to secrete for a second time: correlation with serotonin secretion. A. IgE/DNP-BSA-stimulated RBL cells were incubated for 2 h at 37°C with antibodies 5G10 and 15C8 (control), followed by 3 h at 37°C in antibody-free medium. The cells were stimulated to secrete a second time by incubation for 15 min at 37°C with (+) 4 µg ml⁻¹ A23187. Controls were incubated without (−) A23187. The re-expression of p80-antibody complexes on the cell surface was detected with rhodamine-conjugated goat anti-mouse IgG. The fluorescence intensity of each sample was measured by photography/computerized densitometry as described in Materials and methods. Values are expressed in arbitrary units and represent the mean of 10 determinations. B. Cells were treated as in A, except that [³H]serotonin (2 µCi ml⁻¹) or ¹²⁵I-labelled HRP (1 × 10⁴ cts min⁻¹ ml⁻¹) were present in the 2 h incubation with antibody 5G10. The amount of radioactivity released into the medium after treatment for 15 min without (−) or with (+) 4 µg ml⁻¹ A23187, was expressed as the percentage of the total serotonin or HRP accumulated by the cells. Values are means of duplicate determinations.

Fixation of cells with cold methanol, instead of paraformaldehyde, caused swelling of the vesicles containing the antibody. Their appearance as fluorescent rings (Fig. 3L), indicated the presence of the p80-antibody complex in the membrane of the vesicles. These experiments suggested that the internalized p80 was, at least partly, recycled to LSG.

In similar experiments in which stimulated RBL cells were incubated with either normal mouse IgG, monoclonal antibody 8D12 (recognizing MAP1 in the cytoplasm and the mitotic spindle-associated protein p280 in the nucleus; Bonifacino et al. 1985) or monoclonal antibody 15C8 (reacting with a cis-medial Golgi integral membrane protein; Yuan et al. 1987), no significant uptake of antibodies by the cells was detected. These results indicated that internalization of antibody 5G10 was mediated by the 5G10 antigen exposed on the cell surface and was not produced by fluid-phase pinocytosis or binding to surface Fc receptors.
and a 2.7-fold difference between the surface fluorescence intensities of stimulated and unstimulated cells. Substitution of antibody 5G10 by equivalent amounts of mouse IgG or a series of 10 ascitic fluids containing none, or unrelated, monoclonal antibodies resulted in no increase of surface fluorescence after the second stimulation of secretion. One of these controls, performed with the anti-Golgi antibody 15C8, is shown in Fig. 5A.

The increased surface expression of the p80-antibody complex was correlated with an increase in the secretion of $[^{3}H]$serotonin contained in secretory granules (Fig. 5B). By contrast, secretion of $^{125}$I-labelled HRP contained in lysosomes was not significantly affected by the Ca$^{2+}$ ionophore (Fig. 5B) (see Discussion).

The increased surface expression of the p80-antibody complex was correlated with an increase in the secretion of $[^{3}H]$serotonin contained in secretory granules (Fig. 5B). By contrast, secretion of $^{125}$I-labelled HRP contained in lysosomes was not significantly affected by the Ca$^{2+}$ ionophore (Fig. 5B) (see Discussion).

**Ultrastructural pathway of endocytosis**

The pathway of endocytosis of p80 expressed on the surface of secreting cells was studied by electron microscopy using an immunoperoxidase pre-embedding procedure (Brown & Farquhar, 1984; Tougard et al. 1980). In agreement with the studies performed by immunofluorescence microscopy, the antigen was initially found in patches of 0.2-0.7 μm distributed throughout the surface of stimulated RBL cells (Fig. 6A). It was also frequently localized in coated pits (Fig. 6B). After 2 min of incubation with the antibody the internalized antigen was located in coated pits, coated vesicles (Fig. 6C) and more frequently on the membrane of 0.2-0.6 μm smooth-surface vesicles that are frequently seen in association with tubules (Fig. 6D). The charac-

---

**Fig. 6.** Localization of the p80 exposed on the surface of stimulated RBL cells and morphological characterization of the pathway of endocytosis using immunoelectron microscopy. A,B. Localization of the p80 exposed on the surface of secreting RBL cells. Stimulated cells were incubated for 1 h at 4°C with antibody 5G10 and fixed with 0.1% glutaraldehyde/2% paraformaldehyde prior to incubation with HRP-conjugated F(ab')$_2$ goat anti-mouse IgG. C-H. Internalization of the p80 exposed on the cell surface. Stimulated cells were incubated for 2 min at 37°C with antibody 5G10 and then for 0 min (C,D), 10 min (E-G), 30 min (H) in antibody-free medium, before being fixed with 0.1% glutaraldehyde/2% paraformaldehyde in PBS and studied by the pre-embedding immunoperoxidase method described in Materials and methods. Bars, 0.25 μm.
Fig. 7. Recycling of internalized p80 to LSG and delivery to lysosomes. A, B. RBL cells were stimulated to secrete with IgE/DNP-BSA, incubated for 2 min at 37°C with antibody 5G10 and for 4 h in antibody-free R medium. After fixation with 2% paraformaldehyde/0.1% glutaraldehyde, and permeabilization, the internalized antibody was detected with HRP-conjugated goat anti-mouse IgG. C, D. RBL cells were fixed with PLP, permeabilized with 0.1% saponin, incubated with the monoclonal anti-serotonin YC5/45 and stained with HRP-conjugated goat anti-rat IgG. Note that fixation of the cells is poorer than in A. E–I. Unstimulated RBL cells incubated for 30 min at 4°C with antibody 5G10, then for 30 min at 4°C with 15 nm gold-conjugated goat anti-mouse antibody, washed, incubated for 3 h at 37°C, fixed with PLP, permeabilized with 0.15% saponin and sequentially incubated with YC5/45 anti-serotonin antibody, rabbit anti-rat IgG and 5 nm gold-conjugated protein A, before being fixed with 1.5% glutaraldehyde. Note the expression of p80 in lysosomes (arrowhead), LSG (arrow) and the existence of p80-negative vesicles containing serotonin (star). Bars, 0.22 μm.

J. S. Bonifacino et al.
characteristics of these vesicles correspond to those described for endosomes (Marsh et al. 1986). The presence of internalized antibody in endosomes, at this time, was consistent with the co-localization of antibody 5G10 and HRP observed by immunofluorescence microscopy (Fig. 3A,B). By 10 min after internalization, most of the antigen was still found in these organelles, but some was also found in the cisternae of the Golgi system (Fig. 6E–G). From 30 min to 1 h after internalization, most of the antigen was contained in vesicles that included some multivesicular bodies, and in smaller vesicles in the proximity of the Golgi apparatus (Fig. 6H). Between 1 h and 4 h, the internalized antigen was found in vesicles (diameter 0.2–0.5 μm) whose identity could not be ascertained by morphological criteria (Fig. 7A,B). Similar vesicles were also stained with the anti-serotonin antibody YC5/45 (Fig. 7C,D). To characterize these vesicles further the distribution of internalized 5G10 antibody and serotonin was studied using 15 nm gold-conjugated goat anti-mouse IgG and 5 nm gold-conjugated protein A, respectively, as described in Materials and methods. It can be observed that the internalized p80 was located in vesicles with the characteristics of lysosomes, identified by the absence of serotonin and membrane whirls in their lumen (Fig. 7E, arrowhead), and in LSG containing serotonin (Fig. 7E, arrow, G–1). The latter results indicated that some of the internalized p80 was recycled to LSG (see Discussion). It is noteworthy that in LSG the protein was also associated with the limiting membrane of what appeared to be luminal vesicles (Fig. 7B,G,H). A detailed study revealed that those vesicles probably corresponded to invaginations of the limiting membrane (Fig. 7F,G,1, arrowheads).

Discussion

In this study we have examined the distribution, the pathway of internalization and the recycling of the 80K (p80) protein first found in the membranes of the secretory granules of RBL cells (Bonifacino et al. 1986).

The distribution studies indicate that in resting RBL cells and RNK-7 natural killer lymphocytes p80 is predominantly located in secretory granules and lysosomes. It is important to note that these secretory granules contain lysosomal hydrolases (Sriraganian, 1984; Henkart, 1985) and express three lysosomal integral membrane protein, LIMPs I, II and III, in their membranes. This, and its absence from exocrine and endocrine secretory granules, which are free of the lysosomal hydrolases and LIMPs, indicate that p80 is largely restricted to organelles with lysosomal properties.

The retrieval of the membranes of secretory granules inserted into the plasma membrane during secretion has been extensively studied (reviewed by Farquhar, 1981; Herzog & Miller, 1979; Oliver & Hard, 1981). It happens by endocytosis and, as shown in cells labelled with cationized ferritin (Farquhar, 1978; Herzog & Miller, 1979; Ottosen et al. 1980), lectins (Tougard et al. 1980) or specific antibodies (Patzak et al. 1984; Patzak & Winkler, 1986), the internalized membranes can be traced to Golgi cisternae, condensing vacuoles, secretory granules, multivesicular bodies and lysosomes.

As part of the study of the pathway of internalization of p80 we have examined its distribution on the plasma membrane of stimulated RBL cells. It is important that the protein is not distributed uniformly throughout the cell surface but that it is found in randomly distributed small patches. The presence of these patches in cells fixed with glutaraldehyde/paraformaldehyde before being incubated with the antibody indicates that they are not artifacts produced by antibody-induced aggregation, and suggests that after its insertion p80 is not free to diffuse within the plasma membrane. Furthermore, since the size of the patches (0.2–0.7 μm) is significantly smaller than the average perimeter of the secretory granules (0.6–1.5 μm) but larger than the perimeters of coated pits and coated vesicles (0.12–0.28 μm), it seems likely that the membranes of LSG are fragmented while in the plasma membrane. Also relevant is the presence of p80 in coated pits, which suggests that the LSG membranes are retrieved by internalization via coated pits.

The involvement of endosomes in the endocytic pathway of p80 is suggested by the early co-localization of the internalized 5G10 antibody with the fluid-phase marker HRP in the same cytoplasmic vesicles. The rest of the pathway appears to be similar to that of other membranes internalized by endocytosis, including the involvement of the Golgi apparatus (Farquhar, 1978, 1981; Herzog & Miller, 1979; Snider & Rogers, 1985, 1986; Woods et al. 1986), and probably ends with the arrival of the protein to LSG and lysosomes. The recycling of p80 to LSG is suggested by the presence of internalized antigen–antibody complexes in serotonin-containing vesicles and their re-expression on the cell surface. Although bivalent antibodies have sometimes been shown to cause the internalization of proteins exposed on the cell surface and their degradation in lysosomes, it appears highly unlikely that they could be responsible for the recycling of membrane components.

The presence of internalized 5G10 antibody in vesicles containing no serotonin and its slow but significant degradation, both suggest that the p80–5G10 antibody complexes are partly delivered to lysosomes. Whether this is the result of cross-linking by the antibody, or reflects the physiological transport of LSG membranes to lysosomes, is not known.

We thank Mrs Katy Perry and Carmen Hermoso for excellent secretarial assistance.

References


Recycling of serotonin-containing granules 711


(Received 18 October 1988 - Accepted 23 December 1988)