Coated Vesicles from Thyroid Cells Carry Iodinated Thyroglobulin Molecules

FIRST INDICATION FOR AN INTERNALIZATION OF THE THYROID PROHORMONE VIA A MECHANISM OF RECEPTOR-MEDIATED ENDOCYTOSIS

Françoise Bernier-Valentin, Zdenek Kostrouch‡, Rachida Rabilloud, Yvonne Munari-Silem, and Bernard Rousset§

From the Institut National de la Santé et de la Recherche Médicale Unit 197, Faculté de Médecine Alexis Carrel, 69372 Lyon, Cédex 06, France

We have tried to identify iodinated thyroglobulin molecules in purified thyroid-coated vesicles to determine whether the internalization of the thyroid prohormone could proceed via a mechanism of receptor-mediated endocytosis. Coated vesicles isolated from pig thyroids by differential centrifugation and centrifugation on 2H2O-sucrose cushion were characterized by transmission electron microscopy and analyses of the polypeptide composition by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and Western blot using anti-clathrin heavy chain and anti-thyroglobulin antibodies. Clathrin and thyroglobulin (Tg) appeared as the two major components of the purified thyroid coated vesicles (TCV). Purified TCV fraction was homogeneous when analyzed by isopycnic centrifugation on 30% Percoll gradient. TCV had an apparent buoyant density of 1.035 g/ml. The presence of Tg molecules inside TCV was ascertained by (a) immunogold labeling on cryosections of TCV pellet and (b) identification by gel electrophoresis and radioimmunoassay of a definite fraction of Tg (3–5% of total protein) in TCV treated by Triton X-100. The detergent-treated TCV also contained protein-bound iodine: 0.5–0.7 µg of iodine/mg protein. Pulse-chase experiments on in vitro reconstituted thyroid follicles have been used to further document the presence of iodinated Tg molecules in coated vesicles. TCV were isolated from reconstituted thyroid follicles previously labeled with [125I]iodide to radioiodinate Tg of the follicular lumen (the pre-endocytic compartment) and incubated with or without thyrotropin or dibutyryl cyclic AMP to activate intraluminal 125I-Tg endocytosis. Autoradiographic analyses revealed the presence of 125I-Tg in purified TCV and Triton X-100-treated TCV. 125I-Tg present in TCV represented 1–2% of the total intracellular protein-bound radioactivity. Thyrotropin and dibutyryl cyclic AMP increased 2–3-fold the 125I-Tg content of TCV. Our results clearly show that iodinated Tg, the molecular form of the thyroid prohormone known to be internalized, is present into TCV. The data suggest that coated vesicles are involved in the uptake and transport of Tg from the follicular lumen to the lysosomal compartment and therefore, that the internalization of Tg could proceed, at least for a part, via a mechanism of receptor-mediated endocytosis.

Thyroid hormone synthesis implies bidirectional transport of the prohormone thyroglobulin (Tg).1 After synthesis and maturation, Tg is released into the intrafollicular lumen and iodinated into this extracellular compartment. Hormones containing Tg molecules are then taken up by the cells and conveyed to lysosomes for final processing and generation of free thyroid hormones. Two different pathways of Tg endocytosis have been reported involving the formation of either large vesicles, colloid droplets or small vesicles, pinocytic vesicles (1). The former process also named macropinocytosis share morphological characteristics with phagocytosis; it implicates the formation of pseudopods by the apical plasma membrane. The second pathway or micropinocytosis implies the formation of vesicles by invagination of the cell membrane. The macropinocytosis process enables the cell to take up large amounts of Tg whereas micropinocytosis may offer selectivity in the Tg uptake. The relative importance of macro- and micropinocytosis for the secretion of thyroid hormones is still debated. Micropinocytosis has been well documented in rat and mouse thyroid tissue stimulated by thyrotropin (TSH) (1). The number and the size of pseudopods, as well as the number of endocytotic vesicles or colloid droplets, appear to be related to the degree of TSH stimulation. It has been suggested (2) that micropinocytosis could be the major pathway of Tg uptake in normal gland, i.e. thyroid in a low activation state. In this situation, colloid droplets are rare or absent and the amount of Tg required for the maintenance of normal plasma levels of thyroid hormone could be internalized via micropinocytotic vesicles. A process of micropinocytosis was also postulated in hyperactive glands (after multiple TSH injections) in which the thyroid hormone release is not dependent on the appearance of pseudopods and the formation of colloid droplets (3).

Tg molecules stored in the follicular lumen present a large heterogeneity in terms of iodine or hormone content (4). It has been reported that Tg molecules with a high iodine content are taken up more actively and degraded more rapidly

---

1 The abbreviations used are: Tg, thyroglobulin; CV, coated vesicle; TCV, thyroid coated vesicle; TSH, thyrotropin; RTF, reconstituted thyroid follicles; SDS, sodium dodecyl sulfate; Mes, 4-morpholinee-thanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)tetraacetic acid; Pipes, 1,4-piperazinedithanesulfonic acid.

---

* This work was supported in part by Grant 87C 0572 from the Ministère de la Recherche et de l'Enseignement Supérieur. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Recipient of a postdoctoral fellowship from the Institut National de la Santé et de la Recherche Médicale, France.

‡ To whom correspondence should be addressed.
than low iodinated newly synthesized Tg (5, 6). The micropinocytic process seems to be compatible with the existence of such a selective uptake of Tg. The mechanism of selection of Tg molecules would require the presence of specific binding sites at the site of endocytosis, i.e. the apical plasma membrane. Attempts have been made to identify such binding sites on thyroid slices (7), purified membrane preparation (8), or open thyroid follicles. Data obtained with intact cell systems are consistent with plasma membrane binding activity of Tg with an apparent Kd of 1–5 μM. Purified thyroid membrane preparations are capable of specific recognition of Tg, and the interaction process depends on the iodination level of the ligand but also on the presence or the absence of sialic acid (8, 9). It is not known, however, whether these Tg-binding sites of asialoglycoprotein receptor type (10) are expressed on the cell surface or located on intracellular membranes. Additional work is needed to assign a role of these binding sites in the process of Tg internalization. A mechanism of micropinocytosis characterized by the capacity of sorting, internalization, and addressing of Tg molecules to the lysosome compartment (11, 12) could follow to a large extent the general pathway of receptor-mediated endocytosis (13, 14). Receptor-ligand complexes form on specialized domains of the plasma membrane (coated pits) and enter the cell via coated vesicles. Soon after entering the cell, clathrin coats are shed from coated vesicles. The resulting vesicles deliver receptor-ligand complexes to endosomes. Depending on the ligand, receptor-ligand complexes dissociate or not, and receptor and/or ligand are transported into different cell compartments (plasma membrane, lysosomes). The first type of organelles involved in the selective ligand endocytosis coated vesicles (16) have been identified in thyroid cells by ultrastructural approaches (16–20). It is not known whether Tg is present in thyroid-coated vesicles. The only study (21) devoted to the analysis of the protein component of purified thyroid-coated vesicles has yielded negative results; the small amount of Tg found in the coated vesicle preparations was outside the vesicles associated to the clathrin coat. We have decided to reexamine this question, taking advantage of a method of tissue disruption (22) which permits elimination of more than 98% of the enormous amount of soluble Tg coming from intracellular stores and thus greatly decreases possible artifactual association of Tg with subcellular organelles. This procedure successfully used to purify lysosomes (22) and analyze intralysosomal Tg and intermediate degradation products (23) has now been employed for the isolation of coated vesicles from pig thyroid glands. By combining biochemical and immunological methods, we have obtained evidence for the presence of Tg inside thyroid-coated vesicles. Using an in vitro model of differentiated thyroid cells, the reconstituted thyroid follicles (RTF), we have tried to determine whether Tg molecules, transported by coated vesicles, are iodinated and could correspond to internalized Tg species. Pulse-chase experiments have been used to analyze whether the activation of labeled Tg endocytosis by thyrotropin is associated with an increase of the labeled Tg content of coated vesicles.

**EXPERIMENTAL PROCEDURES**

**Materials**

Mes, EGTA, phenylmethylsulfonyl fluoride, Triton X-100, and sodium dodecyl sulfate, dibutyl cyclic AMP, methimazole, and thyrotropin (TSH) were obtained from Sigma. Bovine serum albumin (fraction V) was purchased from Miles Laboratories. Percoll was from Pharmacia LKB Biotechnology Inc. Acrylamide, N,N′-methylenebis-acrylamide, tetramethylethylenediamine, ammonium persulfate were from Bio-Rad. [125I]Iodide was purchased from Amer sham Corp., and Pansorbin® from Calbiochem. Nitrocellulose paper (HAHY) was from Millipore. H2O (99% pure) was purchased from 'Commissariat à l‘Energie Atomique (France). F-12 culture medium and calf serum were obtained from Flow Laboratories.

**Purification of Coated Vesicles**

Pig thyroid glands were obtained from the local slaughterhouse transported on ice to the laboratory, and processed within less than 90 min after the death of the animals. Forty to 50 glands were trimmed free from fat and connective tissue chopped into small pieces (total wet weight 500–600 g). A tissue fraction named "the open follicle fraction" depleted in intracellular Tg stores was prepared according to Alquier et al. (22) in 100 mM Mes, 2 mM MgCl2, 0.1 mM EGTA 0.5 mM phenylmethylsulfonyl fluoride, 3 mM NaN3, pH 6.4 (the CV isolation buffer or MMEP buffer). Briefly, the open follicle fraction was obtained by forcing thyroid fragments through a 300-μm mesh sieve. The cellular material was collected by centrifugation at 800 × g for 20 min at 4 °C, extensively washed in the same buffer to eliminate residual Tg and homogenized with a glass-Teflon Potter homogenizer rotated at 1500 rpm for six up-and-down strokes. Coated vesicles were purified according to Nandi et al. (24) with slight modifications. The cellular homogenate was centrifuged at 800 × g for 30 min at 4 °C and the resulting supernatant (S1) was centrifuged at 13,300 × g for 30 min at 4 °C; heavy vesicles of the pellet (P2) were eliminated and the supernatant (S2) was centrifuged at 140,000 × g for 60 min at 4 °C. The 140,000 × g pellet (P3) was resuspended in MMEP buffer, homogenized with the glass-Teflon Potter homogenizer and resubmitted to a centrifugation at 13,300 × g for 30 min to remove aggregated material (pellet P4), and the supernatant (S4) was centrifuged at 140,000 × g for 60 min at 4 °C. The final 140,000 × g pellet (P5) was resuspended in 24 ml of MMEP buffer. The protein concentration of this light vesicle suspension ranged from 2 to 3 mg/ml. Six ml of the suspension were layered over 6 ml of a 6% sucrose/3H2O solution prepared in MMEP buffer and centrifuged at 140,000 × g for 90 min at 20 °C. The pellet, which contained the purified thyroid-coated vesicles (TCV) was resuspended in MMEP buffer at a concentration of about 1 mg/ml and stored at 4 °C.

**Thyroid Cell Culture**

Pig thyrocytes were prepared by the discontinuous trypsinization procedure (25). Freshly dispersed cells were cultured as previously reported (26). Cells were suspended in F-12 medium containing 10% calf serum, penicillin (200 units/ml), streptomycin (200 μg/ml), amphotericin B (0.5 μg/ml), and TSH (1 milliunits/ml) at a final concentration of 3 mM EDTA. The treatment in the calcium-free medium caused the opening of the lumen of RTF and the dissociation of cells forming the follicles. The complete recovery of cells was obtained by scraping the cells still attached to the dishes with a rubber policeman. Cells were separated from the intracellular-soluble material by centrifugation at 100 × g for 5 min at 4 °C. The cell pellet was washed and homogenized in the CV isolation buffer (MMEP buffer) using a glass/Teflon Potter homogenizer with a tight-fitting pestle rotated at 3000 rpm for 10 up-and-down strokes. Cell homogenates were centrifuged at 800 × g for 16 min at 4 °C. The resulting supernatant was submitted to the procedure for isolation of CV (see above).

**Labeling and Incubation of Reconstituted Thyroid Follicles**

After 3 days of culture, reconstituted thyroid follicles (RTF) were shifted to a culture medium deprived in TSH for 16 h. On day 4, RTF were washed and incubated with [125I]iodide (10 μCi/dish) in F-12 medium for 1 h at 20 °C. At the end of the labeling period, RTF were washed in F-12 medium supplemented with 1 mM sodium perchlorate and 1 mM methimazole to induce the release of intracellular non-labeled iodide to prevent further organification of residual iodide, respectively. Labeled RTF were then incubated in the same medium with or without TSH (10 milliunits/ml) or 3 mM dibutyl cyclic AMP for 15 min at 20 °C. At the end of the incubation period, RTF were washed and incubated in phosphate-buffered saline supplemented with 3 mM EDTA. The treatment in the calcium-magnesium free medium caused the opening of the lumens of RTF and the dissociation of cells forming the follicles. The complete recovery of cells was obtained by scraping the cells still attached to the dishes with a rubber policeman. Cells were separated from the intracellular-soluble material by centrifugation at 100 × g for 5 min at 4 °C. The cell pellet was washed and homogenized in the CV isolation buffer (MMEP buffer) using a glass/Teflon Potter homogenizer with a tight-fitting pestle rotated at 3000 rpm for 10 up-and-down strokes. Cell homogenates were centrifuged at 800 × g for 16 min at 4 °C. The resulting supernatant was submitted to the procedure for isolation of CV (see above).

**A. Guerrier and B. Rousset, unpublished data.**
**Transport of Thyroglobulin by Coated Vesicles**

### RESULTS

#### Biochemical and Morphological Analyses of Isolated TCV—

The combination of differential centrifugation steps and a centrifugation on a 
H2O sucrose cushion yielded highly purified coated vesicle preparations. One to 3 mg of TCV protein (mean = 1.7 ± 0.4, n = 6) were obtained from 100 g wet weight pig thyroid tissue. The protein content of the starting cell homogenate (depleted in intrafollicular Tg stores) was 1010 ± 50 mg/100 g of thyroid tissue (n = 4). The light vesicle fraction (pellet P5) obtained at the last differential centrifugation step accounted for only 1% of total protein of the homogenate. About 20% of the particulate material of the P5 pellet were recovered as the purified CV fraction sedimenting through the sucrose–H2O cushion. The purity and the structure of TCV were examined by transmission electron microscopy on ultrathin sections after resin embedding (Fig. 1). Ninety % or more of the vesicles could be identified as CV. At high magnification (× 150,000), CV appeared as electron dense vesicles exhibiting the bristle border characteristic of the clathrin coat. The TCV population was rather homogeneous in size, the average diameter being about 75 nm.

The polypeptide composition of purified TCV analyzed by SDS-polyacrylamide gel electrophoresis is reported in Fig. 2A. The main component had an apparent molecular mass of 180 kDa. The analysis by Western blot (Fig. 2B) shows that 90 kDa polypeptide corresponded to the clathrin heavy chain. It was estimated from densitometric tracings that clathrin could account for 30-40% of total TCV protein, a value in agreement with previous reports on CV protein composition (32).

#### Ultrastructural Analyses

**Procedure for Resin Embedding—**CV pellets were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, and postfixed with 1% osmium tetroxide in the same buffer. After dehydration, pellets were embedded in Epon 812 epoxy resin. Ultrathin sections were post-stained with uranyl acetate.

**Procedure for Immunogold Labeling on Cryosections—**Pellets of isolated CV were fixed in 4% (w/v) paraformaldehyde, 0.5% (v/v) glutaraldehyde in 0.2 M Pipes, pH 7.5, for 2 h. Pellets were then washed in 0.2 M Pipes, pH 7.2, and immersed for 60 min in 0.2 M Pipes, 2.3 M sucrose, pH 7.2. The pellets, cut to give pyramids with a base of about 0.25 mm2, were mounted on a cryoholder and frozen in liquid nitrogen. Thin sections were obtained using a Reichert Ultracut E equipped with a cryo-system FC-4. Cryo-sectioning and immunolabeling were performed according to Griffiths et al. (27). For Tg immunolabeling were performed as described previously with monoclonal antibody and protein A complexes (a kind gift from G. Griffiths, European Molecular Biology Laboratory, Heidelberg).

Ultrathin sections embedded in Epoxy or processed for immunogold labeling were examined in a Jeol 1200 X electron microscope (Centre Commun de Microscopie Electronique, Faculté de Médecine Alexis Carrel, Lyon).

#### Isopycnic Centrifugation on Autogenerated Percoll Gradients

Percoll was made iso-osmotic by adding 9 volumes of Percoll to 1 volume of 2.5 M sucrose in 10 mM Tris-HCl, pH 7.4. This stock solution was diluted to 30% (v/v) Percoll with 10 mM Tris-HCl, 0.5% Pipes, pH 7.4 (TS buffer). About 1 ml of purified CV (0.5-2 mg of protein) or crude vesicle fractions was mixed with 22 ml of 30% Percoll and centrifuged at 25,000 rpm for 30 min at 4 °C in a fixed angle Beckman Ti-50.2 rotor. Fractions of 1 ml were collected from the top to the bottom. The density profile was determined by using density-marker beads (1.036–1.146 g/ml).

**Radioimmunoassay of Tg**

Tg was purified by velocity sedimentation on sucrose gradients from the protein solution obtained after mechanical opening of pig thyroid follicles on the metal sieve (23). Purified Tg was labeled with [125I]iodide using chloramine T (11). The same purified Tg preparation was used as standard to generate the reference curve. The liquid-phase radioimmunoassay was conducted as previously reported (11) using protein A absorbent (Pansorbin suspension) to collect immune complexes.

**Protein Analysis by Polyacrylamide Gel Electrophoresis and Western Blot**

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed as reported earlier (28). Western blot analysis was carried out as described previously (29) using anti-rabbit IgG, a heavy chain mouse monoclonal antibody (Boehringer Mannheim, Federal Republic of Germany). Antigen–antibody complexes on nitrocellulose sheet were detected using a secondary antibody (goat anti-rabbit or anti-mouse Ig antibodies from Antibodies Inc., Davis, CA) and 125I-labeled protein A. Autoradiography was carried out using X-Omat AR Kodak film.

**Other Methods**

Protein was assayed by the Lowry method, using bovine serum albumin as standard. Samples containing particulate material were pretreated with 1% (w/v) sodium deoxycholate to solubilize membrane protein. Protein in sucrose gradients was assayed according to Bradford (9). Depending on the amount of protein analyzed on the gradient, aliquots of 10–20 μl of each fraction were mixed with 0.5 or 1 ml of the Coomassie reagent. Results are given as absorbance at 595 nm. Since Percoll interferes in the Lowry and Bradford assays, the vesicle distribution on Percoll gradient fractions was estimated by turbidimetric measurements at 350 nm. Isolation of isolated CV fractions was assayed by the method of Sandell and Kolthoff (31) using a Technicon AutoAnalyzer.

Peroxidase activity was assayed using guaiacol as substrate (22). A guaiacol unit is defined as the amount of enzyme causing a change of 1 unit of absorbance at 470 nm/min.
Transport of Thyroglobulin by Coated Vesicles

**FIG. 1.** Electron micrographs of thyroid-coated vesicle preparations. The pellet of TCV resulting from centrifugation on sucrose-1H2O cushion was fixed and embedded in resin. Observations were made at different magnifications. The bars represent 200, 50, and 50 nm, respectively, from the left to the right.

**FIG. 2.** Analysis of the polypeptide composition of purified TCV by SDS-polyacrylamide gel electrophoresis (A) and Western blot (WB) (B). A, protein samples were analyzed on 6% acrylamide gels. The first lane on the left contains molecular mass markers, the sizes of which are given in kDa; middle lane (H) is thyroid cellular homogenate (90 μg of protein). The lane on the right (CV) contains purified thyroid coated vesicles (90 μg of protein). The arrows indicate the mobility of thyroglobulin monomer, Tg, and clathrin heavy chain (Cl). Numbers on the right of panel A identify three groups of polypeptides of 100-110 kDa, 45-55 kDa, and about 35 kDa reproducibly found in purified TCV. B, samples of 90 μg of protein of purified TCV were fractionated on 6% acrylamide gels and transferred onto a nitrocellulose sheet. Immunoreactive Tg and clathrin (Cl) were identified using polyclonal anti-pig Tg antibodies and a monoclonal anti-clathrin heavy chain antibody. Immune complexes were detected using 125I-labeled protein A and autoradiography. The middle lane corresponds to a portion of the acrylamide gel which was stained with Coomassie Blue.

**FIG. 3.** Sedimentation properties of TCV on 30% Percoll gradients. Purified TCV (about 1 mg of protein) or a crude thyroid vesicle fraction sedimenting between 8 X 10^6 g × min to 9 X 10^6 g × min (10 mg of protein) were mixed with 30% Percoll and centrifuged at 60,000 g for 30 min. Fractions of 1 ml were collected from the top to the bottom and the vesicle distribution was determined by A_{280} measurements. Open circles, purified TCV; closed circles, crude thyroid vesicle fraction. The fractions of the gradient containing crude thyroid vesicles were pooled as indicated on the figure (pool I–VI) and centrifuged at 150,000 g for 60 min. The pellets were resuspended in TS buffer and assayed for protein; aliquots of 100 μg of protein were fractionated on 6% acrylamide gels and transferred onto nitrocellulose. Immunoreactive clathrin was detected as in panel B of Fig. 2. The top of the figure contains the portion of the autoradiogram of the nitrocellulose sheet corresponding to the mobility of clathrin heavy chain. The horizontal bars and the dotted line indicate the location of density marker beads and the density profile, respectively.

and mitochondria, sedimented as a broad peak with density ranging from 1.04 to 1.06 g/ml; lysosomes present in fraction VI had a density ranging from 1.08 to 1.14 g/ml (22).

**Localization of Tg in Purified TCV Fractions**—The possibility of an artifactual association of Tg to TCV along the purification steps appeared very unlikely. Indeed, purified TCV did not exhibit any binding activity for exogenous labeled Tg, whatever the Tg concentration, from 80 pg/ml to 4 mg/ml (Table I).

Immuno-gold labeling with anti-Tg antibodies and chemical treatments have been used to precise the location of Tg in purified TCV preparations. The electron microscope observations of cryosections of isolated TCV further documented the structure of the vesicles. Using this sample preparation procedure, the membrane of the vesicles is clearly visible (Fig. 4). The average internal diameter of the vesicle was close to 50 nm whereas the mean diameter of the vesicle (with its coat) was about 85 nm. The interior of the vesicle appeared electron dense. Immuno-gold labeling with anti-Tg antibodies was used to visualize the presence of Tg into TCV. Anti-Tg antibody-protein A-gold complexes were observed either inside the vesicles or over the coat (Fig. 4). The specificity of the labeling was demonstrated by the absence of gold particles on TCV when anti-Tg antibodies were preabsorbed with purified Tg (result not shown). The location of gold particles over the coat does not necessarily indicate that Tg molecules are outside the vesicles. Indeed, due to the length of the immunoglobulin cross-bridge, a protein A-gold particle bound to a Tg-Anti-Tg antibody complex located on the internal side of the CV membrane can be found either in the center of the vesicle or on the other side of the membrane, on the coat.

Biochemical approaches were used to further ascertain that TCV actually contain Tg molecules. TCV were submitted to three different treatments: (i) high ionic strength treatment (1.5 M NaCl) to eliminate peripheral membrane proteins associated to particles on the basis on charge-charge interactions; (ii) a treatment by 0.5 M Tris, pH 7.0, or 10 mM Tris, pH 8.5, to induce the dissociation of the clathrin coat and therefore the removal of protein artifically attached or entrapped into the coat structure; and (iii) a treatment by...
Absence of association of soluble Tg to thyroid coated vesicles

Purified TCV (100 μg of protein) were incubated with 125I-Tg (60,000 cpm, 20 μg) without or with unlabeled Tg (0.15 or 1 mg) in 250 μl of MMEP buffer for 30 min at 4 °C. At the end of the incubation period, the mixture was layered on an 8% sucrose-2H2O cushion and centrifuged at 140,000 × g for 30 min at 4 °C. The supernatant corresponding to the H2O and 2H2O phases was separated as completely as possible from the pellet, and the two fractions were counted for radioactivity. An aliquot of 125I-Tg in absence of TCV was treated similarly. Tg was labeled using lactoperoxidase and glucose-glucose oxidase as H2O2 generating system. The specific radioactivity of 125I-Tg was 2 μCi/mg and the iodination led to an incorporation of three iodine atoms/molecule of Tg. Results are the mean of duplicate incubations.

| Conditions                      | 125I distribution after centrifugation on sucrose-2H2O cushion | %  | Pellet |
|---------------------------------|---------------------------------------------------------------|----|--------|
| 125I-Tg alone                   |                                                              | 98.2 | 1.8  |
| TCV + 125I-Tg                   |                                                              | 98.1 | 1.9  |
| TCV + 125I-Tg + Tg (0.15 mg)    |                                                              | 98.8 | 1.2  |
| TCV + 125I-Tg + Tg (1 mg)       |                                                              | 98.4 | 1.6  |

0.06% Triton X-100 to solubilize contaminating membrane or vesicles which could contain Tg. Coated vesicles are supposed to be not or only slightly affected by such a detergent treatment (35). As shown in panel A of Fig. 5, the polypeptide composition of purified TCV was not altered by the 1.5 M NaCl treatment. As expected, vesicles which sedimented after 0.5 M Tris treatment, no longer contained clathrin. Tg was still present in the resulting uncoated vesicle fraction. In contrast with previous treatments, the exposure of TCV to MMEP buffer containing 0.05% Triton X-100 yielded a sedimentable vesicle fraction with the same amount of clathrin, but with a reduced Tg content (panel B of Fig. 5). Intact Tg was recovered in the detergent supernatant together with some other components but no clathrin. The Triton X-100 treatment induced the release of soluble Tg and/or the solubilization of membrane-bound Tg present in contaminating non-coated vesicles. Indeed, under the conditions used, Triton X-100 was capable of solubilizing Tg from uncoated vesicles obtained by treatment of TCV by 10 mM Tris, pH 7.0, or 0.5 M Tris, pH 7.0 (for uncoating).

Attempts have been made to quantify the amount of Tg retained in Triton X-100-treated TCV using a Tg radioimmunoassay (Fig. 7). Results of panel A of Fig. 7 show that increasing amounts of TCV fractions (supernatants, or vesicles lysed by freezing-thawing) gave competition curves which were roughly parallel to the standard curve generated with pure Tg. TCV resuspended in MMEP buffer or 0.5 M Tris and pelleted by centrifugation at 140,000 × g had almost the same Tg content as untreated TCV; less than 10% was recovered in the supernatant. The Triton X-100 treatment induced the release of 50–60% of immunoreactive Tg; a value in keeping with the observations made on acrylamide gels. It must be noted that the total amount of immunoreactive Tg was increased about 1.5-fold in detergent-treated TCV as compared with that of TCV which were only freezed-thawed (column D versus column A of Fig. 7B). Assuming that the detergent treatment plus the freezing-thawing step allowed detection of all the Tg present, we estimated that Tg accounts...
TCV and TCV subfractions. A, competition curves between \(^{125}\)I-Tg and unlabeled pure Tg or TCV fractions in a soluble-phase radioimmunoassay. Labeled Tg (30,000 cpm, 2 ng) was incubated with increasing amounts (0.3-3,000 ng) of Tg or TCV fraction (1-30 \(\mu\)g) in the presence of 4 \(\mu\)l of a rabbit anti-Tg immune serum in a total volume of 500 \(\mu\)l of phosphate-buffered saline, supplemented with 5 mg/ml bovine serum albumin. After 18 h at 4 °C, immune complexes were collected using protein A adsorbent (Pansorbin). Prior to the assay, TCV or vesicle fractions resulting from TCV treatment were lysed by freezing-thawing to release Tg. The curves reported on the figure correspond to pure Tg (*) used as standard, supernatant (A), and pellet (B) of TCV resuspended in MMEP buffer (control medium) and centrifuged at 140,000 \(\times\) \(g\) for 30 min; supernatant (D) of TCV treated by 0.05\% Triton X-100 in MMEP buffer. \(^{125}\)I-Tg binding values (B) obtained in the presence of various amounts of Tg or TCV extracts are expressed as the percentage of the maximal binding value (Bmax) obtained in the absence of competitor. Each point represents the mean of triplicate. B, autoradiographic detection of \(^{125}\)I-Tg in CV prepared from prelabeled RTF-derived cells (lane 3). Pure Tg (lane 2) and CV prepared from intact thyroid tissue (lane 1) were used for comparison. Each sample contained about 20 \(\mu\)g of protein. Lanes 4, 5, and 7, Triton X-100; lanes 6 and 8, control buffer. Numbers on the right of the figure indicate the molecular mass in kDa of marker proteins.

Identification of Iodinated Tg in CV: Pulse-Chase Experiments on in Vitro RTF—Thyroid cells cultured in the presence of TSH in plastic Petri dishes allowing cell attachment reorganized in histotypic structures—thyroid follicles with morphological characteristics and metabolic activities corresponding to those of the intact tissue. The properties of in vitro RTF to iodinate Tg stored in the neoformed follicular lumina and to internalize iodinated Tg from that compartment, will be described in a separate report. For this study, we have taken advantage of this in vitro system to label Tg molecules which are subjected to the endocytotic process. Tg was metabolically labeled by incubating RTF with \(^{125}\)I-iodide for 1 h. After addition of methimazole, a peroxidase inhibitor, RTF-Thyroid cells cultured in the presence of TSH were resuspended in MMEP buffer (control) or in MMEP buffer containing 0.05\% Triton X-100. After 30 min at room temperature, the mixtures were centrifuged at 140,000 \(\times\) \(g\) for 45 min. Supernatants and pellets resuspended in MMEP buffer were assayed for iodine. Results were expressed for 100 \(\mu\)g of protein of purified TCV.

Identification of Iodinated Tg in TCV: Pulse-Chase Experiments on in Vitro RTF—Thyroid cells cultured in the presence of TSH in plastic Petri dishes allowing cell attachment reorganized in histotypic structures—thyroid follicles with morphological characteristics and metabolic activities corresponding to those of the intact tissue. The properties of in vitro RTF to iodinate Tg stored in the neoformed follicular lumina and to internalize iodinated Tg from that compartment, will be described in a separate report. For this study, we have taken advantage of this in vitro system to label Tg molecules which are subjected to the endocytotic process. Tg was metabolically labeled by incubating RTF with \(^{125}\)I-iodide for 1 h. After addition of methimazole, a peroxidase inhibitor, RTF were incubated for a short period of time (15 min) without or with TSH or dibutyryl cyclic AMP to activate \(^{125}\)I-Tg internaliza-
tation. The stimulation of endocytosis resulted in an increase of 125I-Tg in the cells and a corresponding decrease in the luminal compartment (see "Experimental Procedures" for the procedure used to separate cells from the intrafollicular material). The CV isolation procedure used to separate cells from the thyroid tissue both in terms of yield and material. The CV isolation procedure used up to now on intact thyroid tissue have been applied to labeled RTF-derived cells to answer three questions. (a) Can we find internalized 125I-Tg molecules in CV? (b) How much of the internalized labeled prohormone is present in purified TCV and Triton X-100-treated TCV? (c) Does TSH and/or dibutyryl cyclic AMP increase the amount TCV-associated 125I-Tg?

The electrophoretic analysis reported in panel A of Fig. 8 indicates that the CV fraction extracted from RTF-derived cells exhibited a purity comparable to that of CV extracted from the thyroid tissue. The fraction could be identified by the presence of clathrin. TCV prepared from RTF-derived cells had a low Tg content; no Tg band was visible after Coomassie Blue staining. The autoradiograms shown on panel B of Fig. 8 demonstrate the presence of intact 125I-Tg molecules in TCV and Triton X-100-treated TCV extracted from either untreated or TSH- or dibutyryl cyclic AMP-treated RTF. The amount of 125I-Tg in purified TCV was clearly augmented by both TSH and the cyclic AMP derivative. The quantitative analysis of 125I-Tg distribution in cell subfractions and isolated CV is reported in Table III. Purified TCV collected as pellets after differential centrifugation and centrifugation on the 3H2O-sucrose cushion contained 1.6-3.0% of the cell-associated 125I-Tg and accounted for 20-30% of 125I-Tg present in the vesicle fraction which sedimented at 140,000 x g. The treatment of TCV by 0.05% Triton X-100 led to a partial release of radioactivity; however, 60-70% of 125I-Tg remained bound to sedimentable vesicles after the detergent treatment. The radioactivity released by Triton X-100 mainly corresponded to labeled components with a molecular weight lower than that of Tg (panel B of Fig. 8). The amount of 125I-Tg present in Triton X-100-treated TCV expressed in percentage of total cell radioactivity was 2 fold higher in stimulated than in untreated cells (Table III).

**DISCUSSION**

The method of Nandi et al. (24) initially developed for the purification of brain CV gave satisfying results when applied to the thyroid epithelial tissue both in terms of yield and purity. The overall yield for TCV (1.7 mg of protein/100 g wet weight of tissue) was 2-3 fold lower than the yield for brain CV or CV from a number of sources (25). The difference is likely related to the presence of a very large non-cellular compartment, follicular lumina, in the thyroid. Indeed, intrafollicular soluble Tg stores represent 60-80% of total proteins in normal glands. The analyses of (a) the structure of purified vesicles by transmission electron microscopy, (b) the polypeptide composition of the total vesicle fraction by polyacrylamide gel electrophoresis, and (c) the dispersity of the vesicle population by sedimentation on Percoll density gradients, give evidence for the purity of TCV preparations. Considering the 180-kDa clathrin heavy chain as a CV marker and assuming that clathrin represents 0.15% of cellular proteins (37), one can estimate from the proportion of clathrin among purified TCV proteins (about 30-40%) that TCV were purified 200-300-fold as compared with the homogenate.

**TABLE III**

Effect of TSH and dibutyryl cyclic AMP (DBcAMP) stimulation on the 125I-Tg content of CV purified from prelabeled RTF-derived cells

| Cell treatment | Cells (A) | 800 x g supernatant | 140,000 x g pellet | Purified TCV | Triton X-100-treated TCV | B/A x 100 |
|----------------|----------|---------------------|-------------------|-------------|------------------------|---------|
| None           | 760 ± 43 | 275 ± 4             | 61 ± 2            | 12.4 ± 1.2 | 6.8 ± 0.1              | 0.90    |
| TSH, 10 milliunits/ml | 826 ± 30 | 301 ± 0             | 63 ± 2            | 10.0 ± 3.0 | 13.8 ± 2.6             | 1.67    |
| DBcAMP, 3 mM   | 847 ± 15 | 318 ± 2             | 80 ± 4            | 25.0 ± 1   | 15.8 ± 0.1             | 1.87    |

The Triton X-100 treatment of purified TCV did not change the amount of vesicle-associated clathrin but decreased by 40-60% (depending on the preparation) the Tg content of TCV. From these data, we are inclined to think that purified TCV preparations contained a small proportion of non-coated vesicles with a high Tg content. These vesicles could correspond to the scarce structures with very electron dense content observed on ultrathin sections prepared from resin-embedded samples (panel A of Fig. 1). Such vesicles might represent Tg-rich exocytotic or endocytotic structures. Tg molecules which remained associated to Triton X-100-extracted TCV and accounted for 3-4% of the detergent-insoluble protein fraction meet the criteria for a CV-transported protein. They remain into the vesicles resulting from Triton buffer-mediated TCV uncoating and are extracted from the uncoated vesicles by a Triton X-100 treatment. The amount of Tg transported into CV could be somewhat underestimated if one considers that all the Tg extracted by the
detergent exclusively corresponds to Tg present in contaminating vesicles. Indeed, some authors (38) think that the resistance of CV to Triton X-100 treatment could vary from tissue to tissue and that in some cases, the detergent could remove some receptors and proteins present inside CV.

Results of organic iodine assay in TCV and detergent-treated TCV are in keeping with the presence of iodinated Tg molecules inside TCV. If we assume that all the TCV-associat

The identification of radiiodinated Tg in TCV prepared from prelabeled RTF to indicate the data obtained on TCV isolated from intact thyroid tissue. The Tg content of TCV preparations obtained from RTF was almost insensitive to isolated from intact thyroid tissue. The Tg content of TCV containing vesicles seems to be higher in the former than in the latter situation. In the same way, the low Tg content of iodination level of Tg would range from 60 to 80 iodine of Tg. These molecular species could likely correspond to Tg labeled components with molecular weights lower than that of Tg. These molecular species could likely correspond to Tg degradation products generated in post-endocytotic contaminating organelles. The difference of effect of Triton X-100 on TCV isolated from intact tissue and TCV isolated from RTF was not unexpected since, potential contamination by Tg-containing vesicles, seems to be higher in the former than in the latter situation. In the same way, the low Tg content of CV extracted from RTF-derived cells as compared with that of CV purified from intact tissue could be related to differences in the concentration of Tg in the pool from which Tg is internalized, i.e., the follicular lumina. Indeed, although Tg accumulates in the follicular lumina of RTF, the concentration of Tg which is achieved after 4 days of culture is much lower than that reached in follicles in intact tissue.

The pulse-chase type of experiments conducted on RTF not only demonstrate the transfer of iodinated Tg molecules into CV, but clearly show that the process is hormonally regulated. TSH and dibutyryl cyclic AMP are shown (a) to be activators of the endocytosis of prelabeled lz51-Tg in the RTF system (the endocytosis being evidenced by the decrease of luminal 125I-Tg and the concomitant increase of cell-associated 125I-Tg) and (b) to increase the 125I-Tg content of CV. Interestingly, TSH and dibutyryl cyclic AMP had a more marked effect on the labeling of CV than on either the labeling of the bulk of vesicles or on the overall cell labeling. The relative low amount of 125I-Tg found inside CV (1-2% of intracellular radioactivity) seems to be compatible with the short half-life of CD45 or the short residence time of the ligand in CV (39, 40). It must be noticed that experiments on RTF were conducted at 20 °C to decrease post-endocytotic events and to try to increase CV accumulation (41). Since CV are supposed to carry only very few molecules (1-4) of internalized ligand (35, 42), it is likely that the TSH-induced increase of the labeling of CV increases to an increase in the number of CV rather than an increase of the 125I-Tg content of the CV. In conclusion, our observations show for the first time that the endocytosis of the thyroid prohormone Tg could proceed via a mechanism involving coated vesicles. It is there fore reasonable to think that Tg internalization, at least in part or in certain situations, brings into play a process of receptor-mediated endocytosis. Efforts will now be made to try to characterize the Tg receptor(s) which should mediate the sorting and the selective uptake of Tg molecules from the follicular lumen.

Acknowledgments—We would like to thank Dr. Hubert Borne (Pacifique de Medicin Grange Blanche, Universite Claude Bernard Lyon) for the iodine measurements and Drs. Alain Pauloin and Christophe Thurelbe (Laboratoire des Proteines, URA 1, CNRS Paris) for their valuable advices for CV isolation.

REFERENCES
1. Ericson, L. E. (1981) Mol. Cell. Endocrinol. 22, 1-24
2. Seljelid, R., Reith, A., and Nakken, K. F. (1970) Lab. Invest. 23, 595-605
3. Morell, A. G., Gregorjadis, G., Scheinberg, L. H., Hickman, J., and Ashwell (1971) J. Biol. Chem. 246, 1461-1467
4. Van den Hove, M. F., Couveure, M., De Visser, C., and Salvatore, G. (1982) Eur. J. Biochem. 212, 415-422
5. Van den Brandenbroeck, M. F., De Nayer, A., Hervey, J. P., and De Visser, M. (1981) Endocrinology 104, 383-399
Coated vesicles from thyroid cells carry iodinated thyroglobulin molecules. First indication for an internalization of the thyroid prohormone via a mechanism of receptor-mediated endocytosis.

F Bernier-Valentin, Z Kostrouch, R Rabilloud, Y Munari-Silem and B Rousset

*J. Biol. Chem.* 1990, 265:17373-17380.