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Thyroglobulin Molecules Internalized by Thyrocytes Are Sorted in Early Endosomes and Partially Recycled Back to the Follicular Lumen

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ABSTRACT

Thyroglobulin (Tg) molecules stored in thyroid follicle lumens are heterogenous in terms of iodine and hormone contents. It has been suggested that thyroid hormone is preferentially produced from the most highly iodinated Tg molecules and that thyrocytes are capable of selecting these molecules. The cellular localization as well as the molecular basis of such a selection process are not known. The present work was undertaken to determine whether there is selectivity at the step of endocytosis and, if not, to discover other possible mechanisms. Studies were conducted on reconstituted thyroid follicles (RTF) in culture. We compared the ability of thyrocytes to internalize Tg and an exogenous protein, BSA, which is neither iodinated nor glycosylated. To identify the protein, Tg and BSA were coupled to gold particles of different size and microinjected in a fixed ratio into the lumen of RTF. Neither of the two protein gold probes detected by transmission electron microscope bound at the cell surface, and both entered the cells at a similar rate and were concentrated in early endosomes. After 20 min, both Tg-G and BSA-G were segregated into distinct vacuolar structures. At 60 min, the intracellular content of BSA-G (mainly in prelysosomes and lysosomes) was 2- to 3-fold higher than that of Tg-G. At the same time, there was a marked reduction in the BSA-G/Tg-G ratio in the lumen. The differences between the Tg-G and BSA-G distribution patterns that were amplified in TSH-treated RTF are in keeping with a back-transfer of internalized Tg toward the lumen. The existence of a cell to lumen transport of previously endocytosed Tg was further documented using intralumenal [^{125}I]Tg as a marker. RTF pulse labeled with tracer amounts of [^{125}I]iodide were shortly incubated with TSH to induce [^{125}I]Tg endocytosis, and the fate of internalized [^{125}I]Tg was studied in a chase incubation period of up to 4 h. At 20 C, where the degradation of internalized Tg is blocked, we observed a time-dependent decrease in intracellular [^{125}I]Tg and a corrresponding increase in the lumenal [^{125}I]Tg content. This cell to lumen [^{125}I]Tg transfer was inhibited by primaquine.

In conclusion, our data show that 1) the thyroid apical endocytic process does not exhibit selectivity for Tg; 2) the thyrocyte possesses a sorting machinery for endocytosed ligands; and 3) internalized Tg molecules can be recycled back to the follicular lumen. These findings led us to propose a mechanism for the preferential utilization of well iodinated Tg in thyroid hormone production; high and low iodinated Tg molecules internalized at random would be sorted inside the cells, the former being conveyed to lysosomal compartments and the latter being recycled to the lumen for completion of hormone synthesis. This postulated pathway requires direct experimental groundings. (*Endocrinology* **132:** 2645-2653, 1993)

THE ENDOCYTOSIS of thyroglobulin (Tg) from the follicle lumen can take place through two different mechanisms: macropinocytosis or pseudopod-dependent endocytosis, leading to colloid droplet formation and micropinocytosis (1), or coated-vesicle dependent endocytosis (2). Depending on the animal species and the functional activity of the thyroid gland, the relative contributions of each of these two modes of Tg internalization vary considerably. The first mode of endocytosis, which enables the cell to take up large amounts of Tg, has clearly been demonstrated in the rat thyroid. In that species, there is indeed a need for a high capacity endocytic system. It can be easily calculated that the daily Tg utilization rate in rats, corrected for size, is 40–50 times higher than that in humans (3). The second mode of endocytosis is expected to be the preponderant operative system in the thyroid of the larger mammals (human, pig, *etc.*) under physiological situations. In these species, however, changes in thyroid function giving rise to an elevated and durable demand for Tg could require the involvement of the macropinocytic process.

Tg molecules stored in thyroid follicle lumens that are subjected to endocytosis are heterogeneous in terms of iodine and hormone contents. Besides the interfollicular heterogeneity related to the size and activity of the different follicles, there is an intrafollicular heterogeneity. In a given follicle, Tgs at different degrees of maturation (in regard to level of iodination and hormone content) coexist. This raises an important question for the overall thyroid economy: are these different molecules used (endocytosed and degraded) indifferently or specifically by thyrocytes? Attempts to answer this question by *in vivo* studies based on radioiodine turnover measurements have raised conflicting data (see Ref. 4 for a review) largely due to the interfollicular heterogeneity of the Tg pool.

In vitro studies conducted on rat hemilobes led to the conclusion that the more iodinated Tg molecules are internalized and degraded more actively than less iodinated molecules (5). This selectivity has been explained by a preferential uptake of the most iodinated Tg molecules. Two different mechanisms have been proposed, one applies to macropinocytosis and the other to micropinocytosis. Al-

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though not selective in nature, macropinocytosis, through the projection of pseudopods toward the center of the follicle lumen, could collect protein at some distance from the apical cell surface and, thus, avoid internalization of the most newly synthetized and probably the less iodinated Tg molecules located close to the apical cell surface. Micropinocytosis, which brings into play coated vesicles, could operate a positive selection of mature Tg molecules through apical membrane receptors in a process ressembling receptor-mediated endocytosis. Alternatively, the sorting of Tg molecules to internalize could result from a negative selection, with the low iodinated molecules being protected from endocytosis by binding to plasma membrane. This mechanism has been termed selective fluid pinocytosis (5).

Despite a number of studies of radioiodinated Tg binding to thyroid slices, isolated open follicles, and membrane fractions, the existence of Tg-binding sites on the apical plasma membrane of the thyrocytes has not yet been established. Using open follicle preparations (obtained by collagenase treatment of pig thyroid tissue) in which apical plasma membrane Tg-binding sites should be preserved and accessible to an exogenous labeled probe, such as Tg complexed to gold particles, we have been unable to find any specific interaction of the ligand with the apical border of the thyrocytes (unpublished data). This failure could be related to a difference between the natural intralumenal physicochemical environment required for Tg binding and the artificial external medium we used. To overcome this potential limitation, we decided to reinvestigate the question using *in vitro* reconstituted thyroid follicles and Tg-gold complexes microinjected into intact follicle lumens. This experimental approach has given us the opportunity to determine whether Tg binds to the apical plasma membrane and to further analyze the Tg endocytic pathway and the intracellular fate of internalized Tg. A control exogenous molecule, BSA, complexed to gold particles of a different size was comicroinjected with the Tg-gold probe to test the specificity or selectivity of the Tg transport steps. Contrary to what was expected, we did not obtain evidence for a selective endocytic process, but we found that thyrocytes possess an intracellular sorting machinery, which could be involved in the preferential utilization of the more mature Tg molecules for hormone production.

Materials and Methods

Thyroid cell culture

Reconstituted thyroid follicles (RTF) were obtained as previously reported (2, 6). In brief, thyroid cells isolated from pig glands by discontinuous trypsinization were cultured in F-12 medium supplemented with 10% calf serum and TSH (1 mU/ml) in tissue culture-treated petri dishes under an 95% air-5% CO_2 atmosphere at 37 C. Follicles formed within 36–48 h were used on day 3 or 4 of culture.

Preparation of gold probes

Colloidal gold suspensions were prepared according to the method of Slot and Geuze (7). Pig Tg was purified by velocity sedimentation on sucrose gradient from the protein solution obtained after mechanical opening of thyroid follicles by passing tissue fragments through a metal seive (8). Tg-gold (Tg-G) and BSA-gold (BSA-G) complexes were prepared from 9- or 15-nm gold particles, as previously reported (9). The number of Tg or BSA molecules bound per gold particle was determined using radioiodinated Tg or BSA of known specific radioactivity. Tg and BSA were labeled with [^{125}I]iodide using lactoperoxidase and glucose-glucose oxidase as the hydrogen peroxide-generating system. The concentration of gold particles in the final preparations was estimated from the ratio between the number of Tg- or BSA-gold particles and latex beads in a mixture spread on Formvar- and carbon-coated grids. Latex bead suspensions with a known concentration of particles were kindly provided by Dr. Michael Wurtz (University of Basel, Switzerland) or were purchased from Interfacial Dynamic Corp. (Portland, OR). The average numbers of protein molecules per 9- and 15-nm gold particles were, respectively, 3.3 and 4.5 for Tg and 17.2 and 50.6 for BSA. When two types of protein-gold particles of different size were mixed, the exact ratio between the two probes was controlled in two different ways. First, the mixture was sprayed over Formvar- and carbon-coated grids, as mentioned above, for the determination of gold-particle concentration. Second, an aliquot of the mixture was diluted in water and mixed with 2% (wt/vol) methylcellulose (viscosity at 25 C, 25 centipoises). Electron microscope grids were placed on drops of methylcellulose-gold particle mixtures and removed using a wire loop. The methylcellulose film covering the grid was air dried, and the grids were examined in a JEOL 1200 EX electron microscope (Centre commun de Microscopie Electronique, Faculté de Médecine Alexis Carrel, Lyon, France). Particle counting was made on randomly taken micrographs at a magnification of ×15,000. A minimum of a 1000 gold particles was counted for each Tg-gold/BSA-gold mixture.

Microinjection and quantitative analysis of protein-gold probe distribution

Protein-gold probes. Tg-G and BSA-G were comicroinjected into the lumen of RTF, as previously described (6). The concentrations of Tg-G and BSA-G were between 10^{14}-10^{13} particles/ml. To control the quality of the microinjection, the distribution of fluorescein isothiocyanate-labeled dextran added to the gold-particle suspension was visualized under fluorescence illumination. The conditions for cell incubation and sample processing for electron microscope observations were similar to those reported previously (9). Nine to 12 follicles were examined in each condition. Ultrathin sections of 70 nm (50–150 sections/follicle) through the microinjected follicles were examined, and those containing gold particles were photographed. All of the gold particles (small and large) found over the cells were counted. In all experimental conditions, gold particles remaining in the lumens were also counted on randomly taken photographs. Particle counting was performed on at least 50 different photographs/condition.

Metabolic labeling of intralumenal Tg and quantitative analysis of its transport inside the follicles

Three-day-old RTF were deprived of TSH for 16 h. On day 4, intralumenal Tg was radioionated, as previously described (2). RTF were incubated with carrier-free [^{125}I]iodide (~10 μCi/dish) in F-12 medium for 60 min at 20 C. After removal of medium and washing in F-12 medium containing 1 mM methimazole and 1 mM sodium perchlorate to block subsequent iodination, RTF were incubated in the latter medium for 20 min at 20 C in the presence of TSH (10 mU/ml) to stimulate [^{125}I] Tg internalization. RTF were washed again and incubated (chase period) in F-12 medium supplemented with the above-mentioned inhibitors for up to 4 h at 20 or 37 C. To analyze the [^{125}I]Tg distribution between cells and lumen, RTF were washed with ice-cold F-12 medium and incubated in PBS supplemented with 3 mM EDTA and 1 mM methimazole for 30 min at 0–4 C. This treatment causes the disassembly of tight junctions (6) and the release into the medium of the intralumenal material. Cells were gently detached from the dish using a rubber policeman, and the cell suspension was centrifuged at 100 × g for 5 min at 4 C to separate cells from the released intralumenal soluble material. Protein-bound radioiodine ([^{125}I]PBI) in cells or medium was measured as the radioactivity precipitated by 10% trichloroacetic acid using BSA as carrier protein. Polyacrylamide gel electrophoresis in the presence of sodium

dodecyl sulfate and autoradiography demonstrated that more than 80% of the [^{125}I]PBI actually correspond to [^{125}I]Tg.

Statistical analyses

The statistical significance of the changes in the relative distributions of Tg-G and BSA-G either in the cells or over the lumina under various circumstances was analyzed by the χ^2 test. For example, the numbers of Tg-G and BSA-G particles found in lumina 60 min after microinjection of control RTF (see Table 1) were compared to the corresponding numbers obtained in the lumina of acutely TSH-stimulated RTF in a 2 × 2 table.

The statistical analysis of data deriving from radioactivity measurements was performed using Student's *t* test.

Results

Electron microscope follow-up of Tg-G and BSA-G microinjected into the lumen of RTF: identification of an intracellular sorting process

Tg-G (15-nm particles) and BSA-G (9-nm particles) introduced in a 1:1 ratio into the lumen of RTF by microinjection remained dispersed into this space. Whatever the time after microinjection, the number of Tg-G found on or close to the apical plasma membrane of the thyrocytes delimiting the follicle lumen was low and not different from the number of BSA-G on or close to the membrane. The same result was obtained with Tg-G (9 nm) and BSA-G (15 nm). Within 5 min after microinjection, Tg-G and BSA-G were found in small vesicles just beneath the apical plasma membrane. Many vesicles exhibited the typical structure of coated vesicles. Tg-G and BSA-G were internalized as single particles, but were also found together in the same vesicle (Fig. 1). Small vesicles containing one or few particles were observed at all incubation times (up to 120 min) at 37 C. The entry of gold particles inside the cells was blocked when RTF were incubated at 4 C. Twenty minutes after microinjection, Tg-G and BSA-G appeared in larger vacuolar structures with the morphological characteristics of early endosomes (EE). EE were the first structures in which Tg-G and BSA-G accumulated (Fig. 2A). Gold particles were mostly localized at the periphery of the endosome in either narrow clefts or distended tubules or cisternae bordering the vacuole and surrounding an electron lucent internal space (Fig. 2, A, B, and D). In some rare structures, there was an accumulation of a very high number of particles of both types (Fig. 2C). After 60 min, gold particles appeared in vacuoles located deeper in the cytoplasm with an electron-dense interior; these structures probably corresponded to late endosomes and lysosomes previously characterized (9) (Fig. 2E). Already at 20 min and more frequently at later times, we observed vacuolar structures of different sizes containing only one type of particles, Tg-G or BSA-G, in the vicinity of vacuoles containing both probes (Fig. 3). The segregation of Tg-G was more evident than that of BSA-G. Surprisingly, the gold particles that were predominantly observed in thyrocytes 60 min after Tg-G and BSA-G microinjection were those complexed to BSA. Tg-G in small vesicles or in vesicles resembling EE were found in the vicinity of late endosomes and lysosomes that mainly contained BSA-G. The same observation was made when Tg was complexed to 15-nm particles

TABLE 1. Quantitative analysis of the distribution of BSA-G and Tg-G in the lumenal and cellular compartments of follicles microinjected with the two probes and incubated for 60 min at 37 C

TSH treatment	No. of gold particles in				BSA-G to Tg-G ratio in	
	Lumen		Cells		Lumen	Cells
	BSA-G	Tg-G	BSA-G	Tg-G		
None	2488	1484	974	245	1.67	3.97
Acute	2967	1966[a]	596	88[a]	1.51	6.77
Chronic	1009	719[b]	1179	167[b]	1.40	7.06

Tg and BSA were coupled to 15- and 9-nm gold particles, respectively. The BSA-G/Tg-G ratio in the microinjected mixture was 1.8. RTF used in these experiments were either deprived of TSH for 16 h (no treatment), deprived of TSH and then incubated with 10 mU/ml TSH during the experimental period (acute treatment), or maintained in the standard culture conditions, *i.e.* in the presence of 1 mU/ml TSH (chronic treatment). Gold particle counting was performed on at least 50 different cell sections in each condition. The statistical significance of the TSH-induced changes in the relative distribution of BSA-G and Tg-G in either the lumenal space or over the cells was analyzed by the χ^2 test. In each experimental situation, the relative distribution of BSA-G and Tg-G in the lumen was statistically different from that over the corresponding cells ($P < 0.001$).

[a] $P < 0.025$ *vs.* BSA-G.

[b] $P < 0.001$ *vs.* BSA-G.

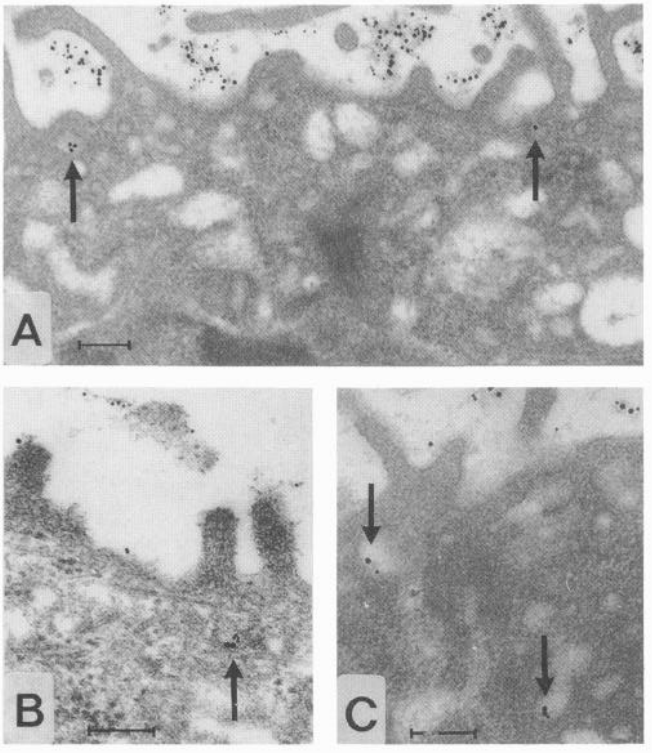
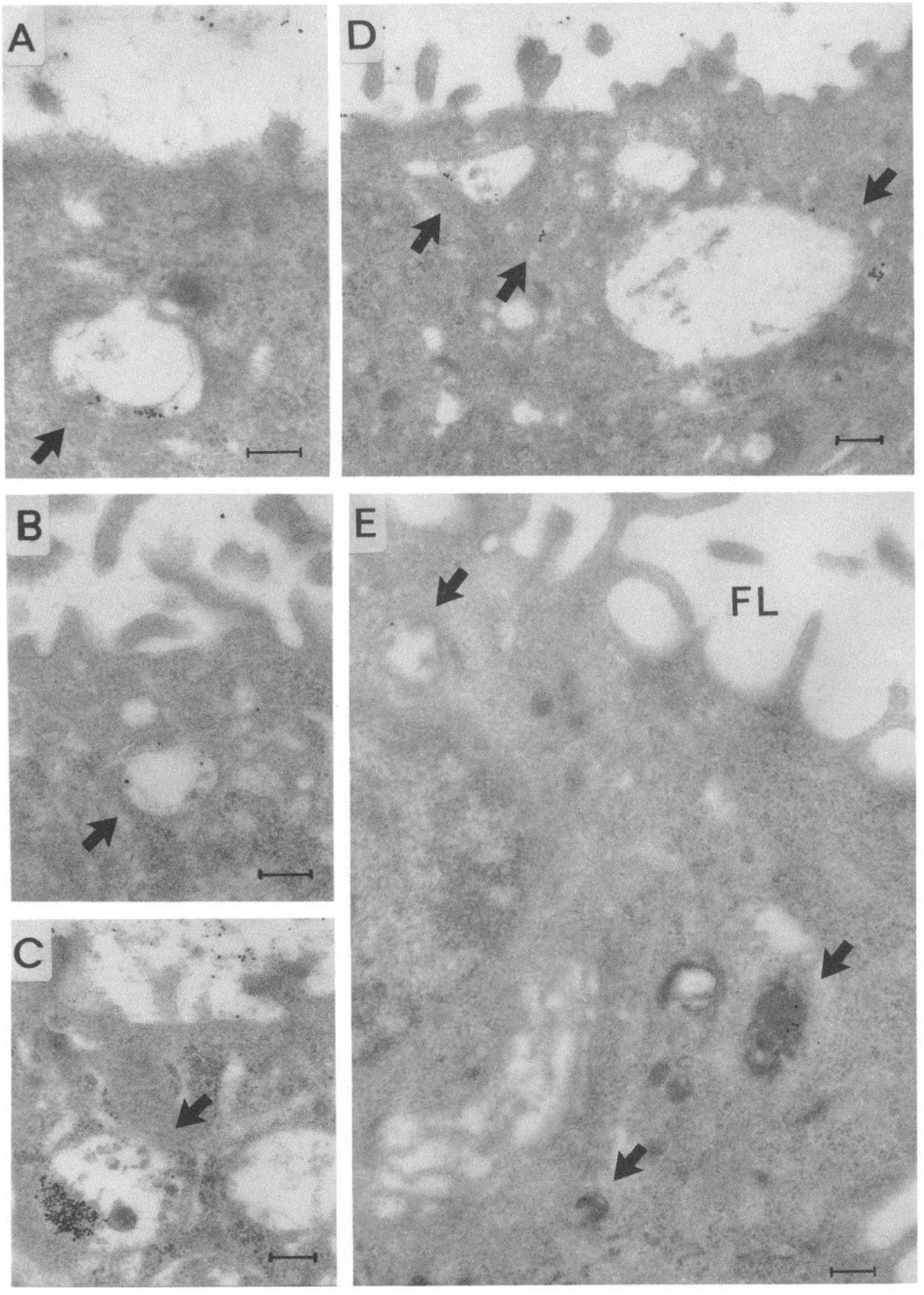


FIG. 1. Intracellular localization of Tg-G and BSA-G 5 min after microinjection of the two protein-gold probes in the lumen of RTF. Tg and BSA were complexed to 15- and 9-nm gold particules, respectively. A 1:1 mixture of the two types of particles was microinjected at 20 C, and RTF were incubated for 5 min at 37 C. Large particles alone or together with small particles are present in plasma membrane invagination (A) and subapical vesicles (A–C). The vesicle (in B) probably corresponds to a coated vesicle; it contains both BSA-G and Tg-G. *Arrows* identify vesicles that contain gold particles. *Bars* = 200 nm.



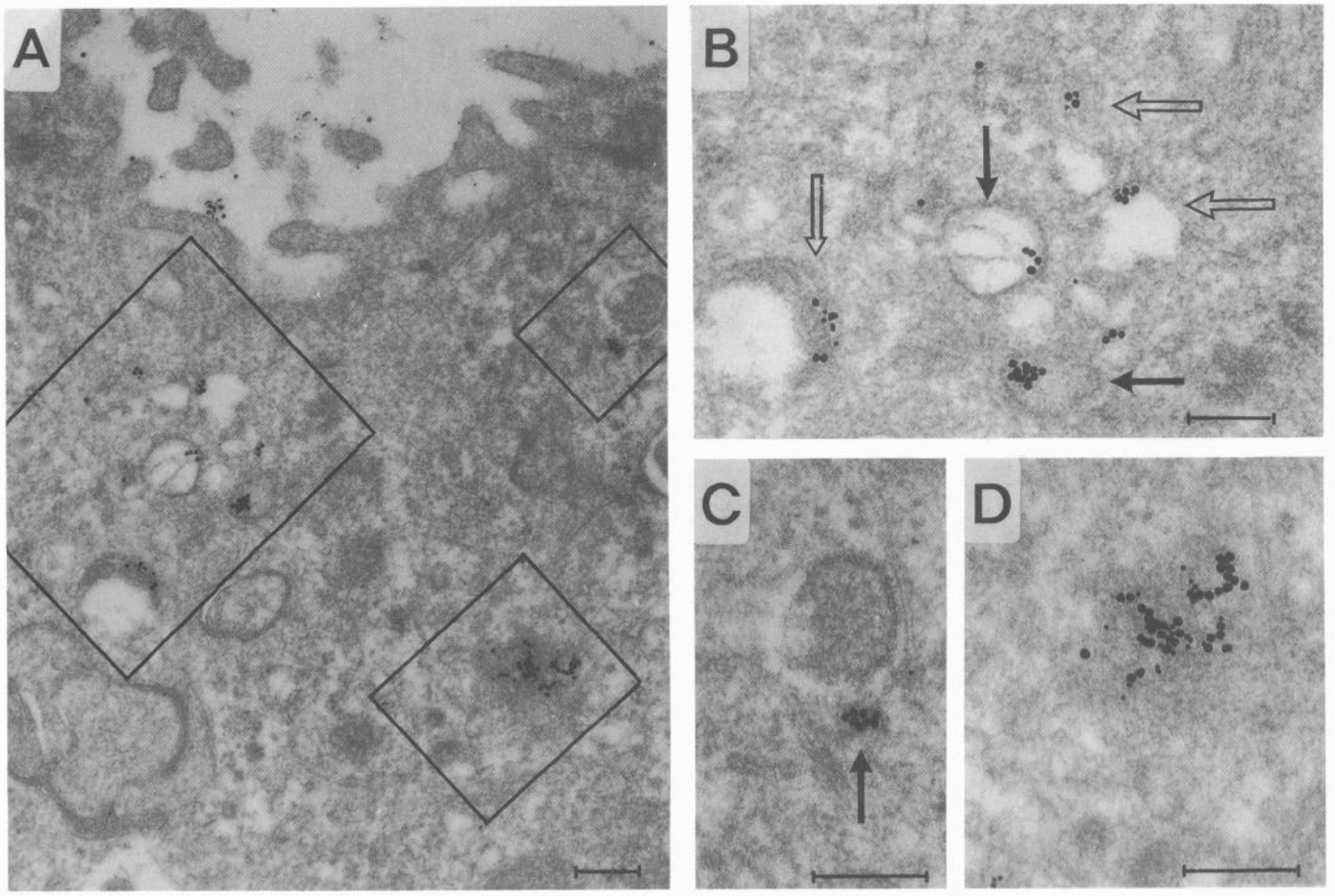


FIG. 3. Ultrastructural evidence for an intracellular sorting of internalized Tg-G and BSA-G. Tg-G (15 nm) and BSA-G (9 nm) were comicroinjected in a 1:1 ratio into the lumen of RTF. RTF were then incubated for 60 min at 37 C. A shows a section through a cell that contains gold particles in different types of vesicular structures. The three distinct regions of the cytoplasm delimited by frames in A are presented at a higher magnification in B, C, and D. On the *left side* of B, a typical early endosome contains both BSA-G (small gold particles) and Tg-G (big gold particles). In the vicinity of this structure, vacuoles or vesicles of different sizes and electron densities appear to contain either one gold probe (*closed arrows*) or the two probes together (*open arrows*). C shows another case where Tg-G appears segregated in a vesicle; a small particle (BSA-G) is present in a tubular structure nearby. In contrast, the electron-dense structure (enlarged in D), possibly corresponding to a late endosome, contains a mixture of Tg-G and BSA-G. *Bars* = 200 nm.

and BSA to 9-nm particles and when Tg was complexed to small and BSA to big gold particles.

Quantitative analysis of the internalization and intracellular accumulation of Tg-G and BSA-G: evidence for a process of recycling

To determine whether the differential intracellular behavior of BSA-G and Tg-G was related to a difference in the rate of internalization of the two probes, we performed a quantitative time-course analysis of Tg-G and BSA-G uptakes. Lumens of RTF were microinjected with Tg-G and BSA-G in a 1:1 ratio. Results are reported in Fig. 4. It is apparent that the internalization of Tg-G and BSA-G occurred at the same rate. The numbers of Tg-G and BSA-G particles per 100-cell sections were the same for up to 20 min. In accordance with what was observed (but not quantified) in the previous experiments, there was a marked difference between the Tg-G and BSA-G cell contents after 60 min of incubation. Such a result could be explained by a selective transport of Tg-G out of the cells either toward the basolateral pole in the culture medium or toward the apical

FIG. 2. Intracellular distribution of Tg-G and BSA-G 20 min (A–C) or 60 min (D and E) after microinjection of the two probes in the lumen of RTF. Tg and BSA were respectively complexed to 9- and 15-nm gold particles in A, D, and E and to 15- and 9-nm gold particles in B and C. In all cases, a 1:1 mixture of the two types of particles was microinjected at 20 C, and RTF were incubated for 20 or 60 min at 37 C. Gold particles are found in electron-translucent vacuoles corresponding to early endosomes and at later times (60 min) in electron-dense structures, prelysosomes (or late endosomes) and lysosomes. Early endosomes represent sites of concentration of gold particles of both sizes (see A). An extreme but rare example of concentration is reported in C. D shows the presence of gold particles in tubular extensions or vesicles in the vicinity of early endosomes. E illustrates a common observation: late endosomes and lysosomes predominantly contain BSA-G (big gold particles). *Arrows* identify structures that contain gold particles. *Bars* = 200 nm.

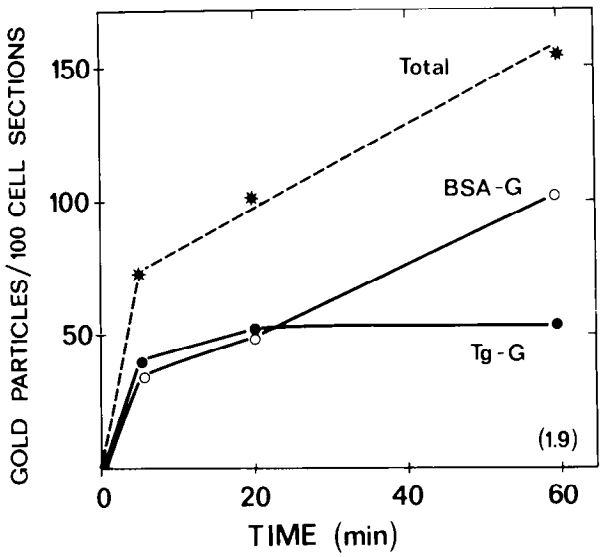


FIG. 4. Time-course analysis of intracellular accumulation of Tg-G and BSA-G. A 1:1 mixture of Tg and BSA, respectively, complexed to 15- and 9-nm gold particles was microinjected into the lumen of RTF. RTF were then incubated for 5, 20, or 60 min at 37 C. At each time point, 150 cell sections containing nuclei were examined under the electron microscope, and each area containing gold particles was photographed. The total number of particles (*) and the number of particles of each size (○, BSA-G; ●, Tg-G) were expressed per 100-cell sections. The *numbers in parentheses* indicate the BSA-G/Tg-G ratios at 60 min.

pole in the follicle lumen. It has been possible to test the second hypothesis experimentally. Indeed, a significant retrotransport of Tg-G into the lumen should result in a detectable decrease in the BSA-G to Tg-G ratio in this compartment. We have tried to detect such changes in RTF under different conditions of stimulation: RTF deprived of TSH for 16 h (basal state), and RTF deprived of TSH and acutely stimulated by TSH and RTF under chronic TSH treatment (Table 1). In the three experimental situations, the relative distributions of BSA-G and Tg-G inside the cells were different from those observed in the lumen. There was an intracellular accumulation of BSA-G compared to Tg-G, and this phenomenon was more marked in TSH-treated RTF; the intracellular BSA-G/Tg-G ratio increased from about 4 in control follicles to 7 in either chronically or acutely TSH-treated follicles. Opposite changes were found in the lumen. The intralumenal BSA-G/Tg-G ratio, after 60 min of incubation of RTF under basal conditions, was slightly lower than the BSA-G/Tg-G ratio at time zero, *i.e.* the BSA-G/Tg-G ratio in the microinjected mixture. The intralumenal BSA-G/Tg-G ratio decreased in response to TSH stimulation and became significantly lower than the initial BSA-G/Tg-G ratio (1.51 and 1.40 in acutely and chronically TSH-treated follicles *vs.* 1.8 in the microinjected mixture). The decrease in the intralumenal BSA-G/Tg-G ratio was of lower amplitude than the increase in the intracellular BSA-G/Tg-G ratio because the changes in the lumen were buffered by the presence of noninternalized BSA-G and Tg-G. Similar results were obtained using Tg coupled to 15-nm particles and BSA coupled to 9-nm particles or *vice versa* (data not shown).

Demonstration of a cell to lumen transfer of endogenous metabolically labeled [^{125}I]Tg

To validate the results obtained with exogenous Tg coupled to gold particles, we analyzed the fate of endogenous Tg metabolically labeled with tracer amounts of [^{125}I]iodide. The design of the experiment has been based on the Tg endocytosis assay system previously described (2). The experimental protocol was divided into three steps: 1) Tg that accumulates into the lumen of RTF was pulse labeled with [^{125}I]iodide; 2) intralumenal [^{125}I]Tg was internalized by thyrocytes in response to a short term (15-min) TSH treatment of RTF; and 3) the fate of intracellular [^{125}I]Tg was studied during a chase period of 4 or 5 h (Fig. 5), during which radioiodination of Tg was blocked.

When the chase incubation was performed at 37 C, the amount of [^{125}I]Tg inside the cells decreased with time, whereas the intralumenal [^{125}I]Tg content remained rather constant. About 65% of [^{125}I]Tg present in RTF (cells plus lumen) disappeared in 5 h. This is in keeping with our previous observations showing that at 37 C, internalized [^{125}I]

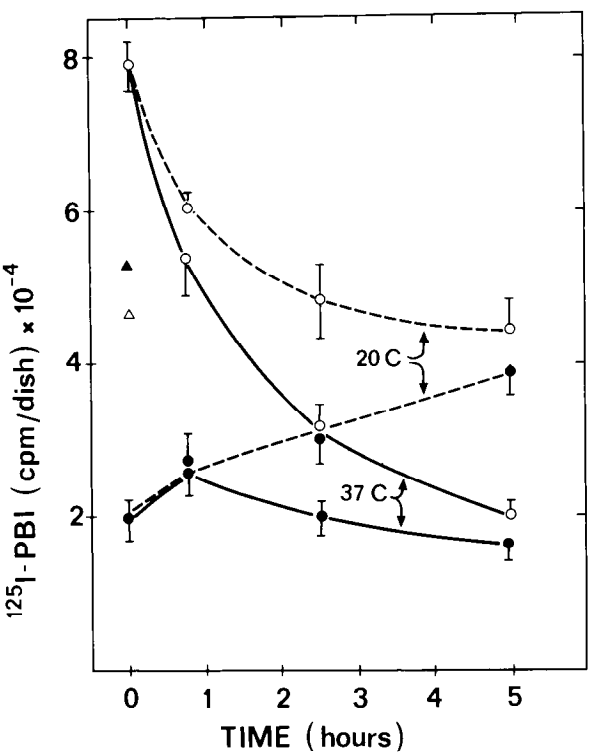


FIG. 5. Fate of internalized [^{125}I]Tg in reconstituted thyroid follicles. Evidence for a cell to lumen transfer or recycling process. Three-day-old RTF deprived of TSH for 16 h were pulse labeled with [^{125}I]iodide for 60 min at 20 C (intralumenal Tg labeling) and preincubated with TSH (10 mU/ml) for 20 min at 20 C in a medium containing 1 mM methimazole and 1 mM sodium perchlorate (Tg endocytosis). RTF were then incubated for 45 min to 5 h at 20 or 37 C (chase period). Lumenal and cellular [^{125}I]Tg (^{125}I-PBI) contents were determined after a 30-min treatment of RTF in PBS and 1 mM EDTA at 0–4 C. The cell-associated (△) and intralumenal (▲) [^{125}I]Tg contents of RTF at the end of the labeling period are shown. ○, Cell-associated [^{125}I]Tg; ●, intralumenal [^{125}I]Tg. ○ and ● at time zero give the cell-associated and intralumenal [^{125}I]Tg contents, respectively, after short term TSH treatment. TSH induced a 5-fold increase in the endocytic index (2). —, 37 C; - - -, 20 C.

Tg is subjected to proteolysis, and there is production of free thyroid hormones, which are secreted into the medium (2). When the chase incubation was performed at 20 C, which blocks entry of internalized ligands into lysosomes (10), we observed a decrease in the intracellular [^{125}I]Tg content and a concomitant increase in intralumenal [^{125}I]Tg. This result shows the existence of a cell to lumen transport or recycling pathway for previously internalized Tg molecules. The total amount of [^{125}I]Tg present in RTF after 5-h incubation at 20 C was decreased by about or less than 15%. The sorting and recycling of various types of receptors or ligands in different cells have been shown to be inhibited by weak bases such as primaquine (11). The results summarized in Table 2 show that the cell to lumen transport of Tg was abolished when RTF were incubated in the presence of such a compound during the chase period. The primaquine inhibitory action was evident after 2 h and was more marked after 4 h (compare lines 2 and 3 and lines 4 and 5 of Table 2). Primaquine not only prevented the reappearance of [^{125}I]Tg into the lumens, but caused a depletion of [^{125}I]Tg in the lumens. Indeed, the intralumenal [^{125}I]Tg content after 2 or 4 h of chase was lower than that at time zero (the onset of the chase). The same observation was made when the chase incubation was performed at 20 or 37 C. The primaquine-induced changes in the [^{125}I]Tg distribution pattern are also apparent using the ratio between intracellular and intralumenal labeled Tg contents; an increase in this ratio indicates an intracellular accumulation, and a decrease a depletion. At the end of the TSH treatment applied to induce the internalization of Tg, the ratio was equal to 6.6; it reached a value of 9.3 in RTF treated with primaquine for 4 h, whereas it declined to 2.6 in untreated RTF (Exp I of Table 2).

Discussion

The morphological and functional properties of pig RTF have been described in recent reports (2, 6, 9). This experimental system has allowed us to better characterize the Tg internalization process known as micropinocytosis (2) and to define the cellular compartments, the endosomes (9) involved in the transport of Tg internalized via coated vesicles. Part of this work on the implication of endosomes in Tg traffic has been based on the use of Tg-gold complexes microinjected into the lumen of thyroid follicles. This technique has now been used to analyze the ability of polarized thyrocytes in the form of RTF to "handle" two different molecules: Tg and an exogenous protein, serum albumin. Albumin is known to be naturally present in low amounts in thyroid follicles. Recent data from De Vijlder *et al.* (12) clearly indicate that the so-called thyroid albumin originates from the blood. Therefore, the use of serum albumin as a marker for nonselective endocytosis bears an intrinsic interest. Indeed, albumin that could enter the follicle lumen by basal to apical transcytosis (13) is expected to be cleared from or metabolized by thyrocytes. We found that the apical thyroid membrane does not differentiate between Tg and BSA. This finding has an important implication; it allows further definition of the process of Tg internalization. The coated vesicle-dependent endocytosis of Tg, therefore, is related to fluid phase endocytosis. Such a mechanism is in keeping with the fact that the Tg molecules to be internalized are highly concentrated into the follicle lumen and can enter coated pits without the intervention of receptors. The amount of Tg internalized by thyrocytes, therefore, would depend on the concentration of Tg in the lumen and the rate of formation of endocytic vesicles, a process regulated by TSH (2, 14). Endocytic vesicles internalizing both BSA-G and Tg-G delivered their content to early endosomes. These endocytic structures appeared as sites of concentration and sites of sorting of internalized ligands. Since BSA-G and Tg-G enter the cells at the same rate, and since protein-gold complexes are not metabolized by the cells, the different distributions of BSA-G and Tg-G imply the existence of a differential treatment of the two ligands by the cells. The observation of a relative enrichment of Tg-G compared to BSA-G in the follicle lumen provides an explanation for the relative depletion of the Tg-

TABLE 2. Inhibitory effect of primaquine on the cell to lumen transfer of internalized [^{125}I]Tg molecules

Exp no.	Chase incubation conditions			[^{125}I]Tg (cpm) × 10^{-2}		Cells/lumen
	Duration (h)	Temperature (C)	Primaquine	Lumen	Cells	
I	0			525 ± 44	3475 ± 67	6.6
	2	20	−	726 ± 12[a]	3021 ± 205	4.2
	2	20	+	419 ± 39[b]	3019 ± 320	7.4
	4	20	−	934 ± 78[a]	2447 ± 140[a]	2.6
	4	20	+	346 ± 15[a,b]	3228 ± 229[b]	9.3
II	0			809 ± 43	3383 ± 218	4.2
	2.5	20	−	1036 ± 46[a]	3198 ± 163	3.1
	2.5	20	+	489 ± 38[a,b]	3504 ± 379	7.2
	2.5	37	−	827 ± 38	2381 ± 162[a]	2.9
	2.5	37	+	330 ± 14[a,b]	2453 ± 170[a]	7.4

Three-day-old RTF were deprived of TSH for 16 h and pulse labeled with [^{125}I]iodide for 60 min at 20 C. ^{125}I-Labeled RTF were washed and preincubated with TSH (10 mU/ml) for 20 min at 20 C to induce [^{125}I]Tg internalization. RTF were washed and incubated without or with 1 mM primaquine for 2 or 4 h at 20 C (Exp I) or for 2.5 h at 20 or 37 C (Exp II). At the indicated time (time zero of the chase period or at the end of the incubation), the [^{125}I]Tg contents of the lumens and cells were determined after 30-min treatment of RTF in a calcium-free medium at 0–4 C. Results are the mean ± SEM of triplicate determinations.

[a] Statistically different from the values obtained at time zero (no incubation), $P < 0.01$.

[b] Statistically different from control values (without primaquine), $P < 0.01$.

G intracellular content and demonstrates the existence of a phenomenon of transport of internalized Tg from the cell to the lumen, *i.e.* a phenomenon of recycling of Tg. These new aspects in the complex pathway of Tg traffic are of potential importance for understanding intrathyroidal iodine metabolism. Indeed, intracellular sorting and recycling could represent the processes by which thyrocytes exert a selectivity in Tg utilization. To verify these results, we used a different labeling procedure, radioiodination instead of complexation to gold. The data from the second experimental protocol confirmed the ability of thyrocytes to transfer back into the follicle lumen previously internalized Tg molecules. The proportion of endocytosed [^{125}I]Tg undergoing recycling was rather high when the chase incububation was performed at 20 C, a temperature at which the transfer to prelysosomes or late endosomes and lysosomes is blocked. Recycling of [^{125}I]Tg also took place at 37 C, as can be seen when comparing the lumenal [^{125}I]Tg content with and without primaquine treatment of RTF. In the presence of primaquine, there was a marked reduction of the lumenal [^{125}I]Tg content, indicating that the constant level of the lumenal [^{125}I]Tg content in untreated RTF could result from equilibrated fluxes in (endocytosis) and out (recycling) of the cells. Internalization and recycling of macromolecules are common and well known processes in many cell types. They involve vesicle shuttling from the plasma membrane to endosomal compartments and from endosomal compartments to the plasma membrane. In every cell, the transport of molecules in and out of the cell is coupled with the maintenance of the membrane pool of each cell compartment. In thyrocytes, the loss of apical plasma membrane surface due to the formation of endocytic vesicles could be compensated for in part by the addition of the membrane of recycling vesicles transporting Tg molecules.

Both electron microscope observations of the Tg-gold particle distribution and biochemical analyses of the fate of metabolically labeled Tg indicate that only part of the Tg molecule would be subjected to recycling. Indeed, internalized Tg molecules reached lysosomal compartments; Tg-G particles were found to accumulate together with BSA-gold particles into prelysosomes and lysosomes, and radioiodinated Tg was degraded, leading to the generation of free thyroid hormones. A partition of Tg between progression in the endocytic pathway and recycling is in keeping with the known Tg heterogeneity, *i.e.* the existence of Tg at different degrees of iodination and hormone content. The obvious hypothesis that can thus be proposed is that high and low iodinated Tg molecules internalized at random are sorted inside the cells, with high iodinated Tg conveyed to lysosomal compartments for hormone production and low iodinated Tg recycled to the lumen, during which process further iodination can occur. This hypothesis, which is based on the existence of an intracellular sorting machinery, is substantiated by certain data in the literature. It was reported some time ago by Consiglio *et al.* (15–17) that preparations of membranes from bovine thyroid (probably composed of plasma membranes and membranes from intracellular organelles) contained receptors for Tg. These receptors bound low iodinated Tg (which also contained more exposed *N*-acetyl glucosamine residues) more tightly than highly iodinated Tg with a lesser content of exposed *N*-acetyl glucosamine residues. These results were extended by Miquelis *et al.* (18), who identified a 45-kilodalton protein with *N*-acetyl glucosamine-binding activity. Cytochemical analysis with fluorescently labeled *N*-acetyl glucosamine-BSA indicated that the receptor protein is mainly located in subapical compartments. Binding was maximal at an acidic pH. Our results permit a cytological explanation of these biochemical results. We found that there was no specificity in the uptake of Tg by plasma membrane receptors, since a nonspecific protein such as BSA, which has no sugar, is taken up identically to Tg. Furthermore, both proteins, for as long as 20 min, pass into identical vacuolar structures, early endosomes. It is only at this step that binding of poorly iodinated Tg, presumably because of its exposed *N*-acetyl glucosamine residues, can occur. The binding can be favored by the acidic pH of the endosomal compartment. After this step of recognition, receptor-ligand complexes would be seggregated and transported to the apical surface; at this site, dissociation and release of low iodinated Tg into the lumen can occur because the pH of the lumenal fluid is expected to be neutral. Meanwhile, nonspecific proteins, such as BSA and highly iodinated Tg, are transported to late endosomes and lysosomes, where they are hydrolyzed. Such biochemical and cellular events are known to occur in many cell types for many macromolecules and/or their receptors. The above-developed molecular and cellular mechanisms that could explain the selectivity in Tg utilization remain to be further tested experimentally.

As presented in the introduction of this paper, depending on the physiological situations and the animal species, thyrocytes internalize Tg by two different processes: macropinocytosis and micropinocytosis. The concept of sorting and recycling developed above applies to Tg molecules internalized by micropinocytosis or coated vesicle-dependent endocytosis, the operative system in our experimental model; it could also apply to Tg entering the cells in large vacuoles by macropinocytosis. The requirement would be the fusion between the vacuoles of resorption or colloid droplets and endosomes containing the molecular components involved in Tg sorting. A functional relationship between colloid droplets and endosomes has been proposed by Herzog (19). If that is the case, the intracellular steps of Tg processing would be independent of the mode of Tg internalization.

In conclusion, we report the experimental basis of a new mechanism that can explain how thyrocytes exert a selection of Tg molecules to use for hormone production, a phenomenon often discussed but still not understood.

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