

# Intracellular Routing of GLcNAc-bearing Molecules in Thyrocytes: Selective Recycling through the Golgi Apparatus

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**Abstract.** Previous experiments led us to speculate that thyrocytes contain a recycling system for GlcNAc-bearing immature thyroglobulin molecules which prevents these molecules from lysosomal degradation (Miquelis, R., C. Alquier, and M. Monsigny. 1987. *J. Biol. Chem.* 262:15291–15298). To confirm this hypothesis, the fate of GlcNAc-bearing proteins after internalization by thyrocytes was monitored and compared to that of fluid phase markers.

Kinetic internalization studies were performed using  $^{125}\text{I}$ -GlcNAc-BSA and  $^{131}\text{I}$ -Man-BSA. We observed that the apparent intake rate as well as the amount of hydrolyzed GlcNAc-BSA are smaller than the corresponding values for Man-BSA. These differences were reduced by GlcNAc competitors (thyroglobulin and ovomucoid) or a weak base (chloroquine). Part of the internalized GlcNAc-BSA was released into the extracellular milieu at a higher rate and shorter half life ( $t_{1/2} = \sim 30$  min) than the Man-BSA ( $t_{1/2} = \sim 8$  h).

Subcellular homing was first studied by cell fractionation after internalization using  $^{125}\text{I}$ -ovomucoid and

$^{131}\text{I}$ -BSA. During Percoll density gradient fractionation, endogenous thyroperoxidase was used to separate subsets of organelles involved in the biosynthetic exocytotic pathway. Incubation of the cell homogenate in the presence of DAB and  $\text{H}_2\text{O}_2$  before cell fractionation give rise to a shift in the density of organelles containing 3.5 times more ovomucoid than BSA. Discontinuous sucrose gradient showed that: (a) thyroperoxidase was colocalized with galactosyltransferase-containing organelles in Golgi-rich subfractions; and (b) that at every time studied from 10 to 100 min, the ovomucoid/BSA ratio was higher in these organelles than in other subfractions. Finally we also observed that: (a) ovomucoid sequestered in the Golgi-rich subfraction incorporated  $^3\text{H}$ galactose; and (b) that part of internalized ovomucoid was localized on the Golgi stacks as well as elements of the *trans*-Golgi, as revealed by immunogold labeling on ultrathin cryosections. These data prove that in thyrocytes GlcNAc accessible sugar moieties on soluble internalized molecules are sufficient to trigger their recycling via the Golgi apparatus.

**T**HYROID follicles are lined by a single layer of thyrocytes which synthesize and release thyroglobulin, a tissue-specific prohormone glycoprotein, into the follicular lumen. Thyroglobulin in follicular lumens, referred to as colloid, can reach concentration up to 200 mg/ml. During and after exocytosis, thyroglobulin molecules undergo posttranslational modifications involving iodination and assembly of certain tyrosyl residues into thyroid hormones (Nunez, 1980; Bjorkman and Ekholm, 1990). Iodination and hormonogenesis which takes place at the interface between the apical membrane and the colloid are catalyzed by thyroperoxidase (Ekholm and Wollman, 1975). Hormonal secretion involves internalization and transport of thyroglobulin to lysosomes for cleavage of the hormones from protein backbone and release into the

venous flow (Vandenhove-Vandenbrouke, 1980; Bjorkman and Ekholm, 1990).

Since thyroglobulin molecules in follicular lumens show a high degree of heterogeneity in terms of iodine and hormone content, it can be assumed that there is a mechanism that either preferentially targets iodine-rich molecules to lysosomes or prevents catabolism of iodine-poor molecules. In vitro studies of cultured thyrocytes demonstrated that mature iodine-rich molecules disappear from the incubation medium faster than immature molecules (Vandenbrouke et al., 1976; Cortese et al., 1976). In vivo kinetic studies and subcellular fractionation show that all thyroglobulin molecules are internalized, but a subset, characterized by a low turnover rate, is not delivered to lysosomes but rather recycled back to the follicular lumen (Simon et al., 1979; Bastiani et al., 1980; Miquelis and Simon, 1980). This observation suggests that thyrocytes may contain a retention system for immature thyroglobulin molecules.

Up to now despite numerous studies (Wollman, 1989;

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Kostrouch et al., 1991; Lemansky and Herzog, 1992), no receptor specific for mature thyroglobulin has been found to account for selective internalization. Conversely, a specific signal has been found for iodine-poor molecules which are characterized by the presence of exposed N-acetylglucosamine residues. These GlcNAc-bearing thyroglobulin molecules interact with a membrane component of thyrocytes (Consiglio et al., 1981; Miquelis et al., 1987). We have identified and characterized this component as an endogenous lectin strictly selective for GlcNAc and having the novel ability to recognize ligands at acid pH and release them at neutral pH. Based on these observations we speculated that the GlcNAc receptor is for intracellular selection of mature and immature molecules since it can recognize and bind immature thyroglobulin molecules in acid pH compartments such as endosomes and release them in adjoining neutral pH compartments such as Golgi and/or follicular lumens. We have named this selective process "receptor-mediated exocytosis" (Miquelis et al., 1987).

In the context of a study of this selective process, we have shown that the functional location of the GlcNAc receptor in healthy animal and human thyroids was, as expected for a thyroglobulin conveyor, mainly apical and subapical, facing the lumen (Miquelis et al., 1987; Alquier et al., 1988; Thibault et al., 1993). The aim of the present study was: (a) to establish if accessible GlcNAc sugar residues are sufficient to trigger a selective recycling of internalized GlcNAc-bearing molecules; (b) to give an estimation of the kinetic parameters of this recycling; and (c) to determine what intracellular compartments are involved.

## Materials and Methods

### Reagents

BSA, ovomucoid, type IV collagenase, CNBr-activated Sepharose 4B, protein A-Sepharose CL-4B, and protease inhibitors were purchased from Sigma Immunochemicals (St. Louis, MO). Triton X-100 was from Boehringer (Mannheim, GmbH); iodogen was from Pierce (Rockford, IL) and mannose-, galactose-, glucose-, fucose-, and GlcNAc- $\beta$ -O-(2-carbomethoxyethylthio/ethyl) derivatives of BSA were from Sockerbolaget (Arlow, Sweden).  $^{125}\text{I}$ -labeled Na,  $^{131}\text{I}$ -labeled Na, [ $^3\text{H}$ ]galactose (35–39 Ci/mmol) and UDP-[ $^{14}\text{C}$ ]galactose (297 mCi/mmol) were from Amersham (Les Ulis, France). All reagents were of the best commercially available grade.

### Radiolabeling

GlcNAc-BSA neoglycoprotein (100  $\mu\text{g}$ ) and Man-BSA neoglycoprotein (100  $\mu\text{g}$ ) were iodinated with  $^{125}\text{I}$ -labeled-Na using iodogen (Salacinski et al., 1981). The iodination mixtures were chromatographed on PD10-Sephadex columns (Pharmacia Fine Chemical Co., Piscataway, NJ) and the radioactive material appearing in the void volume was pooled. The yield of neoglycoprotein was 70% with a specific activity of 4.5 to 5.0  $\mu\text{Ci}/\mu\text{g}$  of protein. Radiiodinated molecules were extensively dialysed against 500 ml Eagle's medium and 20 ml Hepes, pH 7.4, supplemented with 2 mM mercaptomethylimidazole. Addition of 15% TCA achieved >98% precipitation from all preparations. Labeled neoglycoproteins were stored at 4°C and used within 4 d. For internalization studies, labeled molecules were diluted to the final specific activity with appropriate neoglycoproteins. Ovomuroid and BSA were iodinated with  $^{125}\text{I}$ -labeled Na and  $^{131}\text{I}$ -labeled Na, respectively using the above procedure to obtain a specific activity of 0.9  $\mu\text{Ci}/\mu\text{g}$ .

### Antibodies

The polyclonal antibody against the N-acetylglucosamine receptor has been described elsewhere (Thibault et al., 1993). The anti-ovomuroid antibodies

were obtained as follows. Rabbits were immunized by four subcutaneous injections of 300  $\mu\text{g}$  ovoinhibitor-free ovomucoid (T 2011; Sigma Immunochemicals). The resulting antiserum was purified by affinity chromatography on ovomucoid coupled to CNBr-activated Sepharose (8 mg/ml; 10 ml column).

### Preparation of Thyroid Follicle Fragments

Open porcine thyroid follicle fragments were prepared using a modified version of established techniques (Herzog and Miller, 1979) as follows. Thyroids were obtained within 10 min after death from a local slaughterhouse. After removal of connective tissue, the glands were minced with razor blades and transported to the laboratory in an ice-cold medium composed of DME (GIBCO-BRL, Gaithersburg, MD) supplemented with 2.4 g/liter of 4 (2-hydroxyethyl)-1-piperazineethane sulfonic acid, pH 7.6, and 0.2 g/liter of  $\text{NaHCO}_3$  (medium 1). In the laboratory, tissue pieces were placed in freshly siliconed 25 ml Erlenmeyer flasks (150 mg/flask) containing 10 ml of medium 1 supplemented with 1.5 mg/ml of collagenase, 0.1 mg/ml soybean trypsin inhibitor, 2  $\mu\text{g}/\text{ml}$  DNase and  $\text{CaCl}_2$  (5 mM final concentration). After incubation in a shaking water bath at 37°C for 30 min, the tissue was subjected to mechanical disruption by gentle pipetting through siliconed Pasteur pipettes. Tissue fragments and large follicle clusters on the bottom of the flask were submitted to an additional collagenase digestion with fresh collagenase medium. Follicle fragments were removed by centrifugation at 70 g for 30 s and washed twice with medium 1 supplemented with 0.1% BSA (medium 2). Dissociation of thyroid tissue into opened follicles was checked by observation under an Olympus microscope (model SZ-TN). The viability of isolated follicles was 80 to 90% as determined by Trypan blue exclusion.

### Studies of Endocytosis and Recycling with Neoglycoproteins

**Determination of Internalization and Hydrolysis.** Follicle fragments freshly isolated from about 600 mg of porcine thyroids were incubated at 37°C (or 4°C for controls) in medium 1 supplemented with 2 mM mercaptomethylimidazole. The minimal estimated cell concentration in the incubation medium was estimated at  $5.10^5$  cells/ml. After addition of  $^{125}\text{I}$ -labeled GlcNAc-BSA (950 cpm/ng, final concentration 3.13  $\mu\text{g}/\text{ml}$ ) and  $^{131}\text{I}$ -labeled Man-BSA (200 cpm/ng; final concentration 3.13  $\mu\text{g}/\text{ml}$ ), 2-ml aliquots were collected at various times and centrifugated at 1,000 g for 5 min at 4°C. Pellets were washed twice with 5 ml of ice-cold medium 1 and then treated with 1 ml of 0.1 M NaOH. Protein determination was performed on 100- $\mu\text{l}$  aliquots. 1,500  $\mu\text{l}$  were added to 500  $\mu\text{l}$  of 50% (wt/vol) TCA and incubated for 30 min at 4°C. The precipitate was removed by centrifugation and washed twice with 5% TCA. Degradation of neoglycoproteins to acid-soluble material was assessed as follows. To a 1.5-ml aliquot of the TCA supernatant were added 15  $\mu\text{l}$  of 40% (wt/vol) KI and 50  $\mu\text{l}$  of 30%  $\text{H}_2\text{O}_2$ . After incubation at room temperature for 5 min, free iodine was removed with 2 ml HCl (Goldstein and Brown, 1974). The  $^{131}\text{I}$  and  $^{125}\text{I}$  content of each fraction was measured in a Crystal Gamma Counter (Packard Instruments, Meriden, CT).

**Assessment of Neoglycoprotein Release and Exocytosis.** Follicle fragments were incubated at 37°C in medium 2 supplemented with 2.5 mM  $\text{CaCl}_2$ , 2 mM mercaptomethylimidazole, penicillin (100 U/ml), and streptomycin (100  $\mu\text{g}/\text{ml}$ ).  $^{125}\text{I}$ -labeled GlcNAc-BSA and  $^{131}\text{I}$ -labeled MAN-BSA (both at a final concentration of 30  $\mu\text{g}/\text{ml}$ ) were added to the follicle suspension ( $\sim 4.10^6$  cells/ml) for 90 min. Then epithelia were centrifuged in a silicone-coated conical tube at 80 g for 15 min and washed twice with 10 ml of medium 2. The pellet was resuspended in a medium containing MEM supplemented with 24 mM  $\text{NaHCO}_3$ , 2 mM mercaptomethylimidazole, penicillin, and streptomycin, and then maintained in a humidified incubator at 37°C for 3 min to allow the cells to recover. At various times after the end of incubation (time zero), aliquots (100  $\mu\text{l}$ ) of the medium were removed and centrifugated in an Eppendorf centrifuge (Brinkman Instruments Inc., Westbury, NY) to remove contaminating cells. TCA was added to supernatants and acid soluble and insoluble fractions were obtained as described above and counted in the Crystal Gamma Counter.

### Subcellular Fractional

**Percoll Gradient and DAB Cytochemistry.** After incubation in medium 1 containing  $^{125}\text{I}$ -labeled ovomucoid (50  $\mu\text{g}/\text{ml}$ ) and  $^{131}\text{I}$ -labeled BSA (50  $\mu\text{g}/\text{ml}$ ), follicle fragments were washed five times with 40 ml of PBS sup-

plemented with ovomucoid (500  $\mu\text{g/ml}$ ) and BSA (500  $\mu\text{g/ml}$ ), and two times with 20 ml of PBS. After centrifugation at 1,000 rpm for 3 min at 4°C, the follicle fragments were resuspended in 1.5 ml homogenization buffer (0.25 M sucrose, 2 mM,  $\text{CaCl}_2$ , and 10 mM Hepes/NaOH, pH 7.2) and homogenized with an Ultra-Turax TP 2 N homogenizer (Janke and Kunkel, GmbH). Nuclei were removed by centrifugation for 10 min at 350 g. The postnuclear supernatant was divided into two equal fractions. One fraction was incubated with 60  $\mu\text{l}$  of DAB solution (10 mg/ml in homogenization buffer) and 0.02%  $\text{H}_2\text{O}_2$ , for 30 min at room temperature according to Courtoy et al. (1984) in order to increase the density of thyroperoxidase containing organelles. The other fraction was incubated with 60  $\mu\text{l}$  of homogenization buffer alone. Both fractions were treated with 60  $\mu\text{l}$  of a proteinase K solution (2 mg/ml of homogenization buffer) and then 0.5 ml of each were overlaid on 8 ml (15%) Percoll solution in 0.25 M sucrose, 2 mM  $\text{CaCl}_2$ , 10 mM Hepes-NaOH, pH 7.2. A density gradient was formed by centrifugation at 33,000 g for 25 min at 4°C in a Centrikon T-1055 superspeed centrifuge (Kontron Instruments) using a 50 Ti rotor (Beckman Instruments, Palo Alto, CA) and fractionated by downward displacement into fractions of about 0.4 ml.  $^{131}\text{I}$  and  $^{125}\text{I}$  were counted in an autogramma counter. Aliquots of 50, 100, and 150  $\mu\text{l}$  were taken from each fraction for protein determination (Micro BCA assay; Pierce), thyroperoxidase activity assay (Szoka et al., 1978), and total acid phosphatase activity assay (Bastiani and Simon, 1977), respectively. The density distribution in the gradient formed was measured using density marker beads (Pharmacia Fine Chemicals).

**Differential Centrifugation.** Follicular fragments were incubated in the presence of  $^{125}\text{I}$ -labeled ovomucoid and  $^{131}\text{I}$ -BSA, washed as described above and homogenized in a buffer containing 0.4 M sucrose and 0.1 M phosphate at pH 7.4. The postnuclear supernatant was centrifuged at 10,000 g for 10 min at 4°C. The resulting pellet was saved and the supernatant was further centrifuged at 226,000 g for 1 h at 4°C. Microsomes were subfractionated by flotation using a slightly modified version of the methods proposed by Chabaud et al. (1974) and Saraste et al. (1986) as follows. The pellet from the 226,000 g centrifugation was resuspended in 0.8 ml 0.1 M phosphate buffer, 1.15 M sucrose, pH 7.4, and then deposited on the bottom of a centrifugation tube and overlaid with a discontinuous sucrose gradient in 0.1 M phosphate buffer, pH 7.4, i.e., successively with 2 ml of 1.3 M sucrose buffered solution (density 1.174 g/ml), 2 ml of 1.15 M sucrose buffered solution (density 1.154 g/ml), 2 ml of 1.05 M, sucrose buffered solution (density 1.14 g/ml), and 1.6 ml of 0.4 M sucrose-buffered solution (density 1.05 g/ml). After centrifugation at 144,000 g for 4 h at 4°C, the interfaces were collected from the discontinuous gradient. The gradient pellet and the 10,000 g pellet were resuspended in 0.5 ml of 0.1 M phosphate buffer, pH 7.4. Aliquots of these fractions were then used for determination of protein content, phosphatase acid activity, thyroperoxidase activity,  $^{131}\text{I}$  content and  $^{125}\text{I}$  content as described above. A galactosyltransferase assay was performed with minor modifications of established methods (Freilich et al., 1975, Howell et al., 1978, Bergeron et al., 1982). The incubation medium contained 50 mM Mes buffer (pH 6.5), 20 mM  $\text{MnCl}_2$ , 0.5% saponin, UDP ( $^{14}\text{C}$ ) galactose ( $7.10^5$  cpm, 1.12 nmol), 5  $\mu\text{g}$  ovomucoid, and cell fraction proteins to a final volume of 0.1 ml. Incubations were carried out for 30 min at 37°C and labeled ovomucoid was quantitatively recovered by immunoprecipitation as described below and counted (68% efficiency) on a liquid scintillation counter (LS 3801; Beckman Instruments).

### Galactosylation of Internalized Ovomucoid

Follicle fragments were incubated for 1, 3, or 6 h at 37°C in a MEM medium containing 50  $\mu\text{g/ml}$  ovomucoid and 200  $\mu\text{Ci}$  of D [ $^3\text{H}$ ]galactose (Amersham) per sample. Controls were done with ovomucoid for 6 h at 4°C and without ovomucoid for 6 h at 37°C. At the end of each incubation period the follicle fragments were washed and homogenized and the postnuclear supernatant was centrifuged at 10,000 g. The resulting supernatant was sedimented at 226,000 g for 1 h and fractionated on a 0.4–1.3 M discontinuous gradient as described above. The top two bands (bands 1 and 2) corresponding to the enriched *trans*-Golgi fraction were pooled and sedimented at 226,000 g for 1 h. The pellet was resuspended in 1 ml PBS, sonicated three times for 10 s at 100 W (Ultrasons Apparatus; Annemasse, France) and diluted to 1:1 with a PBS buffer containing 0.5% saponin, 10 mM EDTA, and 20 mM N-acetylglucosamine. Monospecific anti-ovomucoid IgG (100  $\mu\text{g}$  in 50  $\mu\text{l}$  PBS) was added to the sonicated and permeabilized Golgi membranes, and the immune complex was formed by an overnight incubation at 4°C. Then, 0.1 ml of preabsorbed protein A-Sepharose suspension was added and incubated for 1 h at 4°C. The protein A-Sepharose suspension was pretreated by incubation for 2 h at 4°C with an unlabeled

226,000 g Golgi pellet. The protein A-Sepharose beads were removed by centrifugation. After addition of anti-ovomucoid IgG and protein A-Sepharose, the supernatant was reprocessed to ensure complete removal of ovomucoid and immune complexes.

After washing five times with 200  $\mu\text{l}$  of PBS and two times with 100 ml of 50 mM Tris HCl, pH 6.8, the beads were suspended in 100 ml of 50 mM Tris HCl, pH 6.8, 2% SDS, 2%  $\beta$ -mercaptoethanol, and boiled for 3 min to elute the immune complexes. Sepharose was removed by centrifugation and aliquots of the sample were either subjected to the SDS-PAGE as a control or placed in 15 ml of Pico-fluor 15 (Packard Instruments Company) shaken with 1 ml of distilled water and counted in a liquid scintillation counter (LS 3801; Beckman Instruments).

### Cryo-ultramicrotomy and Immunogold Labeling

Epithelium fragments were fixed for 1 h with 4% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, on ice. Cells were embedded as a pellet in 0.1 M phosphate buffer containing 10% gelatin at 37°C. After solidification at 4°C, small blocks of  $\sim 1$  mm<sup>3</sup> were cut, infiltrated with 2.3 M sucrose, mounted on Leica specimen stubs and frozen in liquid nitrogen. Ultrathin sections were cut at  $-110^\circ\text{C}$  on a Ultracryomicrotome (Ultracut E with a Fc4 cryoattachment; Reichert, Vienna, Austria). Frozen sections were transferred to Formvar-coated grids, immunogold labeled, stained with 2% uranyl acetate, and then embedded with methylcellulose. After thawing the sections were incubated for 1 h at room temperature with anti-ovomucoid monospecific polyclonal antibodies and subsequently with either 15 nm or 5 nm antirabbit antibodies. Sections observed and photographed in a Phillips CM10 electron microscope at 80 kW.

## Results

### Accessible GlcNAc Sugar Residues on Internalized Molecules Are Sufficient to Trigger Selective Recycling to the Extracellular Milieu by Thyrocytes

Since the present study was aimed at evaluating interactions implicating GlcNAc residues, BSA-derived neoglycoproteins differing only with regard to sugar residues were used as markers. We choose GlcNAc-BSA neoglycoprotein which was previously used to characterize the receptor (Miquelis et al., 1987) as the specific marker and Man-BSA as the fluid phase marker, Man-BSA was chosen over BSA or Gal-BSA which gave identical results (not shown): (a) because Man-BSA do not interact with the N-acetylglucosamine receptor (Miquelis et al., 1987) or with thyroid membranes at acid or neutral pH (not shown); and (b) because the fact that MAN-BSA has the same protein backbone as GlcNAc-BSA and the same charge (same number of modified lysine residues: 35 mol/mol BSA) enables direct comparison of the intracellular fate and hydrolysis rates of the two markers.

In a first set of experiments, we analyzed the fate of cell-associated neoglycoproteins. Fig. 1 A shows that while  $^{125}\text{I}$ -labeled GlcNAc-BSA and  $^{131}\text{I}$ -labeled Man-BSA were both internalized by thyroid cells, the apparent uptake rates were different. With  $^{125}\text{I}$ -labeled GlcNAc-BSA a steady state plateau was attained within 60 min. With  $^{131}\text{I}$ -labeled Man-BSA internalization continued until 120 min. Twice as much  $^{131}\text{I}$ -labeled Man-BSA was internalized as  $^{125}\text{I}$ -labeled GlcNAc-BSA (see bottom of Fig. 1 A). Since  $^{125}\text{I}$ -labeled GlcNAc-BSA does not interact with thyroid membrane at neutral pH and thus is probably initially internalized at the same rate as the fluid phase marker, i.e.,  $^{131}\text{I}$ -labeled Man-BSA, these observations suggest a temperature-dependent selective mechanism, (Fig. 1 A, upper part), that minimizes intracellular retention of internalized GlcNAc-BSA.

The hydrolysis rate of internalized neoglycoproteins was

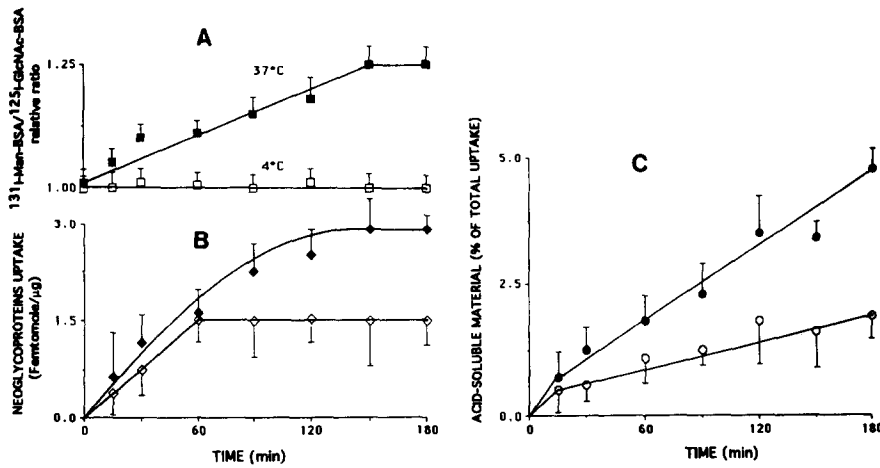


Figure 1. Time course of neoglycoproteins uptake and degradation by thyroid cells. Follicle fragments were incubated in the presence of neoglycoproteins (3, 13  $\mu\text{g}/\text{ml}$  for each one) at either 4° or 37°C and processed as described under Materials and Methods. At the indicated time follicle fragments were washed, the amount of the radioactivity of labeled neoglycoproteins bound to the cells was measured and content of acid-soluble material was determined. Nonspecific uptake and degradation were assessed by incubation at 4°C under identical conditions and substrated from total radioactivity. (A, top) temperature dependence of the relative uptake of neoglycoproteins with time. The results were expressed in arbitrary units with  $^{131}\text{I}/^{125}\text{I} = 1$  at the beginning of the experiment (time zero). (A, bottom) time course of neoglycoprotein internalization with time.  $\blacklozenge$  and  $\diamond$  stand for  $^{131}\text{I}$ -labeled Man-BSA and  $^{125}\text{I}$ -labeled GlcNAc-BSA, respectively. (B) Time course of neoglycoprotein degradation, cell-associated acid-soluble material was expressed as percent of total radioactivity bound to the cell.  $\bullet$  and  $\circ$  stand for acid-soluble material from  $^{131}\text{I}$ -labeled Man-BSA and  $^{125}\text{I}$ -labeled GlcNAc-BSA, respectively. All results were mean values of three or four determinations. Mean cellular protein content averaged 0.5 mg (range 0.34–0.92 mg).

assessed by measuring cell-associated acid-soluble material and expressed in terms of percentage of associated total radioactivity. Fig. 1 B shows that acid-soluble material accounted for only 1 to 5% of the bound radioactivity. The hydrolysis rate of both neoglycoproteins during the 180-min experiment appeared to be linear but  $^{131}\text{I}$ -labeled Man-BSA was preferentially hydrolyzed (Fig. 1 B). Since these results were also obtained with BSA and Gal-BSA (see above), they suggest an intracellular mechanism preventing lysosomal homing of GlcNAc-bearing molecules.

Both intracellular retention and lysosomal homing of GlcNAc-BSA were proven to be GlcNAc dependent. As shown in Table I, addition of GlcNAc-bearing molecules, i.e., ovomucoid (Paz-Parente et al., 1982, 1983; Yamashita et al., 1982) or thyroglobulin (Consiglio et al., 1981; Miquelis et al., 1987; de Waard et al., 1991), led to 68 and 51% increase of the amount of cell-associated GlcNAc-BSA, respectively. In the same way, these competitors led to 58 and 28% increase, respectively, in cell-associated hydrolysis products of GlcNAc-BSA. By contrast, GlcNAc competitors did not affect either the total uptake or hydrolysis of  $^{131}\text{I}$ -

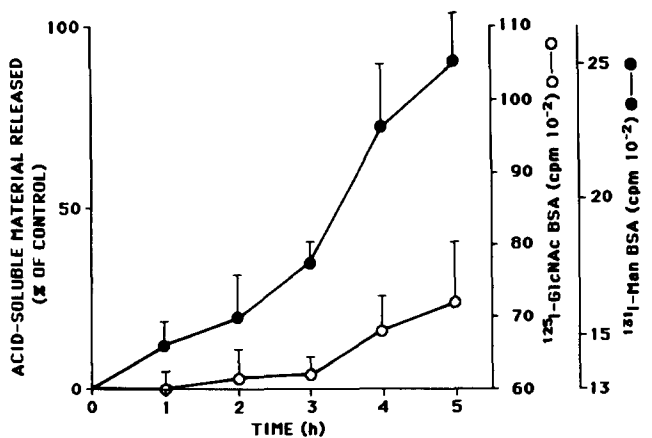
labeled Man-BSA. An additional control experiment using fully glycosylated fetuin indicated that these molecules with no accessible GlcNAc residues had no effect on the apparent uptake or hydrolysis of GlcNAc-BSA. As shown in Table I, selection of GlcNAc-bearing molecules also involves acid intracellular compartments such as endosomes (Gruenberg and Howell, 1989) since chloroquine, a weak base that inhibits the action of the proton pump in the prelysosomal and lysosomal compartments (Gonzalez-Noriega et al., 1980), increases cell-associated GlcNAc-BSA (+38%). Chloroquine had little effect on uptake of  $^{131}\text{I}$ -labeled Man-BSA as a fluid phase marker.

In a second set of experiments, we analyzed the extracellular release of degraded and undegraded molecules from follicle fragments previously incubated in the presence of neoglycoproteins. Monitoring the time course of release of acid soluble material into the extracellular milieu confirmed that the fluid phase marker was preferentially hydrolyzed. As shown in Fig. 2, acid-soluble products from  $^{131}\text{I}$ -labeled Man-BSA appeared immediately while acid-soluble products from  $^{125}\text{I}$ -labeled GlcNAc-BSA appeared 3 h later.

Table I.

Inhibitors		Neoglycoproteins			
		$^{131}\text{I}$ -Man-BSA		$^{125}\text{I}$ -GlcNAc-BSA	
		Internalization	hydrolysis	Internalization	hydrolysis
Ovomucoid	(500 $\mu\text{g}/\text{ml}$ )	< +3%	+7%	+68%	+53%
Thyroglobulin	(500 $\mu\text{g}/\text{ml}$ )	+3%	+5%	+51%	+28%
Fetuin	(500 $\mu\text{g}/\text{ml}$ )	< +3%	< +3%	+ 3%	+ 4%
Chloroquine	(1 mM)	+6%	ND	+38%	ND

The internalization and hydrolysis of neoglycoproteins in the presence of inhibitors. Labeled neoglycoproteins (3.13  $\mu\text{g}/\text{ml}$  for each one) were incubated with follicle fragments for 180 min in the presence of GlcNAc inhibitors (ovomucoid, thyroglobulin) or chloroquine as indicated. Controls were performed in the presence of fetuin. At the end of the incubation period, follicle fragments were washed and the content of acid-soluble and -insoluble material was determined. Nonspecific uptake was assessed by identical experiments at 4°C and substrated. Results are mean values of a representative experiment in duplicate. The values given are the difference between the value measured in the presence and those obtained in the absence of inhibitors. ND, not determined.



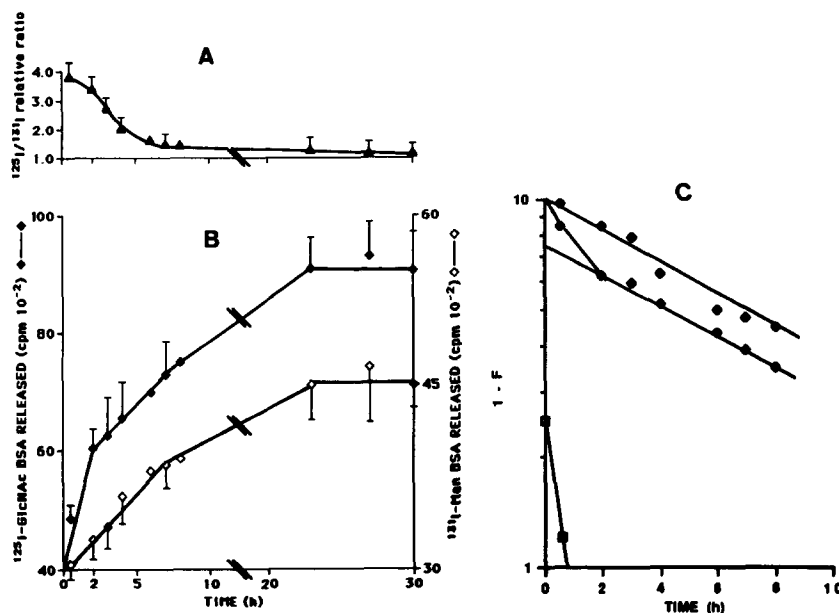
**Figure 2.** Preferential hydrolysis of  $^{131}\text{I}$ -labeled Man-BSA fluid phase marker. Follicle fragments were preincubated in the presence of  $^{131}\text{I}$ -labeled Man-BSA and  $^{125}\text{I}$ -labeled GlcNAc-BSA ( $30\ \mu\text{g}/\text{ml}$  for each one) for a period of 90 min, washed, and resuspended in DME. At this point (time zero) the follicle fragments were further incubated at  $37^\circ$  or  $4^\circ\text{C}$ . At the indicated time, aliquots of the medium were removed, processed as described under Materials and Methods, and content of acid soluble material released was expressed as the difference between the control values obtained at  $37^\circ\text{C}$  and those obtained at  $4^\circ\text{C}$ .

Fig. 3 B shows that part of both internalized neoglycoproteins were spontaneously released into the incubation medium as non-degraded molecules. However, four times more  $^{125}\text{I}$ -labeled GlcNAc-BSA than  $^{131}\text{I}$ -labeled Man-BSA was released at the beginning of the experiment (Fig. 3 A). This preference was no longer observed after 4 hours despite the fact that hydrolysis (see Fig. 2) and membrane transport (see Fig. 3 B) continued for at least 7 h. This is consistent

with selective recycling. Nevertheless, the fact that  $^{125}\text{I}$ -labeled GlcNAc-BSA and  $^{131}\text{I}$ -labeled Man-BSA are subsequently released at the same rate indicates that some  $^{125}\text{I}$ -labeled GlcNAc-BSA escapes this selective rapid pathway and enters a non-specific pathway. Since steady-state labeling was obtained before the experiment, the maximum value for each curve was directly related to the size of the pool of the secreted molecules. Thus we were able to estimate the half-life of each pathway from the data presented in Fig. 3 B. For each curve the function  $1-F(t)$  where  $F$  is the percent at each time of the maximum value at 30 h was plotted on a semilogarithmic scale and a regression line was calculated for each straight line observed. Turnover of the fluid phase marker was monokinetic, with a half life of  $\sim 8$  h (Fig. 3 C, top curve). Turnover of GlcNAc-BSA was biphasic. Peeling (Atkins, 1969) revealed the same slow turnover compartment with a half-life of  $\sim 7$  h corresponding to the non-specific pathway and a rapid turnover compartment with a half life of  $\sim 30$  min corresponding to the selective pathway (Fig. 3 C, mid and lower curves).

### Internalized GlcNAc-bearing Molecules Are Targeted to a Galactosyltransferase-containing Compartment

To determine which compartment is involved in the intracellular routing of GlcNAc-bearing molecules, kinetic experiments followed by subcellular fractionation using either Percoll gradient centrifugation or differential centrifugation followed by discontinuous sucrose gradient were performed. In these experiments we used  $^{131}\text{I}$ -labeled BSA as the fluid phase marker and [ $^{125}\text{I}$ ]ovomucoid as the specific marker for the N-acetylglucosamine receptor. Ovomucoid was chosen not only because it is rich in GlcNAc residues (see above) but also because it was: (a) an adequate substrate for Golgi galactosyl-transferase; and (b) an exogenous ligand which



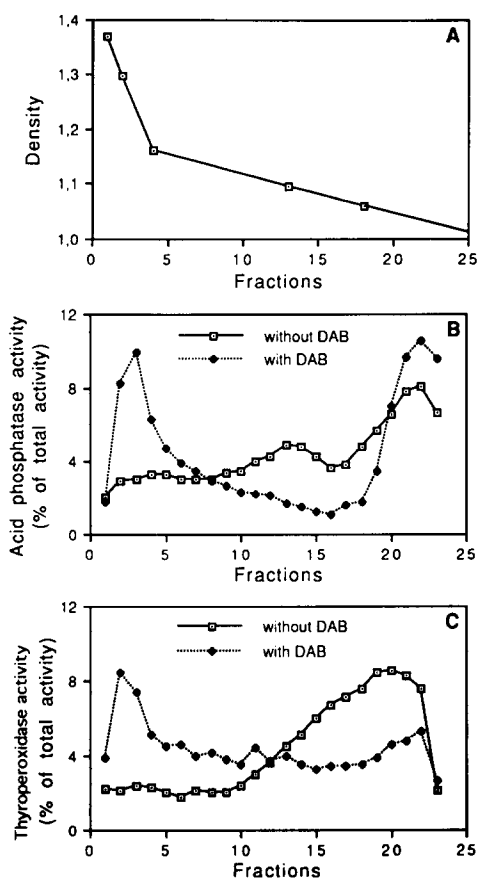
**Figure 3.** Preferential release of undegraded  $^{125}\text{I}$ -labeled GlcNAc-BSA from thyrocytes. Thyroid follicles were preincubated in the presence of  $^{131}\text{I}$ -labeled Man-BSA and  $^{125}\text{I}$ -labeled GlcNAc-BSA ( $30\ \mu\text{g}/\text{ml}$  for each one) during 120 min, and then washed and treated as described in Fig. 2. Acid-insoluble material appearing in the incubation medium with time was determined. (A) Release of neoglycoprotein expressed in arbitrary units using the ratio  $^{125}\text{I}$ -labeled GlcNAc-BSA/ $^{131}\text{I}$ -labeled Man-BSA of the preincubation medium equal to 1. (B) Acid insoluble fractions released, that contained at least 96–98% of undegraded neoglycoprotein as verified by chromatography on Sephadex G25 and SDS-PAGE analysis, were referred to as  $^{131}\text{I}$ -labeled Man-BSA and  $^{125}\text{I}$ -labeled GlcNAc-BSA. Error bars represent SD of five determinations. The data point without error bar is the mean of duplicate determination. (C) The data shown in B were further analyzed on semi-logarithmic plot to determine the apparent turnover rate of  $^{125}\text{I}$ -labeled GlcNAc-BSA and  $^{131}\text{I}$ -labeled Man-BSA.  $^{131}\text{I}$ -labeled Man-BSA, mid curve;  $^{125}\text{I}$ -labeled GlcNAc-BSA. The lower curve was obtained by peeling of the mid curve (Atkins, 1969).

unlike thyroglobulin (presence of endogenous prohormones) and neoglycoprotein (always made from BSA) could be purified and/or identified and thus separated from the "fluid phase" using specific antibodies.

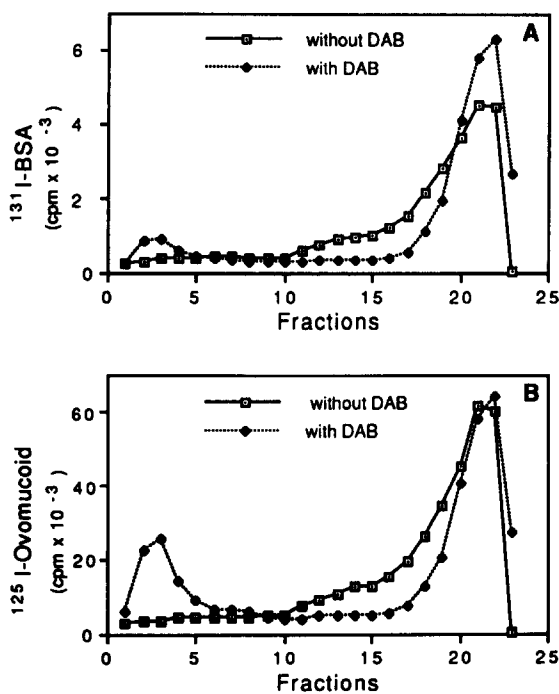
Two enzymatic activities were used to follow the pathways involved in intracellular routing. The first was acid phosphatase which is present in both the biosynthetic and the proteolytic pathways, i.e., Golgi, plasma membrane, endosomes, and lysosomes (Kornfeld and Mellman, 1989; Braun et al., 1989). The second was thyroperoxidase, which is a standard marker for the biosynthetic exocytotic pathway in thyrocytes, i.e., Golgi, exocytotic vesicles, and apical membrane (Ekholm and Bjorkman, 1990).

In Percoll centrifugation experiments, we took advantage of the presence of endogenous thyroperoxidase to further separate and identify the thyroperoxidase-containing vesicles. The usefulness of this method was first demonstrated by Courtney et al. (1984) who showed that incubation of

peroxidase-containing vesicles in the presence of DAB and  $H_2O_2$  before cell fractionation gave rise to a density shift of these organelles due to the peroxidase catalyzed DAB polymerization. As shown in Fig. 4 C, most of the thyroperoxidase-containing organelles ( $\sim 53\%$ ), corresponding to fractions 10 to 23 of the Percoll density gradient (relative density 1.12 to 1.015) shifted to the bottom of the gradient (fractions 1 to 6; 1.38 to 1.15 relative density) after DAB cytochemistry. Interestingly some of these organelles also contained acid phosphatase activity. Indeed, as shown in Fig. 4 B, most acid phosphatase-containing vesicles present in the median fraction of the gradient without DAB (fractions 7 to 16, 1.13 to 1.08 relative density) shifted to the bottom of the gradient after DAB. When follicles fragments were submitted to a 120-min incubation (time necessary to obtain apparent steady state labeling, Fig. 1 A) before homogenization and subcellular fractionation,  $^{131}I$ -labeled BSA and [ $^{125}I$ ]ovomuroid in the organelles were located in the median and light regions of the Percoll gradient density. This type of fractionation experiment is illustrated in Fig. 5 which shows the difference between radioactivity measured by incubation of the follicle fragments at  $37^\circ$  and  $4^\circ C$ . At  $37^\circ C$ , 30% of total radioactivity was located in the median region of the gradient. In contrast the quantity of radioactivity in this region was negligible ( $<3\%$ ) at  $4^\circ C$ . As shown in Fig. 5 A, DAB cytochemistry indicated that most  $^{131}I$ -labeled BSA-con-



**Figure 4.** Percoll gradient density distribution of thyroperoxidase and acid phosphatase activities and the effect of DAB cytochemistry on the density distribution of thyroperoxidase and acid phosphatase containing organelles. Follicle fragments were prepared as described under Materials and Methods, incubated at  $37^\circ C$  for 100 min and homogenized. Half of the postnuclear supernatant was incubated with ( $\bullet$ ) or without ( $\circ$ ) DAB and  $H_2O_2$  before fractionation. (A) The density of the fraction was measured with density marker beads. (B) Density distribution and effect of DAB cytochemistry on the distribution of acid phosphatase-containing vesicles. (C) Density distribution and effect of DAB cytochemistry on the distribution of thyroperoxidase containing organelles.



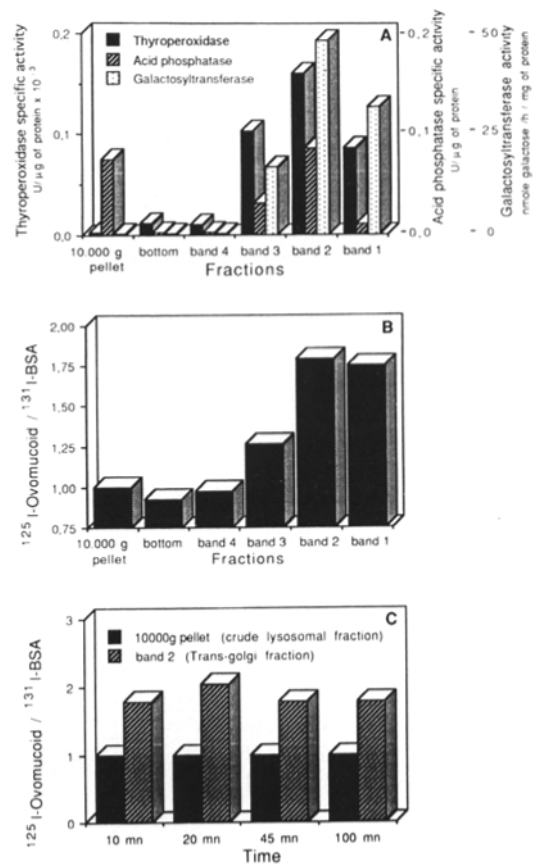
**Figure 5.** Preferential homing of  $^{125}I$ -labeled ovomucoid to thyroperoxidase containing vesicles. The follicle fragments were incubated with  $50 \mu g/ml$   $^{125}I$ -labeled ovomucoid and  $50 \mu g/ml$  of  $^{131}I$ -labeled BSA during 100 min at  $37^\circ C$ . Noninternalized ligand was washed away at  $4^\circ C$  and the cells were homogenized. The postnuclear supernatant was split into two equal portions and treated with ( $\bullet$ ) or without ( $\circ$ ) DAB before fractionation as in Fig. 4. Ligand internalization determined in control experiment at  $4^\circ C$  in parallel was subtracted from all values. The densities in the gradient were in Fig. 4.

taining organelles previously located in the median fraction of the gradient (fractions 10 to 16) shifted to the surface of the gradient (presumably because some  $^{131}\text{I}$ -labeled BSA-associated vesicles become leaky upon DAB cytochemistry and proteinase K treatment). Only a small part of these organelles (4.64% of the total radioactivity) shifted to the bottom. Conversely, [ $^{125}\text{I}$ ]ovomuroid containing organelles from an aliquot of the same postnuclear supernatant shifted exclusively to the bottom of the gradient (Fig. 5 B). This shift accounted for  $\sim 18.2\%$  of  $^{125}\text{I}$ -labeled radioactivity in the gradient, i.e., about 3.5 times more ovomuroid than BSA in 120 min.

To sum up, these results indicated that ovomuroid molecules were diverted from lysosomes by their preferential targeting to a thyroperoxidase and acid phosphatase-containing compartment. As thyroperoxidase is known to be mainly present in the golgi (Bjorkman and Ekholm, 1990) and all sorting and exit of biosynthetic products including lysosomal enzymes is commonly assumed to occur from the *trans* site of the Golgi (Farquhar, 1985; Kornfeld and Kornfeld, 1985; Pfeffer and Rothman, 1987), rerouting through the Golgi apparatus was suspected.

To ascertain this possibility, we performed differential centrifugation yielding a crude lysosomal fraction (i.e., the 10,000 g pellet) and a microsomal fraction (226,000 g pellet) submitted to subfractionation by flotation on a discontinuous sucrose gradient with slight modification of established methods (Chabaud et al., 1974; Sarastre et al., 1986). As stated by Sarastre et al. (1986), bands 4 and 3 at densities 1.17 and 1.15, respectively, could consist of smooth vesicles derived from reticulum and Golgi elements and bands 2 and 1 at densities, 1.14 and 1.13, respectively, could consist of *trans*-Golgi elements associated with higher galactosyltransferase activity. Measurement of the specific activity of thyroperoxidase and acid phosphatase indicated no peroxidase activity in the lysosomal fraction (Fig. 6 A). In contrast, these activities colocalized in fractions from the Golgi complex, particularly in the putative *trans*-Golgi derived vesicles located mainly in band 2 of the sucrose gradient. Measurement of galactosyltransferase (Fig. 6 A) confirmed that this subfraction exhibited the enzymatic characteristics of *trans*-Golgi vesicles.

Preferential targeting of ovomuroid to this subfraction was studied after internalization experiments using  $^{131}\text{I}$ -labeled BSA and [ $^{125}\text{I}$ ]ovomuroid for 10, 20, 30, and 100 min. In three different experiments, we observed that both molecules were present in all fractions at 10 min. Selective routing of GlcNAc-bearing molecules to the Golgi was ascertained by comparing the relative ratio of [ $^{125}\text{I}$ ]ovomuroid and  $^{131}\text{I}$ -labeled BSA entrapped in the Golgi to that in the lysosomal subfraction which was arbitrarily set at 1. Fig. 6 B shows that at 10 min, this ratio was already higher in Golgi fractions and in particular was two times higher in the enriched *trans*-Golgi subfraction (band 2) than the lysosomal fraction. Finally we noted that although the quantity of  $^{125}\text{I}$ -ovomuroid associated with follicle fragments increased with time (from  $\sim 0.2 \mu\text{g}/5.10^6$  cells at 10 min to  $1 \mu\text{g}/5.10^6$  cells at 100 min), 50% of the  $^{125}\text{I}$ -ovomuroid-associated organelles was always found in the 226,000 g microsomal pellet, and, as shown in Fig. 6 C, preferential location of ovomuroid in the enriched *trans*-Golgi subfraction was in the same magnitude i.e., 1.75- to 2-fold higher than the 10,000 g pellet) at each



**Figure 6.** Intracellular distribution of internalized  $^{125}\text{I}$ -labeled ovomuroid and  $^{131}\text{I}$ -labeled BSA in thyrocytes ( $50 \mu\text{g}/\text{ml}$  for each one). Follicle fragments were incubated at either  $37^\circ\text{C}$  or  $4^\circ\text{C}$  in the presence of labeled ovomuroid and BSA molecules in continuous internalization studies ranging from 10 to 100 min, and then washed and homogenized. The postnuclear supernatant was centrifuged at 10,000 g for 10 min and the crude microsomal fraction obtained after centrifugation at 226,000 g for 1 h from the 10,000 supernatant was then subfractionated on a discontinuous sucrose gradient by centrifugation at 144,000 g for 4 h (Kontron T.1055, TST41 Rotor). The 144,000 g pellet (*bottom*) and bands 4, 3, 2, and 1 which correspond to densities of 1.17, 1.15, 1.14, and 1.05, respectively, were collected and aliquot fractions were used for subsequent studies. (A) A typical representation of the distribution of acid phosphatase, galactosyltransferase, and thyroperoxidase activities associated with the different organelle subfractions. (B) Distribution of internalized  $^{131}\text{I}$ -labeled BSA and  $^{125}\text{I}$ -labeled ovomuroid at 10 min in different subsets of organelles. The non-specific cell-associated radioactivity observed at  $4^\circ\text{C}$  was subtracted. The results were expressed in arbitrary unit with  $^{125}\text{I}/^{131}\text{I}$  equal to 1 in the 10,000 g lysosomal pellet to emphasize the preferential location of  $^{125}\text{I}$ -labeled ovomuroid in bands 2 and 1. (C) Kinetic of the relative distribution with time of labeled ovomuroid and BSA in the crude lysosomal fraction and the band 2 subfraction.

time studied. Thus this phenomenon appears to occur within the first 10 min of incubation.

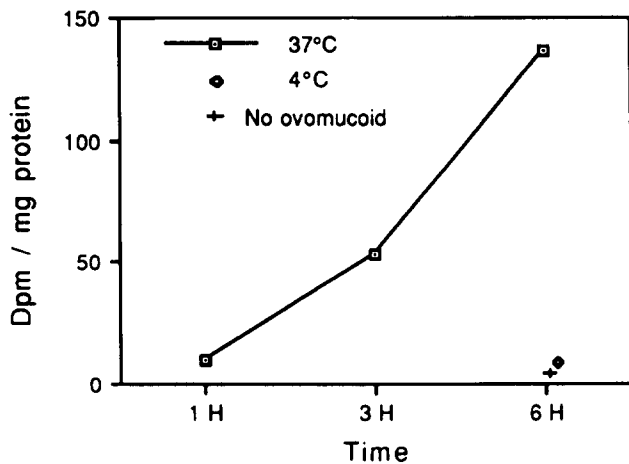
#### ***Internalized GlcNAc-bearing Molecules Are Further Galactosylated***

The fact that ovomuroid and galactosyltransferase comi-

grated in the same band of the discontinuous sucrose gradient is not an absolute proof that they are colocalized in the same organelles. To determine whether or not this was the case, we analyzed if entrapped recycled ovomucoid molecules were further glycosylated. Thus we studied the kinetics of galactosylation of unlabeled ovomucoid by fragment follicles incubated in a medium containing [ $^3\text{H}$ ]galactose. After sonication and then saponin-induced permeation of the organelles from the Golgi fraction, ovomucoid molecules were immunoprecipitated with monospecific anti-ovomucoid IgG. To ensure quantitative recovery of [ $^3\text{H}$ ]galactose-labeled ovomucoid molecules two successive immunoprecipitation experiments were performed for each sample. At each measurement time, the first immunoprecipitation led to recovery of 95 to 98% of total [ $^3\text{H}$ ]galactose-labeled ovomucoid. This finding as well as the observation during control experiments that immunoprecipitated material revealed by SDS-PAGE composed a single homogeneous fraction containing no noticeable contaminant (not shown) proves that ovomucoid was the only galactosylated molecule to be immunoprecipitated. Fig. 7 shows the kinetic curve of galactosylation of ovomucoid molecules associated with the Golgi fraction. Unexpectedly, the curve was linear and no steady state was observed. This suggests either that galactosylated ovomucoid molecules gradually accumulate in the Golgi or that several successive galactosylations occur involving repeated recycling of some ovomucoid molecules through the Golgi.

### Immunogold Localization of Ovomucoid in the Golgi Apparatus

In addition to the biochemical data that we have presented, EM also documented delivery of ovomucoid to the Golgi apparatus. Follicle fragments were incubated with either 500



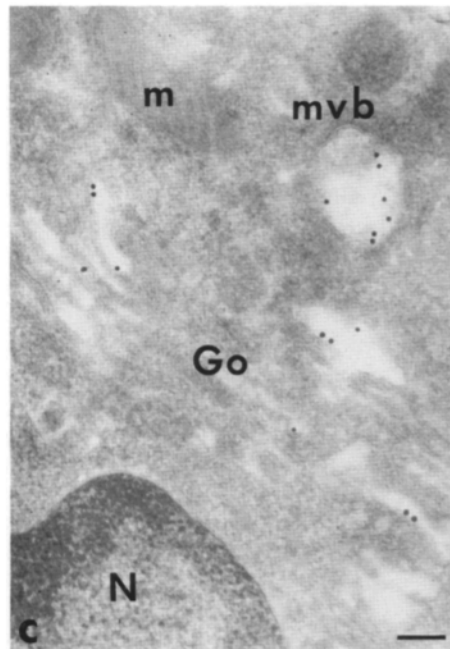
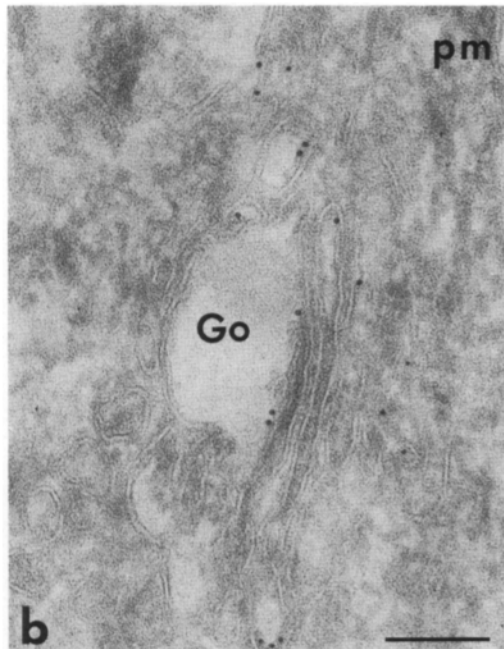
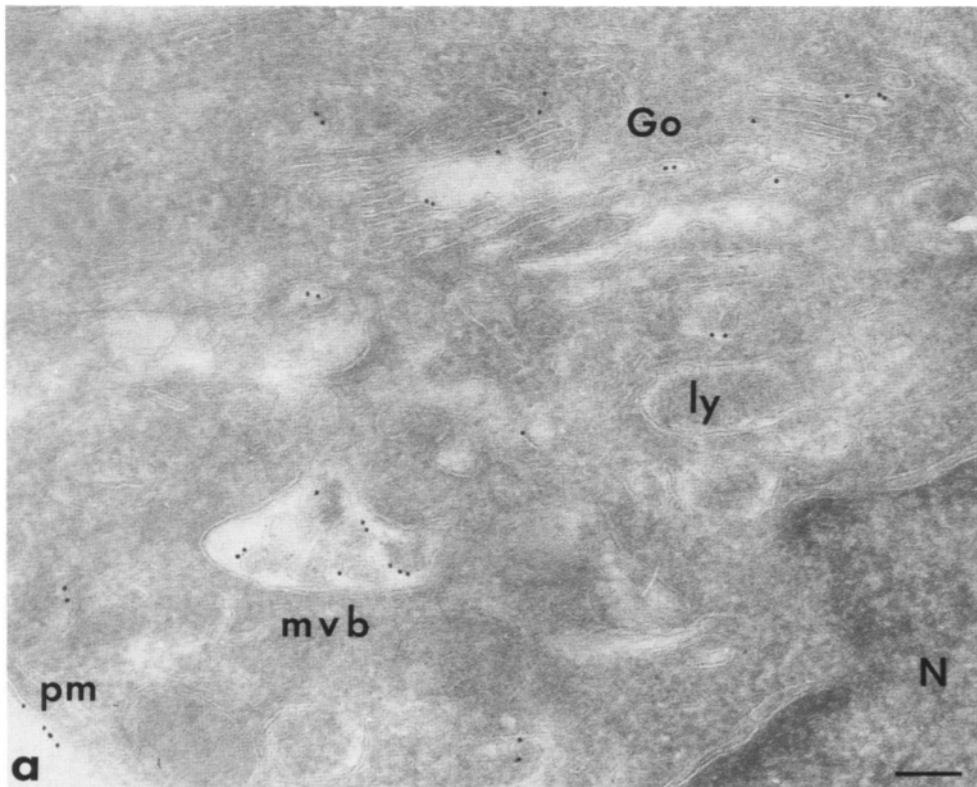
**Figure 7.** Galactosylation with time of internalized ovomucoid in thyrocytes. Follicle fragments were incubated in the presence of 200  $\mu\text{Ci}$  [ $^3\text{H}$ ] galactose/sample and 50  $\mu\text{g}/\text{ml}$  of unlabeled ovomucoid. At the indicated time, follicles were washed, homogenized, and the postnuclear supernatant was subfractionated as depicted in Fig. 6. Bands 2 and 1 were pooled, sonicated, further treated with 0.5% saponin, and ovomucoid molecules associated to these subfractions of organelles were immunoprecipitated by anti-ovomucoid polyclonal antibodies as described under Materials and Methods. Controls were done after incubation at 4°C for 6 h or incubation at 37°C in the absence of ovomucoid for 6 h.

$\mu\text{g}$  of 5 mg of ovomucoid/ml of medium for 60 min. After fixation, ultrathin cryosections were immunogold labeled. Only gold grains associated with Golgi stacks in the plane of section were counted. Despite the resulting overestimation, this method was adopted to ensure that internalized ovomucoid could be visualized if present and therefore that the results could be used as a control. Background counts using preimmune sera were performed for both experimental conditions. Background labeling of the extracellular milieu was low. Similarly background labeling of the cell section was only  $2 \pm 2$  gold particles per cell ( $n = 30$ ). Labeling was essentially localized in the cytoplasm and only occasionally in nuclei. Background labeling was extremely scant on Golgi complexes and was never observed on Golgi stacks, mitochondria, identifiable multivesicular bodies, or secondary lysosomes. Application of anti-ovomucoid polyclonal antibodies to cross sections of epithelia previously incubated in the presence of 500  $\mu\text{g}$  ovomucoid/ml medium enhanced labeling in the plane of the section, i.e., in the extracellular milieu, as well as on cell sections. We counted an average of  $18 \pm 10$  gold particles per cell section ( $n = 103$ ) which represented a significant signal-to-noise ratio. Importantly 88 out of 103 Golgi apparatus observed were positive. Analysis of the relative distribution of the 1,763 gold particles counted on these cell sections indicated that 33% were associated with multivesicular bodies and 25% with the Golgi apparatus, i.e., TGN and the Golgi stacks. No labeling was observed on mitochondria and lysosomes. On cross sections of epithelia previously incubated in the presence of 5 mg of ovomucoid/ml of incubation medium, the average number of gold particles per cell section was  $35 \pm 16$  ( $n = 18$ ). The typical distribution on the subapical cell surface ( $\sim 80\%$  of total labeling on transverse cell section) and the Golgi apparatus are illustrated by the photographs in Fig. 8. Labeling was scant on cell membranes (Fig. 8 *a*) but was observed on endosomes with intravesicular vesicles (Fig. 8, *a* and *c*), also called late endosomes (Stoorvogel et al., 1991). Gold particles were also found in small vesicles surrounding the Golgi apparatus as well as the Golgi stacks (Fig. 8, *a-c*). Analysis of the relative distribution of the 720 gold particles counted indicated that 6% were associated with the plasma membrane, 15% with endocytotic vesicles or tubulovesicular elements located beneath the cell membrane, 21% with multivesicular bodies, 36% with the golgi apparatus, 14% with other vesicles, and 8% over the cytoplasm or nuclei. No labeling was found in secondary lysosomes. Taken together these morphological findings support the notion of ovomucoid delivery to late endosomes and rerouting through the Golgi apparatus.

### Discussion

Based on the findings described above, we concluded: (*a*) that the recycling of internalized molecules that has been reported in vivo in thyrocytes can be reproduced in vitro on follicular fragments; (*b*) that the presence of accessible N-acetylglucosamine sugar residues on internalized molecules is a necessary and sufficient condition to minimize their routing to lysosomes; (*c*) that internalized molecules with accessible N-acetylglucosamine moieties are recycled via a galactosyltransferase-containing compartment. The likely compartments for this unprecedented example of





**Figure 8.** Electron micrographs showing the localization of ovomucoid in both the endosomal compartment and the Golgi apparatus of thyrocytes on ultrathin cryosections. Follicle fragments were incubated for 1 h at 37°C in binding medium containing 5 mg/ml ovomucoid, washed, and fixed, subsequently. Cryosections were indirectly immunolabeled with 5 nm (*a* and *b*) and 10 nm (*c*) anti-rabbit gold particles, respectively, for the demonstration of ovomucoid. Gold-labeled ovomucoid was detected on apical membrane (*a*), in multivesicular bodies (*a* and *c*) and Golgi (*a-c*). *pm*, plasma membrane; *ly*, lysosome; *m*, mitochondria; *mvb*, multivesicular body; *N*, nucleus; *Go*, Golgi. Bars, 0.1  $\mu$ m.

selective retrograde transfer are the *trans*-Golgi stacks and the TGN known to contain galactosyltransferase as a resident membrane protein (Roth and Berger, 1982; Geuze et al., 1985; Nilsson et al., 1991).

The concept of a process minimizing the routing of certain molecules to lysosomes in thyrocytes is not new. It was first described from *in vivo* kinetic experiments (Simon et al., 1979; Bastiani et al., 1980). In our previous working model we proposed that recycling occurs from endosomal compartments either directly back to the apical membrane or via the

Golgi (Simon et al., 1979). The present results do not rule out direct recycling. As discussed below, it can be assumed that direct recycling would be faster than routing via Golgi and thus it would be necessary to perform kinetic experiments lasting <30 min to detect direct recycling. This was not done in the present study. However, we emphasize that linkage of endocytosis and exocytosis via the Golgi would serve two physiologic functions. The first would be to bring GlcNAc-bearing molecules, i.e., immature thyroglobulin, together with thyroperoxidase, the enzyme responsible for

iodination and hormonogenesis at the apical membrane (Ekholm and Wollman, 1975) before thyroglobulin exocytosis. The second function would be to promote elongation of complex N-glycan chains thereby eliminating the GlcNAc retention signal.

We have demonstrated that during their intracellular routing ovomucoid molecules incorporated [<sup>3</sup>H]galactose. Moreover we have noted that the kinetics of galactosylation was linear and no steady state was reached during the 6-h experiment. This suggests either that galactosylated ovomucoid molecules gradually accumulated in the Golgi or that several successive galactosylations occur involving some ovomucoid molecules being repeatedly recycled through the Golgi. Two findings support the latter conclusion. The first is that the dissociation between GlcNAc-bearing molecules and the thyroid N-acetylglucosamine receptor, the potential specific intracellular carrier, is incomplete at near neutral pH. We previously reported that 40% of ligand molecules remained bound after incubating the complex for one hour at pH 7.2 (Miquelis et al., 1987). In this regard, it is important to note that the *trans*-Golgi network is an acid compartment (Anderson and Pathak, 1985). The second finding supporting successive recycling is from *in vivo* observations in human congenital goiter that immature thyroglobulin is not released into follicular lumens, but rather remains tightly bound to microsomes (Monaco et al., 1974).

The present study showed that, while at a slower rate and in smaller quantity, fluid phase markers were also recycled through the Golgi complex. Similar findings have been reported for other cell types (Farquhar, 1983, 1985; Herzog, 1980). The close interconnection between the endosomal system and the Golgi apparatus, as indicated by their identical lipid membrane composition (Van Meer, 1989) suggests that this may be a widespread phenomenon and could account for the fact that under our conditions up to 12% of internalized fluid phase markers was compartmentalized in Golgi-derived vesicles. Regurgitation of fluid phase markers can be rapid or slow (for review see Steinman et al., 1983). Rapid regurgitation ( $t_{1/2} = \sim 5$ –10 min) occurs immediately following uptake. It comes from endosomes and can involve from 5 to 20% (Roberts et al., 1977; Pratten et al., 1977) or 40% (Buktenica et al., 1987) depending on the material internalized. Slow regurgitation ( $t_{1/2} = >3$ –12 h) comes from compartments such as Golgi and lysosomes (Buktenica et al., 1987) and can involve as much as 30 to 40% of material (Roberts et al., 1977; Besterman et al., 1981). In our experiments using Man-BSA as the fluid phase marker, only slow regurgitation was observed ( $t_{1/2} = \sim 8$  h). Further study will be needed to know whether the absence of rapid regurgitation is attributable to our experimental conditions or whether it is characteristic of thyrocytes. In this regard it should be pointed out: (a) that regurgitation is linked to renewal of the plasmic membrane and therefore is highly variable depending on the cell type studied (Silverstein et al., 1977; Steinman et al., 1983); and (b) that renewal of the apical membrane in thyrocytes is ensured by the continuous outflux of exocytotic vesicles which is responsible for thyroglobulin transport (Ekholm and Bjorkman, 1990). This outflux may account for up to 50% of protein synthesis (Dumont et al., 1989) and could explain that no regurgitation was observed from pinocytic vesicles.

The two major findings of this study were the involvement

of a galactosyltransferase-containing compartment in selective recycling and the fact that the half time of transit through this compartment was  $\sim 30$  min, i.e., 16 times faster than the half time of the fluid phase marker. Half times of 30 min or less have only been described for recycling from endosomes to plasma membranes. For example, in HepG2 cells asialo-orosomucoid/Gal-GalNAc receptor complexes are returned to the extracellular medium with a  $t_{1/2}$  of  $\sim 24$  min (Stoorvogel et al., 1987). Similarly recycling to the plasma membrane of the transferrin/transferrin receptor complexes occurs with a  $t_{1/2}$  of  $\sim 2$ –10 min (Stoorvogel et al., 1987, 1988). Conversely recycling via the Golgi is much slower. For example, the half transit time for recycling of the sole transferrin receptor through the Golgi apparatus is  $\sim 2$  h and likely to be partial because of the low efficiency of resialylation of previously deglycosylated receptors (Snider and Rogers, 1985). Duncan and Kornfeld (1988), have reported that mannose phosphate receptors involved in the transport of newly synthesized lysosomal enzymes, recycle only through a sialyltransferase-containing compartment with a  $t_{1/2}$  of  $\sim 3$  h. Rapid transfer kinetics to a galactosyltransferase-containing compartment suggests the existence of a specific mechanism that accelerates and amplifies transport of GlcNAc-bearing molecules through the Golgi complex.

One novelty of the present study is to demonstrate that GlcNAc sugar residues were responsible for a recycling, independently of the thyroglobulin protein backbone. This strongly suggest that the GlcNAc thyroid receptor is implicated. Inhibition of recycling by chloroquine corroborates the involvement of the thyroid lectin which is known to bind ligands at acidic pH. Moreover, using immunofluorescence studies and immunoperoxidase labeling, we were able to locate GlcNAc receptors on the apical membrane of thyrocytes in healthy tissue (Thibault et al., 1993). Consiglio et al. (1981) showed that thyroglobulin specifically bound not only plasma membranes but also Golgi membrane preparations. Our immunogold localization experiments on ultrathin cryosections of follicle fragments demonstrated that the receptor was present both on the apical membrane and in the Golgi complex (Courageot, J., P. Bastiani, and R. Miquelis, unpublished data). These findings indicate that the GlcNAc receptor fulfills the specificity and localization requirements necessary for a receptor involved in the intracellular traffic of GlcNAc-bearing molecules in thyrocytes.

The study of excised pathological human thyroid tissue suggests a close relationship between expression and location of the N-acetylglucosamine receptor, on the one hand, and the degree of stimulation of the gland, on the other. N-acetylglucosamine receptor is undetectable in some types of differentiated cancers such as papillary cancers and its expression and location is modified in certain types of goiter (intracellular subnuclear location) and vesicular cancer (basolateral location) (Thibault et al., 1993). These results indicate that the N-acetylglucosamine receptor is a characteristic feature of the fully differentiated phenotype and that its apical location and thus its potential function is controlled by thyroid-stimulating hormone.

Another potential regulating factor for selective recycling is ligand concentration. Given the positive cooperativity associated with GlcNAc ligand/GlcNAc receptor binding (Miquelis et al., 1987), there is probably a critical concentration of ligand for optimal transfer.

While a great deal of additional work will obviously be needed to demonstrate a common intracellular pathway for GlcNAc-bearing molecules and N-acetylglucosamine receptor complexes, current evidence is sufficient to consider that the thyroid lectin and thyrocytes are useful models for the study of specific recycling from endosomes to the Golgi complex. An especially intriguing question concerns the structural determinants of the receptor and/or the associated molecular machinery in this specific intracellular traffic. Current experiments aimed at molecular cloning and study of N-acetylglucosamine receptor expression may provide insight into these issues.

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*Note Added in Proof:* During the reviewing process of the manuscript, P. A. Huang and M. D. Snider published evidence that confirms recycling of molecules from the plasma membrane to a galactosyltransferase Golgi compartment (Huang, P. A., and M. D. Snider. 1993. Glycoprotein recycling to the galactosyltransferase Golgi compartment of the Golgi complex. *J. Biol. Chem.* 268:9302-9310).

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