An Antibody against Secretogranin I (Chromogranin B) Is Packaged into Secretory Granules

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Abstract. We have investigated the sorting and packaging of secretory proteins into secretory granules by an immunological approach. An mAb against secretogranin I (chromogranin B), a secretory protein costored with various peptide hormones and neuropeptides in secretory granules of many endocrine cells and neurons, was expressed by microinjection of its mRNA into the secretogranin I-producing cell line PC12. An mAb against the G protein of vesicular stomatitis virus—i.e., against an antigen not present in PC12 cells—was expressed as a control. The intracellular localization and the secretion of the antibodies was studied by double-labeling immunofluorescence using the conventional and the confocal microscope, as well as by pulse-chase experiments. The secretogranin I antibody, like the control antibody, was transported along the secretory pathway to the Golgi complex. However, in contrast to the control antibody, which was secreted via the constitutive pathway, the secretogranin I antibody formed an immunocomplex with secretogranin I, was packaged into secretory granules, and was released by regulated exocytosis. Our results show that a constitutive secretory protein, unaltered by genetic engineering, can be diverted to the regulated pathway of secretion by its protein–protein interaction with a regulated secretory protein. The data also provide the basis for immunologically studying the role of luminally exposed protein domains in the biogenesis and function of regulated secretory vesicles.

Some eukaryotic cells—such as exocrine, endocrine, and neuronal cells—contain at least two distinct pathways of protein secretion. In the constitutive pathway, which exists in these and essentially all other eukaryotic cells, certain secretory proteins (e.g., components of the extracellular matrix) are transported to the cell surface without intracellular storage and are released without external regulation of exocytosis. In the regulated pathway, which is a characteristic feature of exocrine, endocrine, and neuronal cells, certain other secretory proteins (e.g., zymogens, peptide hormones, and neuropeptides) are packaged into secretory storage granules from which they are exocytosed in a regulated manner (for reviews see Burgess and Kelly, 1987; Pfeffer and Rothman, 1987).

The specific secretion of constitutive and regulated secretory proteins by the same cell via the constitutive and regulated pathway, respectively, involves the segregation of these proteins from each other. Recently, it has been suggested that the packaging of regulated secretory proteins into secretory granules is a signal-mediated process which is dominant over the post-Golgi route taken by constitutive secretory proteins (Burgess and Kelly, 1987; Pfeffer and Rothman, 1987). The only direct piece of evidence in support of this concept is the observation that a chimeric fusion protein composed of a regulated secretory protein (growth hormone) and the ectodomain of a membrane protein (G protein of vesicular stomatitis virus [VSV-G]) lacking the transmembrane and cytoplasmic domains) was packaged into secretory granules of AtT-20 cells (Moore and Kelly, 1986). However, the possibility cannot be excluded that the truncation of VSV-G and its fusion to growth hormone may have inactivated structural information that otherwise would have resulted in the transport of the fusion protein to the cell surface via the constitutive pathway.

We therefore decided to investigate secretory protein sorting by an alternative approach that would not have the problems inherent in experiments with chimeric fusion proteins and that could complement the latter studies. This approach was to study the intracellular traffic of a noncovalent complex consisting of a regulated secretory protein and a constitutive secretory protein unmodified by genetic engineering. For a regulated secretory protein we chose secretogranin I (Sgl), also called chromogranin B (Eiden et al., 1987), a protein packaged into secretory granules of a wide variety of endo-

Abbreviations used in this paper: NGF, nerve growth factor; Sgl, secretogranin I; SglI, secretogranin II; VSV-G, G protein of vesicular stomatitis virus.
Antibodies

mAbs against rat Sgl were raised as follows. [35S]Sulfate-labeled secreted granulins were purified from PC12 cells by heat treatment, DEAE-Sephacel chromatography, and two-dimensional PAGE as described (Rosa et al., 1985b; Benedum et al., 1987). After electrophoresis, proteins were transferred onto nitrocellulose filters (Rosa et al., 1985a) and the position of Sgl was revealed by autoradiography. Pieces of the nitrocellulose filter containing pure Sgl (either p113 or p105; Lee and Huttner, 1983; Rosa et al., 1985b; 47 ± g as determined by Coomassie blue staining of a duplicate gel) were implanted into the peritoneal cavity of 6-wk-old BALB/c mice. 1 mo later, the mice were boosted with 47 ± g of pure Sgl that was prepared according to standard procedures (Ochiai et al., 1982, Goding, 1983). Hybridoma supernatants were screened by immunoblotting of total PCI2 cell protein, and clones producing antibodies against Sgl were subcloned under the basis for studying the functional role of luminally exposed epitopes of secretory granule proteins by antibody neutralization, analogous to previous work on a Golgi-resident protein (Burke and Warren, 1984). Here we show that a monoclonal anti-Sgl antibody, in contrast to a control antibody, is diverted from the constitutive pathway of secretion and packaged, in an immunocomplex with Sgl, into secretory granules of the neuroendocrine cell line PC12.

Materials and Methods

Antibodies

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Characterization of mAb 219.6

PC12 cells (clone II-251) were grown in DME supplemented with 10% horse serum and 5% FCS. For immunoblotting, a PC12 cell total homogenerate (Lee and Huttner, 1983) and a human pheochromocytoma chromaffin granule fraction, prepared as described for bovine chromaffin granules (Trifaro and Duerr, 1976) with minor modifications, were separated by SDS-PAGE, and the proteins were transferred onto nitrocellulose filters as described (Rosa et al., 1985c). The filters were incubated for 18 h at 4°C in immunoblotting buffer (PBS containing 8% BSA) followed by incubation for 2 h at room temperature either in undiluted hybridoma supernatant containing 10 mM Hepes or mice sera diluted 1:100 in immunoblotting buffer containing 0.3% Tween 20. After several washes in PBS-Tween, filters were incubated for 45 min with peroxidase conjugated to goat anti-mouse IgG and IgM antibodies (Jackson Immuno Research Labo...
intermediate salt wash was omitted and distilled water was used for elution. Poly(A)+ RNA was then precipitated after addition of 0.2 M potassium acetate, 2 M NaCl, and 2 vol ethanol; the precipitate was washed with 75% ethanol and redissolved in water at a final concentration of 1 mg/ml for microinjection. The mRNA coding for heavy and light chain of IgG in poly(A)+RNA preparations was quantitated by in vitro translation using a reticulocyte lysate system in the presence of L-[35S]methionine (NEK-001 translation kit; New England Nuclear, GmbH, Dreieich, FRG) followed by immunoprecipitation of heavy and light chain, SDS-PAGE, and determination of incorporated radioactivity.

Cell Culture and Microinjection

PC12 cells were plated onto either poly(l)lysine- or poly(d,l)ornithine-coated coverslips and grown either for 1 d in the absence of nerve growth factor (NGF) or for 2-3 d in the presence of 200 ng/ml of NGF (a gift from Drs. Y.A. Barde and H. Thoenen, Max Planck Institute for Psychiatry, Martinsried, FRG). Immediately before microinjection, 10 mM Hepes, pH 7.4, was added to stabilize the pH of the medium during microinjection.

Cells were microinjected (1 mg poly(A)+RNA/ml water) at room temperature using the automated capillary microinjection system with computer-controlled positioning of the cells and the capillary (Ansgorge and Pepperkok, 1988). With this system, coordinates of the microinjected cells were stored in the computer, allowing their precise retrieval and hence a reliable statistical analysis of the results (Pepperkok et al., 1988). Depending on the experiment, 150-1,000 cells were injected per coverslip in 10-30 min.

After microinjection, dishes received fresh DME supplemented with serum and containing penicillin/streptomycin before being returned to the incubator for the indicated times. In some experiments, dishes received 10 ng/ml of NGF (from Alexis, Australia) either after microinjection or followed by an 18-h incubation.

In other experiments, dishes maintained for 18 h after microinjection were incubated for 9 min in release medium (Meldolesi et al., 1983) either with or without 3 nM α-latrotoxin purified from female black widow spiders, Latrotoxias tetricimutatus (Meldolesi, 1982; a gift of Dr. J. Meldolesi, University of Milan, Milan, Italy). After incubation under these various conditions, cells were fixed and analyzed by immunofluorescence as described below.

Pulse-Chase Labeling of IgG Expressed in Microinjected PC12 Cells

Approximately 3,000 PC12 cells were plated on 2-mm2 pieces of coverslips coated with poly(l)lysine and grown overnight in 96-well plates in 50 μl of DME supplemented with serum. For microinjection, coverslips were carefully transferred to 3.5-cm dishes containing Hepes-buffered DME. About 1,000 cells per coverslip were microinjected as described above. After microinjection, coverslips were put back into 96-well plates and incubated in 50 μl of methionine-free DME supplemented with 1% dialyzed horse serum and 0.5% dialyzed FCS. Noninjected control coverslips were directly transferred to 96-well plates containing the latter medium. After 3 h, the medium was replaced with 50 μl of methionine- and KCl-free DME containing L-[35S]methionine (1 mCi/ml; Amersham Buchler GmbH) and 7 mM potassium acetate (from the [35S]methionine stock). The cells were labeled for 1 h, and the labeling medium was removed and replaced with chase medium (DME containing a doubling concentration of methionine and supplemented with 1% horse serum and 0.5% FCS). After 4 h of chase, the chase medium was collected, spun for 5 min in a centrifuge (Eppendorf; Brinkmann Instruments Co.), adjusted to 0.3% Triton X-100, and subjected to immunoprecipitation as described above. The cells were washed with cold PBS, solubilized in 50 μl of 50 mM Tris-HCl, pH 7, containing 0.3% Triton X-100, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, spun for 1 min in a centrifuge (Eppendorf; Brinkmann Instruments Co.), and also subjected to immunoprecipitation.

Labeled IgG was immunoprecipitated by incubation for 15 min with 25 μl (packed gel) of goat anti-mouse IgG coupled to agarose. After several washes with 50 mM Tris-HCl, pH 7, 0.3% Triton X-100, 450 mM NaCl, 5 mM EDTA, the immune complex was solubilized under a nonreducing condition by boiling in Laemmli sample buffer without 2-mercaptoethanol and subjected to reducing SDS-PAGE (6% polyacrylamide) followed by fluorography (Lee and Huttner, 1983). For electrophoresis under reducing conditions, gel pieces from the IgG region were excised from the reducing gels, renatured in 30% methanol and 10% acidic acid to remove the salicylate from the fluorography, rinsed in water, and dried. The gel pieces were then reswollen for 15 min in 400 μl of Laemmli sample buffer containing 5% 2-mercaptoethanol, boiled in this buffer for 2 min fol-lowed by immediate removal of excess buffer, and placed in the slots of a 12% SDS-polyacrylamide gel followed by electrophoresis and fluorography. The fluorograms were analyzed quantitatively using an ultroscan XL (LKB Instruments, Inc., Gaithersburg, MD). Absorbance values from the corresponding region of samples from noninjected cells of the same fluorograms were subtracted as background.

Immunofluorescence

For double immunofluorescence, cells were washed with PBS, fixed for 30 min at room temperature with 3% paraformaldehyde in 120 mM phosphate buffer, pH 7.4, permeabilized for 5 min in PBS containing 0.3% Triton X-100, and quenched for 30 min with 0.1 M glycine-Tris, pH 7.5. Cells were then incubated for 1.5 h in 20 mM phosphate buffer, pH 7.3, 450 mM NaCl, 0.2% gelatine (immunofluorescence buffer) containing goat anti-mouse IgG anti-body and 30% of the fluoroscopy, respectively. In several washes in immunofluorescence buffer, cells were incubated for 2 h with the indicated polyclonal rabbit or monoclonal mouse antibodies against either Sgl, SglI, P38/synaptophysin, or the Golgi complex. In the case of immunostaining for lgl20, PBS-washed PC12 cells were fixed for 5 min in methanol at −20°C, washed with PBS and immunofluorescence buffer, and incubated with polyclonal rabbit anti-rat lgl20. All coverslips were then washed again and incubated in immunofluorescence buffer containing the appropriate goat anti-rabbit IgG or anti-mouse IgG secondary antibodies coupled to fluorescein. Coverslips were sequentially rinsed in immunofluorescence buffer, PBS, and distilled water and finally mounted in Mowiol 4.88 (Calbiochem-Behring GmbH). The cells were observed using a confocal microscope (Ultraphot; Carl Zeiss, Inc., Thornwood, NY) equipped with immunofluorescence optics and photographed using Tmax negatives (Eastman Kodak Co., Rochester, NY).

In some experiments, cells after microinjection of 219.6 mRNA were immunostained with mAb 219.6 IgGs purified from ascites and coupled to rhodamine (Brandtzaeg, 1973).

Quantiﬁcation of IgG expression was performed by counting all PC12 cells immunostained for IgG on a coverslip and comparing the number obtained with that of the cells microinjected on this coverslip as recorded by the computer.

Reverse Immunostaining

The presence of monoclonal IgGs expressed in microinjected PC12 cells and the state of occupancy of their antigen-binding sites were investigated by a double-immunostaining procedure which involved the use of anti-IgG antibody and the relevant antigen, referred to as “reverse immunostaining.” PC12 cells microinjected with either 219.6 or P5D4 mRNA were fixed, quenched, and permeabilized as described above. Coverslips were then incubated for 3 h in 100 μl of immunofluorescence buffer containing either 0.125 μg/ml of (a) partially purified Sgl (Sgl purified from PC12 cells through heat treatment and DEAE chromatography [Rosa et al., 1985b; Benedum et al., 1987]) conjugated to rhodamine according to the procedure of Brandtzaeg (1973); or (b) partially purified VSV-G (VSV solubilized in 10 μl of 1% Triton X-100 in PBS before 10-fold dilution with immunofluorescence buffer, equivalent to ~0.03 μg of G protein as determined by dot-blot assay). The heavy chain of the antibody and the relevant antigen, referred to as “reverse immunostaining” (Sälzer et al., 1989). The instrument was operated in the configuration of the confocal microscope used in the present study has been described in detail elsewhere (Stelzer et al., 1989). The instrument was operated in the epifluorescence mode. Fluorescein and rhodamine were excited with the blue (476.5 nm) and green (514.5 nm) lines, respectively, of an argon ion laser. A combination of the dichroic mirror from a K2 Ploemopak (E. Leitz,
Figure 1. Characterization of mAb 219.6 against SgI. For immunoblotting, the total homogenate of PC12 cells (\( \sim 100 \mu g \) of protein) and a chromaffin granule fraction of a human pheochromocytoma (h. PHEO, \( \sim 30 \mu g \) of protein) were immunolabeled with unconditioned cell culture medium (lanes 1 and 3) or cell culture medium conditioned by 219.6 hybridoma cells (lanes 2 and 4), followed by peroxidase. Arrowheads indicate SgI. The immunolabeled bands with faster electrophoretic mobilities than SgI correspond to known proteolytic fragments of SgI formed in secretory granules in vivo (Rosa et al., 1985b). Molecular mass standards are in kilodaltons. For immunoprecipitation: (lane 5, TH) Total homogenate (\( \sim 100 \mu g \) of protein) of \([^{3}H]\)tyrosine-labeled PC12 cells; (lane 6, 219.6) an immunoprecipitate from this homogenate using 219.6 hybridoma medium; and (lane 7, C) an immunoprecipitate from this homogenate using hybridoma medium containing a control IgG1 directed against \( \alpha \)-actinin of Dictyostelium discoideum. (Lane 8) Immunoprecipitation from \([^{3}H]\)tyrosine-labeled PC12 cells (\( \sim 80 \mu g \) of protein) using mAb 219.6 (15 \( \mu l \) of ascites) at the standard pH (pH 7.5) or (lane 9) at pH 6. (Lane 10, TH) Total homogenate (\( \sim 50 \mu g \) of protein) of PC12 cells labeled with \([^{3}H]\)tyrosine in the presence of monensin; (lane 11, 219.6) an immunoprecipitate from this homogenate using mAb 219.6 (15 \( \mu l \) of ascites). Samples were analyzed by SDS-PAGE in 7 or 10\% polyacrylamide gels. Fluorograms of the gels are shown. Arrowheads indicate SgI. Molecular mass standards are in kD.

Inc., Wetzlar, FRG) and an OG515 cut-off filter (Schott, Mainz, FRG) was used when observing the fluorescein signal; the dichroic mirror of an N2 Plomopak (E. Leitz, Inc.) and OG530 and OG550 cut-off filters (Schott) were used when observing rhodamine. The microscope was equipped with a 100\times/1.32 objective (NPL Fluotar; E. Leitz, Inc.). The detector was a photomultiplier (R1463-01; Hamamatsu Photonics K. K., Hamamatsu City, Japan). The pinhole in front of the detector had a size of 50 \( \mu m \) (1.22 \( \times \) \( \left(0.5 \mu m/1.32\right) \times 100 = 46 \mu m\)), which was optimal for the present configuration of the microscope. A single mirror mounted on a turnable galvanometer moved the light spot in the plane of the object. The movement along the optical axis was accomplished by moving the objective of the microscope up and down (Stetzer et al., 1989). Analog electronics were used to drive the scan system in either a horizontal x/y- or a vertical x/z-plane, while a computer (VME-based 68010 microcomputer; ELTEC, Mainz, FRG) monitored the intensity detected by the photomultiplier as a function of the three-dimensional position of the light spot in the object. The sample was illuminated only while intensities were recorded. For this purpose, an acousto-optical modulator driven by the movement of the galvanometer deflected the beam off the sample outside the detection window.

Sample Preparation. The same samples analyzed by double immunofluorescence in the conventional microscope were used for observation in the confocal microscope.

Double Immunofluorescence. The double immunofluorescence experiments were performed by running the laser (model 2020-05; Spectra-Physics, Darmstadt, FRG) in single line and switching back and forth between the 476.5- and the 514.5-nm lines. The cells to be analyzed had previously been selected by using a conventional microscope. The fluorescein signal was observed first. The laser line, the dichroic mirror, and the cut-off filter were then changed and the rhodamine signal was observed without further realignment of the microscope. In a previous set of experiments, PC12 cells double immunostained for the Golgi complex and secretory granules using rhodamine- and fluorescein-conjugated secondary antibody, respectively, had been used to choose the appropriate filters and to establish the conditions that gave optimal discrimination between these two distinct immunofluorescence patterns.

Optical Sections. Z series of x/y-images were recorded with intervals of 0.3 and 0.5 \( \mu m \) along the optical axis. These intervals conformed with the depth discrimination along the optical axis of \( \sim 0.4 \mu m \) full width, half maximum (Wijnaendts-van-Resandt et al., 1985; Stetzer et al., 1989) and guaranteed minimum overlap. Smaller intervals were avoided since they could increase the photoinduced damage to the object.

Images and Photographs. Only the high voltage supply of the photomultiplier could be varied. The gain of the amplifier as well as its offset were fixed. The high voltage supply of the photomultiplier was adjusted to the available amount of light and great care was taken that the analog-to-digital converter was operated in the correct range. As a consequence, the intensity covered a window of \( \sim 245 \) of the 256 available grey levels. All images were stored on the computer. Photographs of the images were taken from a black and white monitor on 50 ASA Ilford Pan F film (Ilford GmbH, Neu-Isenburg, FRG) (van Meer et al., 1987).
Information Content of an Image Obtained by the Confocal Scanning Laser Microscope. Every image recorded by the confocal microscope consisted of 512 × 512 pixels, each capable of storing 256 grey levels. The intensity was linear with the pixel value. The high voltage defining the gain of the photomultiplier had to be set carefully to avoid saturation of the amplifier. The lateral resolution in the configuration was better than 200 nm. A typical image with a field size of 3600 μm² consisted therefore of well-resolved, oversampled pixels. The discrimination against out-of-focus light, defined as the distance from the fluorescent at which the intensity dropped to 50% of its maximum value, was ~0.2 μm. Images recorded with a spacing of 0.5 μm along the optical axis showed almost no overlapping information. The pixel values represented an average of four accesses per pixel. No further image processing/filtering was performed. Photographs of images obtained with the confocal microscope lost quantitative information since it was not possible to push the 256 different grey levels into the ~40 grey tones on a photographic print.

Results

The following approach was taken to express an antibody directed against a secretory granule matrix protein in the secretory pathway of cells capable of forming secretory granules: (a) the generation of an mAb against Sgl, a secretory protein found in secretory granules of many endocrine cells and neurons; (b) the isolation of mRNA coding for the heavy and light chains of the anti-Sgl antibody from the hybridoma cells producing this antibody; and (c) the microinjection of this mRNA into PC12 cells, a neuroendocrine cell line that produces this antibody; and (c) the microinjection of mRNA into PC12 cells, a neuroendocrine cell line that produces Sgl and packages it into secretory granules.

Isolation and Characterization of an mAb against Sgl

Mouse mAbs were raised against rat Sgl, purified from PC12 cells by heat treatment, ion-exchange chromatography, two-dimensional PAGE (Rosa et al., 1985b; Benedum et al., 1987), and immobilized on nitrocellulose filters. Characterization of the hybridoma clones by immunoblotting of total PC12 cell protein revealed two mAbs that specifically recognized rat Sgl. One of these, referred to as mAb 219.6, recognized not only rat but also human Sgl, indicating conservation of the epitope (Fig. 1, lanes 2 and 4). This mAb recognized Sgl under a variety of conditions relevant for the subsequent experiments. First, mAb 219.6 specifically bound to nondenatured Sgl in mild immunoprecipitation conditions (Fig. 1, lane 6). Second, it immunoprecipitated not only the mature, posttranslationally modified form of Sgl, but also both Sgl from monensin-treated PC12 cells (which lack posttranslational modifications such as phosphorylation and sulfation; Lee and Huttner, 1985; Rosa, P., and W. B. Huttner, unpublished data) (Fig. 1, lane II) and Sgl produced by coupled in vitro transription–translation of the cloned Sgl cDNA (Benedum et al., 1987). These observations indicated that mAb 219.6 also recognized the pre-Golgi form of Sgl and that the epitope recognized contained no posttranslational modification of the amino acid residues. Third, immunoprecipitation of Sgl with mAb 219.6 was equally efficient at pH 7.5 and 6 (Fig. 1, lanes 8 and 9), indicating that the immunocomplex between Sgl and mAb 219.6 would not be impaired at the slightly acidic milieu of the trans-Golgi network (Anderson and Pathak, 1985), the site of secretory granule formation (Farquhar and Palade, 1981; Orci et al., 1984; Tooze and Tooze, 1986). Fourth, mAb 219.6 recognized rat Sgl after fixation of PC12 cells (not shown) and could therefore be used to investigate, by direct immunofluorescence, whether or not the epitope on Sgl was occupied by mAb 219.6 expressed in PC12 cells. Finally, mAb 219.6 was an IgG (G1 subclass,  light chain) rather than an IgM (data not shown). We considered this to be an advantage with respect to its assembly into a functional antibody in foreign, nonlymphoid cells and in terms of its size when used as probe in intracellular transport studies. In view of these properties, mAb 219.6 was chosen for expression in PC12 cells.

Expression and Intracellular Localization of Anti-Sgl and Control Antibodies in PC12 Cells

Poly(A)+ RNA enriched in mRNA coding for the heavy and light chains of mAb 219.6 was isolated from the bound polysome fraction of 219.6 hybridoma cells. For control mRNA, the poly(A)+ RNA coding for the heavy and light chains of mouse mAb P5D4, an antibody recognizing an epitope on the cytoplasmic domain of VSV-G (Kreis, 1986), was isolated from the corresponding hybridoma cells. As was expected, this mAb gave no detectable signal in PC12 cells by immunofluorescence (data not shown). Like mAb 219.6, mAb P5D4 belonged to the IgG1 subclass and therefore was an appropriate control antibody. Comparison of the 219.6 mRNA preparation (anti-Sgl IgG) and the P5D4 mRNA preparation (control, anti-VSV-G IgG) by in vitro translation of equal amounts of RNA indicated that the amount of antibody synthesized from the P5D4 mRNA was slightly greater than that synthesized from the 219.6 mRNA (data not shown). In the subsequent microinjection experiments with PC12 cells, in which the intracellular transport of anti-Sgl IgG and control IgG was investigated, the two mRNA preparations were used at identical RNA concentrations.

The 219.6 and P5D4 mRNAs were introduced into the cytoplasm of PC12 cells by automated, computer-assisted microinjection (Ansorge and Pepperkok, 1982; Pepperkok et al., 1988). At various time points after microinjection, cells were fixed, and the intracellular synthesis and transport of the anti-Sgl IgG and the control IgG were analyzed by immunofluorescence. To quantitate the expression and transport of the two antibodies, 150–600 cells were microinjected on each coverslip. The position of each injected cell was recorded by the computer, permitting their retrieval after the immunofluorescence procedure.

Anti-Sgl Antibody. 1.5 h after microinjection of 219.6 mRNA into PC12 cells, immunoreactivity for the anti-Sgl IgG was largely observed in the perinuclear area and colocalized with the Golgi complex, as revealed by double immunofluorescence using the anti-Golgi antibody of Louvard et al. (1982) (data not shown). This indicated that antibody expressed via mRNA injection entered the secretory pathway in PC12 cells.

Fig. 2 shows the intracellular localization of the anti-Sgl IgG 3, 6, and 18 h after microinjection of 219.6 mRNA compared with that of the endogenous Sgl, using double immunofluorescence. 3 h after microinjection, most of the immunoreactivity for the anti-Sgl IgG was detected as diffuse staining in the perinuclear area, indicating the localization of most of the anti-Sgl IgG in the Golgi complex (Fig. 2 a). In addition, a minor portion of the immunoreactivity for the anti-Sgl IgG was seen in structures with a dotted appearance. Some of these punctate structures were present in the periphery of the cell and were indistinguishable in position and appearance from the secretory granules detected by staining for Sgl (Fig. 2, a and a'). 6 h after microinjection, only a minor
Figure 2. Double immunofluorescence micrographs showing the accumulation of the anti-Sgl IgG in PC12 cells microinjected with 219.6 mRNA. PC12 cells were fixed 3 (a and a'), 6 (b and b'), and 18 h (c and c') after microinjection and double immunostained for the localization of anti-Sgl IgG (a'-c', α-Sgl IgG) and for the localization of Sgl (a-c, Sgl) using an anti-mouse IgG coupled to rhodamine and using an affinity-purified rabbit anti-rat Sgl antibody (15 µg/ml) followed by a secondary antibody conjugated to fluorescein, respectively. The asterisks indicate the microinjected cells, some of which are polynucleated. The immunostaining for the α-Sgl IgG 18 h after microinjection is virtually identical to that for Sgl. The immunostaining in the Golgi area is often masked by the abundant immunostaining of secretory granules (see Fig. 6 for immunoreactivity in the Golgi area after depletion of secretory granules). Bar, 10 µm.

portion of the immunoreactivity for the anti-Sgl IgG was found in the perinuclear region where most of it was associated with punctate peripheral structures. The pattern of this peripheral punctate immunoreactivity for anti-Sgl IgG was contained in the pattern of immunoreactivity for Sgl in injected cells and constituted a substantial portion of the latter (Fig. 2, b and b'). 18 h after microinjection, the patterns of immunoreactivity for the anti-Sgl IgG and Sgl itself were almost identical, the pattern for anti-Sgl being contained in the pattern for Sgl and constituting almost all of the latter (Fig. 2, c and c').

Control Antibody. Fig. 3 shows the intracellular localization of the control IgG (anti-VSV-G IgG) 3, 6, and 18 h after microinjection of P5D4 mRNA compared with that of the endogenous Sgl, using double immunofluorescence. At all times after microinjection, the patterns of immunoreactivity for the control IgG and Sgl were clearly distinct. 3 h after microinjection, immunoreactivity for the control IgG had a Golgi-like pattern (Fig. 3 a') like that observed for the anti-Sgl IgG (compare Fig. 2 a'). However, in contrast to the latter, immunoreactivity for the control IgG gave a Golgi-like pattern also 6 and 18 h after microinjection, the intensity be-
Double immunofluorescence micrographs showing the transient presence of the control antibody in PC12 cells microinjected with P5D4 mRNA. PC12 cells were fixed 3 (a and a'), 6 (b and b'), and 18 h (c and c') after microinjection and double immunostained for the localization of the control antibody (a'-c', α-VSV-G IgG) and for the localization of Sgl (α-c, Sgl) using an anti-mouse IgG conjugated to rhodamine and using an affinity-purified rabbit anti-rat Sgl antibody followed by a secondary antibody conjugated to fluorescein, respectively. The asterisks indicate the microinjected cells, some of which are polynucleated. The immunostaining for the α-VSV-G IgG is largely confined to the perinuclear region and is much less intense 18 h after microinjection. Note the difference in immunostaining between Sgl and α-VSV-G IgG at all times after microinjection. Bar, 10 μm.

Quantitation. A quantitative comparison of the expression and intracellular localization of the anti-Sgl IgG and the control IgG is shown in Table I. After microinjection of either 219,6 or P5D4 mRNA, about half of the injected PC12 cells transiently synthesized IgG, as indicated by the percentage of IgG-positive cells at 3 h. In the case of the anti-Sgl IgG, the percentage of IgG-positive cells remained at this level from 3 to 18 h. During this time, the immunoreactivity for the anti-Sgl IgG in the perinuclear area, observed in every IgG-positive cell at 3 h, decreased and concomitantly increased in peripheral punctate structures, which were stained in every IgG-positive cell at 18 h. In contrast, in the case of the control IgG, the percentage of IgG-positive cells decreased from 3 to 18 h to about one third of the original value. Here,
from Microinjected PC12 Cells

Whereas the Anti-Sgl antibody was stored in cellular structures that appeared to be secretory granules.

**Table 1. Quantitation of the Expression of Anti-Sgl IgG and Control IgG in Microinjected PC12 Cells and Analysis of their Intracellular Localization**

| IgG             | Time | IgG-positive microinjected cells | Diffuse perinuclear immunostaining | Dotted peripheral immunostaining |
|-----------------|------|----------------------------------|-----------------------------------|---------------------------------|
|                 | h    | %                                | %                                 | %                               |
| α-Sgl IgG       | 3    | 55                               | 55                                | ++                              |
| α-VSV-G IgG     | 3    | 56                               | 56                                | ++                              |
| α-Sgl IgG       | 6    | 53                               | 45                                | ++                              |
| α-VSV-G IgG     | 6    | 40                               | 40                                | ++                              |
| α-Sgl IgG       | 18   | 50 (3)                           | (3)                               | +                               |
| α-VSV-G IgG     | 18   | 18                               | 18                                | +                               |

Intracellular localization of IgG

PC12 cells were fixed 3, 6, and 18 h after micr0injection of either 219.6 or PSD4 mRNA and double immunostained as described in the legends to Figs. 2 and 3. IgG-positive cells—i.e., cells immunostained for either anti-Sgl IgG (α-Sgl IgG) or control IgG (α-VSV-G IgG)—are expressed as percent of the total cells microinjected, as recorded by the computer. IgG-positive cells with diffuse perinuclear immunostaining and dotted peripheral immunostaining are expressed as percent of the total cells microinjected. The immunostaining is graded by its intensity as well as by the density of labeled organelles. Note that the strong dotted peripheral immunostaining for the anti-Sgl IgG observed 18 h after microinjection may have masked some of the diffuse perinuclear immunostaining, particularly in small cells. Since this may have resulted in an underestimation of the cells showing the latter immunostaining, the corresponding number is given in parenthesis.

**Colocalization of Sgl and Anti-Sgl IgG by Double Immunofluorescence Using the Confocal Microscope**

The apparent identity of the immunofluorescence pattern of endogenous Sgl and that of the expressed anti-Sgl IgG seen 18 h after mRNA injection was further investigated using the confocal microscope (European Molecular Biology Laboratory, Heidelberg, FRG). This allowed the optical sectioning of cells with a section thickness (z-axis) of ~0.4 μm and a resolution (x/y-plane) of ~0.17 μm, which is in the range of the diameter of secretory granules of PC12 cells (Greene and Tischler, 1976). Fig. 4 shows the optical sectioning of a double-immunostained microinjected PC12 cell at five different focal levels. The pattern of secretory granules, as revealed by immunostaining for Sgl, was distinct at each of the five focal levels. At each focal level, the pattern for the expressed anti-Sgl IgG was indistinguishable from that of endogenous Sgl, indicating colocalization of antibody and antigen; i.e., the presence of anti-Sgl IgG in secretory granules.

**The Control Antibody Is Constitutively Secreted from Microinjected PC12 Cells Whereas the Anti-Sgl Antibody Is Stored Intracellularly**

The finding that the intracellular immunoreactivity for the control IgG after microinjection of P5D4 mRNA was transient (Fig. 3) suggested, but did not prove, that this antibody was secreted via the constitutive pathway. Likewise, the massive accumulation of anti-Sgl IgG in secretory granules after microinjection of 219.6 mRNA (Fig. 2) was consistent with the assumption that most of the newly synthesized anti-Sgl IgG was packaged into these storage organelles. To investigate this biochemically, PC12 cells microinjected with either P5D4 or 219.6 mRNA were pulse labeled for 1 h with [35S]methionine and then chased for 4 h in the presence of unlabeled methionine (Fig. 5). Labeled IgG comigrating with unlabeled IgG marker on nonreducing SDS-PAGE was detected after microinjection of either mRNA. After injection of 219.6 mRNA, the synthesized anti-Sgl IgG was only detectable in the cells and not in the medium, whereas after injection of the P5D4 mRNA, the synthesized control IgG was found not only in the cells but also in the medium (Fig. 5a). However, the ratio of IgG in the medium and cells could not be reliably quantitated from these gels because the IgG region of the cell samples also contained other proteins that unspecifically absorbed to the anti-IgG-agarose used for immunoprecipitation (Fig. 5a, compare lanes 219.6 and P5D4 with lane n. i.). Therefore, the IgG region of the cell and medium samples was reelectrophoresed under reducing conditions (Fig. 5b, fluorograms) and the amount of labeled heavy chain of either anti-Sgl IgG (219.6) or control IgG (P5D4) found in the cells and the medium was quantitated (Fig. 5b, graphs). This indicated that 91% of the pulse-labeled control IgG synthesized after injection of P5D4 mRNA was found in the medium after the 4-h chase, showing that the control antibody was indeed constitutively secreted from PC12 cells (Fig. 5b, bottom graphs). In contrast, ~95% of the pulse-labeled anti-Sgl IgG expressed after microinjection of 219.6 mRNA into PC12 cells was stored intracellularly since it was found in the cells after a 4-h chase period (Fig. 5b, top graphs), a time sufficient for the transport of anti-Sgl IgG into secretory granules (cf. Fig. 2). This high efficiency of packaging of the anti-Sgl IgG into secretory granules is consistent with the observation that <90% of pulse-labeled Sgl is stored intracellularly during this time (Tooze, S., and W. B. Huttner, unpublished data).

As a side observation, we noticed that in the case of anti-Sgl IgG, the amount of heavy and light chain recovered after reducing SDS-PAGE was less than expected from the inten-
Figure 4. Double immunofluorescence micrographs, obtained with the confocal microscope (European Molecular Biology Laboratory, Heidelberg, FRG), showing the colocalization of anti-Sgl IgG and Sgl in secretory granules of a PC12 cell microinjected with 219.6 mRNA. PC12 cells were fixed 18 h after microinjection and double immunostained for the anti-Sgl IgG (a'-e', α-Sgl IgG, rhodamine) and for Sgl (a-e, Sgl, fluorescein) as described in Fig. 2. The microinjected cell was scanned in the z-axis at 0.5-μm steps. The pattern of immunostaining is shown at five different focal levels (a and a', 3.5 μm; b and b', 2 μm; c and c', 1.5 μm; d and d', 0.5 μm; e and e', 0 μm = level of coverslip). Overlapping of immunoreactivity for Sgl and anti-Sgl IgG is clearly visible. Note the noninjected cells, labeled only with fluorescein, in the left part of a-e. Bar, 10 μm.
Regulated Secretion of Anti-Sgl IgG Induced by α-Latrotoxin

A diagnostic feature that a secretory protein has been packaged into secretory granules is its exocytosis in response to a secretagogue. Regulated secretion of Sgl from PC12 cells can be achieved in a calcium-dependent manner by depolarization. However, with this (or any other) physiological form of stimulation of secretion, only a minor fraction of the total stored Sgl is released from PC12 cells (Rosa et al., 1985b), as is the case with other regulated secretory proteins (Schweitzer and Kelly, 1985). To investigate whether the majority of the stored anti-Sgl IgG was subject to regulated secretion, we chose as a secretagogue the black widow spider venom α-latrotoxin, which has been shown to induce massive exocytosis of secretory granules in PC12 cells (Melodelsi et al., 1983; Watanabe et al., 1983). The effect of α-latrotoxin on anti-Sgl IgG release from PC12 cells was studied 18 h after microinjection of 219.6 mRNA by immunofluorescence. Fig. 6 shows a representative set of PC12 cells immunostained for the anti-Sgl IgG 18 h after microinjection of 219.6 mRNA. Compared to control cells (Fig. 6, a–d), cells exposed to 3 nM α-latrotoxin for 9 min (Fig. 6, e–h) showed a drastic reduction in the peripheral punctate staining for the anti-Sgl IgG, consistent with the exocytotic release of the anti-Sgl IgG from secretory granules. Interestingly, the perinuclear immunoreactivity for the anti-Sgl IgG, which was seen in a small proportion of PC12 cells 18 h after microinjection (cf. Table I), was not significantly reduced after exposure to α-latrotoxin (Fig. 6f), suggesting that anti-Sgl IgG still present in the Golgi complex was not affected by the secretagogue.

Specificity of the Subcellular Localization of the Anti-Sgl IgG

The specificity of the localization of the anti-Sgl IgG in secretory granules of microinjected PC12 cells was further investigated by comparison with another secretory granule marker and with markers for other post-Golgi organelles. As another secretory granule marker, we studied SgII, another matrix protein of many endocrine and neuronal secretory granules (Rosa et al., 1985a,b; Cozzi et al., 1989) that is co-processed with Sgl in secretory granules of PC12 cells (Griiliths, G., P. Rosa, and W. B. Huttner, unpublished observations). As markers for other post-Golgi organelles, we studied Synaptophysin (p36, p38), an integral membrane protein characteristic of small synaptic vesicles in the nervous system and of related organelles in endocrine cells (Huttner et al., 1983; Jahn et al., 1985; Wiedenmann and Franke, 1985; Navone et al., 1986), and Igpl20, a lysosomal and prelysosomal membrane protein (Lewis et al., 1985; Griffiths et al., 1988). Double immunofluorescence 18 h after microinjection of 219.6 mRNA revealed that the intracellular distribution of the anti-Sgl IgG and SgII were largely identical (Fig. 7, a and

Figure 5. Pulse–chase experiment showing that the control IgG is constitutively secreted from microinjected PC12 cells whereas the anti-Sgl IgG is stored intracellularly. PC12 cells microinjected with mRNA coding for the control antibody (P5D4) or the anti-Sgl antibody (219.6) as well as noninjected PC12 cells (n.i.) were labeled for 1 h with [35S]methionine and then chased for 4 h in the presence of unlabeled methionine. At the end of the chase, IgG was immunoprecipitated from the cells and the medium and subjected to nonreducing SDS-PAGE (6% polyacrylamide). The IgG region of the fluorogram (3.5-d exposure) is shown in a. The position of labeled IgG (arrowheads) was identified by comparison with unlabeled IgG which was electrophoresed in adjacent lanes and detected by Coomassie blue staining. Note both the IgG band in cells injected with 219.6 and P5D4 mRNA, but not in noninjected cells, and the presence of significant amounts of IgG in the medium only after expression of the control antibody (P5D4), but not the anti-Sgl antibody. (The background in the immunoprecipitates of the three cell samples is probably due to both the low amount of expressed IgG relative to the endogenous cell proteins and to the fact that only 1,000 out of 3,000 PC12 cells were microinjected, only 50% of which expressed IgG [Table I].) The dot indicates a cellular protein unspecifically precipitated and present in equal amounts in all three cell samples. For quantitation of the amount of the control IgG and anti-Sgl IgG found in the cells and medium at the end of the chase, the IgG band was reelectrophoresed under reducing conditions (12% polyacrylamide) followed by fluorography for various times (15–31 d), shown for cellular and secreted P5D4 IgG in b on the left and right, respectively. The positions of the heavy (H) and light (L) chain are indicated. Appropriate fluorograms were used for densitometric scanning of the heavy chain region of the cell and medium samples. For either the anti-Sgl antibody (219.6, top graphs) or the control antibody (P5D4, bottom graphs), each of the curves shown was constructed from 200 absorbance values determined over a length of 8 mm (see abscissa); each of the 200 values per curve is expressed as a percent of the total (sum of the 200 absorbance values of heavy chain in the cells plus the 200 absorbance values of heavy chain in the medium). The absolute amount of total 219.6 heavy chain recovered after reducing SDS-PAGE was less than that of P5D4 heavy chain, the 219.6 heavy chain in the medium being at the limit of detection (see text).
Figure 6. Immunofluorescence micrographs showing the effect of α-latrotoxin on the release of anti-Sgl IgG from PC12 cells microinjected with 219.6 mRNA. 18 h after microinjection of 219.6 mRNA, PC12 cells were incubated for 9 min in release medium without (a–d, control) or with (e–h, α-Latrotoxin) 3 nM α-latrotoxin. Cells were then fixed and immunostained with anti-mouse IgG conjugated to rhodamine. For each pair of control and α-latrotoxin–treated cells (a and e, b and f, c and g, d and h), the negatives were taken with the same exposure and the micrographs were printed under identical conditions. A consistent reduction in intracellular anti-Sgl IgG immunoreactivity can be observed after stimulation of exocytosis by α-latrotoxin (compare a–d with e–h). Bar, 10 μm.

α’), suggesting that the secretory granules containing the anti-Sgl IgG also contained SglII. In contrast, the intracellular distribution of the anti-Sgl IgG was clearly distinct from that of p38/synaptophysin which was predominantly seen in a perinuclear location (Fig. 7, b and b’). The pattern of immunoreactivity for the anti-Sgl IgG was also different from that for lgp120 (Fig. 7, c and d). These results indicated that the packaging of the anti-Sgl IgG into secretory granules was specific with respect to other post-Golgi organelles.

Anti-Sgl IgG Forms an Immunocomplex with Sgl

The transport of the anti-Sgl IgG, but not of the control antibody, with Sgl into secretory granules strongly suggested the formation of an immunocomplex between the anti-Sgl IgG and Sgl. To directly demonstrate this, PC12 cells microinjected with mRNA were subjected to reverse immunostaining; i.e., the detection of expressed functional antibody by labeling the fixed and permeabilized cells with exogenous antigen (Fig. 8). PC12 cells microinjected with 219.6 mRNA, coding for anti-Sgl IgG, were incubated with partially purified rat Sgl conjugated to rhodamine to investigate whether any of the expressed anti-Sgl IgG had unoccupied antigen-binding sites. As a positive control, PC12 cells microinjected with P5D4 mRNA, coding for the control anti-VSV-G IgG, were incubated with partially purified VSV-G which was subsequently detected by a polyclonal antibody against VSV-G (Matlin et al., 1982) followed by rhodamine-conjugated secondary antibody. For both PC12 cells microinjected with 219.6 or P5D4 mRNA, the reverse immunostaining obtained with exogenous antigen was compared, using double fluorescence, to the normal immunostaining of the expressed antibody obtained with fluorescein-conjugated antibody against mouse IgG. Reverse immunostaining with exogenous VSV-G as antigen showed the presence of expressed functional control antibody in the Golgi area 3 and 6 h after microinjection of PC12 cells; the pattern was identical to that observed with normal immunostaining (Fig. 8, compare c’ and d’ with c and d). This indicated that the antigen-binding sites of the expressed control antibody were unoccupied, as expected. In contrast, no free antigen-binding sites could be detected on the expressed anti-Sgl IgG since reverse immunostaining with exogenous Sgl as antigen 3 and 18 h after microinjection gave no signal over the Golgi area and secretory granules, respectively, where the expressed anti-Sgl IgG was
Figure 7. Immunofluorescence micrographs showing the intracellular localization of the anti-Sgl IgG in PC12 cells 18 h after microinjection of 2196 mRNA compared with that of SglII, p38/synaptophysin, and lgp120. PC12 cells were immunostained for the localization of the anti-Sgl IgG (a', b', and d, α-Sgl IgG), SglII (a), p38/synaptophysin (b, Syn), and lgp120 (c) using an anti–mouse IgG coupled to rhodamine, an affinity-purified rabbit anti-rat SglII antibody (15 µg/ml), a mouse monoclonal anti-rat p38/synaptophysin antibody (30 µg/ml), and a rabbit anti-rat lgp120 antiserum (1:500 dilution), respectively, followed by appropriate secondary antibodies conjugated to fluorescein (a and b) or rhodamine (c). a and a' and b and b' are double-immunofluorescence micrographs; c and d are different coverslips. The asterisks indicate the microinjected cells, some of which are polynucleated. The intracellular localization of the anti-Sgl antibody is very similar to that of SglII (a and a') but quite distinct from the largely perinuclear localization of p38/synaptophysin (b and b') and lgp120 (c and d). Bar, 10 µm.
Figure 8. Reverse immunostaining showing the presence of an immunocomplex between SgI and the anti-SgI antibody in PC12 cells after microinjection of 219.6 mRNA. PC12 cells were fixed 3 (a and a') and 18 h (b and b') after microinjection of 219.6 mRNA (PC12 expressing α-SgI IgG) and double immunostained using partially purified SgI conjugated to rhodamine (a' and b') to search for free antigen-binding sites on the expressed anti-SgI IgG (reverse immunostaining), and using an anti–mouse IgG antibody conjugated to fluorescein (a and b) to detect the expressed anti-SgI IgG. As a control, 219.6 hybridoma cells (e and f, 219.6 Hy, two different coverslips) were immunostained using the partially purified SgI conjugated to rhodamine (f) or an anti–mouse IgG antibody conjugated to rhodamine (e). PC12 cells were fixed 3 (c and c') and 6 h (d and d') after microinjection of P5D4 mRNA (PC12 expressing α-VSV-G IgG) and double immunostained using partially purified VSV-G followed by polyclonal rabbit anti-VSV-G antibody and a secondary antibody conjugated to rhodamine (c' and d') to demonstrate the presence of free antigen-binding sites on the expressed control antibody (reverse immunostaining) and using an anti–mouse IgG antibody coupled to fluorescein (c and d) to detect the expressed control antibody. As a control, P5D4 hybridoma cells (g and h, P5D4 Hy, two different coverslips) were immunolabeled using the partially purified VSV-G followed by the polyclonal anti-VSV-G antibody and rhodamine-conjugated secondary antibody (h) or an anti–mouse IgG antibody conjugated to rhodamine (g). Note the absence and presence of free antigen-binding sites on the expressed anti-SgI IgG (a' and b') and control antibody (c' and d'), respectively. The pattern of immunostaining for the free antigen-binding sites on the expressed control antibody (c' and d') overlaps with that for the expressed antibody (c and d). Bar, 10 μm.
Figure 9. Double-immunofluorescence micrographs showing the effect of ammonium chloride on the intracellular accumulation of anti-SgI IgG in NGF-differentiated PC12 cells microinjected with 2196 mRNA. NGF-differentiated PC12 cells microinjected with 2196 mRNA were incubated for 18 h without (a, a', and a", Control) or with (b, b', and b", Ammonium chloride) 10 mM NH₄Cl. Cells were fixed and double immunostained for the localization of anti-SgI IgG (a" and b", α-SgI IgG) and of SgI (a' and b') using an anti-mouse IgG conjugated to rhodamine and using an affinity-purified rabbit anti-rat SgI antibody followed by a secondary antibody conjugated to fluorescein, respectively. a and b show Nomarski photographs of the corresponding fields. The asterisks indicate the microinjected cells, some of which are polynucleated. In control cells, dotted immunostaining for both SgI and anti-SgI IgG is observed in the perikaryon, along the cell processes, and in the tips of the processes (a, a', a", arrowheads). In ammonium chloride–treated cells, dotted immunostaining in the perikaryon and cell processes is observed only for SgI, whereas the immunostaining for the anti-SgI IgG is more diffuse and perinuclear, and little, if any, anti-SgI IgG appears to accumulate in the periphery of the perikaryon and in the tips of the cell processes (b, b', b", arrowheads). Bar, 10 μm.
localized as revealed by normal immunostaining (Fig. 8, compare a' and b' with a and b).

As another positive control, 2196 hybridoma cells, which express anti-SgI IgG but not SgI, were subjected to reverse immunostaining with exogenous SgI as antigen. This demonstrated that reverse immunostaining with exogenous SgI allowed the detection of intracellular anti-SgI IgG with unoccupied antigen-binding sites (Fig. 8 f). The reverse immunostaining of 2196 hybridoma cells was similar in pattern and intensity to the reverse immunostaining obtained with exogenous VSV-G as antigen in P5D4 hybridoma cells, which express anti-VSV-G IgG (Fig. 8 h).

The lack of free antigen-binding sites on the anti-SgI IgG expressed in PC12 cells was, therefore, most probably due to the occupancy of these sites by endogenous SgI. This suggested that the endogenous SgI was produced in excess over the expressed anti-SgI IgG. This conclusion was supported by two observations (data not shown). First, mAb 2196 conjugated to rhodamine was used in immunofluorescence of PC12 cells microinjected with 2196 mRNA to label the endogenous SgI via the same epitope that was the target for the expressed anti-SgI IgG. This showed only a small and variable decrease in the rhodamine fluorescence in microinjected cells compared with noninjected cells, suggesting that the amount of expressed anti-SgI IgG was only sufficient to bind to a portion of the newly synthesized SgI. Second, the amount of radioactive SgI and radioactive IgG synthesized during a 1-h pulse with 14C]methionine was quantitated. This, together with the fact that one third of the cells were microinjected, ~50% of which expressed IgG (Table I), allowed us to estimate that labeled SgI exceeded labeled IgG at least fivefold in microinjected, antibody-expressing PC12 cells.

**Packaging of Anti-SgI IgG Is Blocked by Ammonium Chloride**

Drugs that neutralize the acidic pH of intracellular organelles, such as ammonium chloride and chloroquine, are thought to inhibit the packaging of regulated secretory proteins into secretory granules and to divert them to the constitutive pathway of secretion in AFT-20 cells (Moore et al., 1983). Consistent with this notion is the observation that in PC12 cells treated with ammonium chloride, a substantial portion of the newly synthesized SgI and SgII is secreted without stimulation of exocytosis (Touze, S., P. Rosa, P. Baeuerle, and W. B. Huttner, unpublished data). It was therefore of interest to investigate whether the packaging of the anti-SgI IgG was affected when PC12 cells microinjected with 2196 mRNA were treated with ammonium chloride.

For these experiments, PC12 cells were differentiated with NGF and microinjected with 2196 mRNA to label the endogenous SgI (Fig. 9 a'). As in undifferentiated PC12 cells, this punctate pattern overlapped with a major portion of the pattern of secretory granules immunostained for the endogenous SgI (Fig. 9 a'). When NGF-differentiated PC12 cells were incubated for 18 h after microinjection of 2196 mRNA in the presence of ammonium chloride, the perinuclear staining for the anti-SgI IgG was still observed, whereas the punctate staining for the anti-SgI IgG at the periphery of the perikaryon and in the tips of the cell processes was hardly detectable (Fig. 9 b'). Ammonium chloride treatment also partly reduced the punctate immunostaining for the endogenous SgI at the periphery of the perikaryon and in the tips of the cell processes, but not the perinuclear staining in the Golgi area (Fig. 9 b'). These results were consistent with the interpretation that (a) in the presence of ammonium chloride, a major portion of the newly synthesized SgI, and the anti-SgI IgG bound to it, were not packaged into secretory granules but diverted to transport vesicles of the constitutive secretory pathway that were not detected under the present experimental conditions; and (b) the SgI-immunoreactive structures seen in the tips of the cell processes after ammonium chloride treatment represented secretory granules that had been formed before the drug treatment.

**Discussion**

*Rerouting of a Constitutive Secretory Protein to Secretory Granules by Protein–Protein Interaction with a Regulated Secretory Protein*

We have shown that a constitutive secretory protein can be diverted to the regulated pathway of secretion by interacting noncovalently with a regulated secretory protein. This conclusion is supported by several lines of evidence. First, the constitutive secretory protein chosen, a monoclonal anti-SgI antibody, was specifically packaged into secretory granules, as indicated by (a) its colocalization with SgI and SgII, two established secretory granule markers (Rosa et al., 1985a, b), in double immunofluorescence using the conventional as well as the confocal microscope; (b) its virtually complete intracellular storage in pulse–chase experiments; (c) its release from PC12 cells after stimulation of exocytosis of secretory granules by α-latrotoxin; and (d) its absence from other post-Golgi organelles such as prelysosomes, lysosomes, and small synaptic vesicles. Second, the packaging of this constitutive secretory protein into secretory granules was the consequence of its noncovalent interaction with a regulated secretory protein via the formation of an immunocomplex, since (a) a control antibody of the same IgG subclass was not packaged into secretory granules but was secreted constitutively; (b) the antigen-binding sites on the anti-SgI antibody were occupied by endogenous antigen; and (c) diversion of the antigen, SgI, to the constitutive pathway by ammonium chloride treatment of cells also prevented packaging of the anti-SgI antibody into secretory granules.

These results provide direct evidence that the targeting information contained in a regulated secretory protein can dominate over the post-Golgi secretory route usually taken by a constitutive secretory protein. Our observations are consistent with the results of Moore and Kelly (1986), who studied the intracellular transport of a chimeric fusion protein composed of a polypeptide hormone and the ectodomain of a viral membrane protein, and extend their conclusion to an intact constitutive secretory protein unaltered by genetic engineering. Moreover, as far as we are aware, our data provide the first evidence for the rerouting of a newly synthesized protein by a noncovalent protein–protein interaction.
Implications for the Sorting of Regulated Secretory Proteins

At least three mechanisms can be envisaged to explain the sorting of regulated secretory proteins from constitutive secretory proteins, their aggregation, and their packaging into secretory granules (Fig. 10; Burgess and Kelly, 1987; Pfeffer and Rothman, 1987). In the first mechanism (Fig. 10, left), it is assumed that regulated secretory proteins contain sorting signals in the form of specific linear sequences or conformational epitopes and are sorted from constitutive secretory proteins by a receptor-mediated mechanism to a compartment where they aggregate (e.g., a specific region of the trans Golgi network or condensing vacuole) followed by their packaging into secretory granules. In the second mechanism (Fig. 10, middle), it is assumed that regulated secretory proteins segregate from constitutive secretory proteins in a receptor-independent process by their intrinsic ability to form aggregates, either with themselves or with other regulated secretory proteins, which are then packaged into secretory granules by a mechanism involving the recognition of some feature of the aggregate by a receptor (Huttner et al., 1988). In the third mechanism (Fig. 10, right), which combines features of the first two mechanisms, it is assumed that a portion of the regulated secretory proteins specifically interact with a receptor, which triggers the formation of an aggregate of regulated secretory proteins that excludes constitutive secretory proteins, followed by packaging into secretory granules.

The implications of the present data for these possible sorting and aggregation mechanisms are illustrated in Fig. 10. If Sgl is sorted by the first mechanism, the epitope on Sgl occupied by mAb 219.6 cannot be the sorting signal recognized by the receptor (Fig. 10, left). However, such a sorting signal could be identified in Sgl, or any other regulated secretory protein, by searching for an mAb that, when expressed in cells, interferes with the sorting. This immunological approach to studying secretory protein sorting could complement the genetic engineering approach. If Sgl is sorted by the second mechanism, the 219.6 epitope may or may not be involved in the recognition of the aggregate by the receptor since (a) the aggregate contains more potential receptor-binding sites than there are receptors; and since (b) the amount of antibody expressed in the present conditions was at most 20% of that of newly synthesized Sgl and thus not sufficient to occupy the 219.6 epitope of all newly synthesized Sgl molecules (Fig. 10, middle). Such a binding site could be identified in Sgl, or any other regulated secretory protein, using the approach of antibody expression, provided...
that the antibody under study is expressed in excess of the newly synthesized regulated secretory protein. Interestingly, overexpression of an anti-SgI IgG could also be a means of investigating the biological significance of the aggregation step since we find it unlikely that aggregation of SgI would proceed normally if all newly synthesized SgI molecules were complexed to an antibody. If SgI is sorted by the third mechanism, the 2196 epitope again may or may not be involved in the interaction with the receptor, since under the present conditions a sufficient number of newly synthesized SgI molecules uncomplexed to anti-SgI IgG were available to bind to the receptor and to initiate the aggregation (Fig. 10, right).

Implications for Functional Studies on Secretory Granules

The finding that antibodies can be diverted into secretory granules opens new ways of studying secretory granule functions. Antibodies have proven to be powerful tools for studying cell–cell interactions, as well as intracellular function, when applied to the outside of cells (e.g., Müller and Gerisch, 1978) or microinjected into the cytoplasm (e.g., Mabuchi and Okuno, 1977). Given the results of the present study and using more efficient expression systems than microinjection of hybridoma mRNA, it should in principle be possible, by using expressed antibodies as tools, to study the function of any epitope exposed on the luminal side of secretory granules, be it on a granule matrix or membrane protein. For example, the organization of the granule matrix, the processing of prohormones, and the role of ion pumps and other transmembrane transport systems could be investigated by this immunological approach. Moreover, antibodies that block the transport of specific proteins into secretory granules could be used to generate granules selectively depleted of these proteins. Finally, one may think of expressing chimeric antibodies to cross-link secretory proteins and study the functional consequences.

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