Regulated and Constitutive Protein Targeting Can Be Distinguished by Secretory Polarity in Thyroid Epithelial Cells

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Abstract. We have studied concurrent apical/basolateral and regulated/constitutive secretory targeting in filter-grown thyroid epithelial monolayers in vitro, by following the exocytotic routes of two newly synthesized endogenous secretory proteins, thyroglobulin (Tg) and p500. Tg is a regulated secretory protein as indicated by its acute secretory response to secretagogues. Without stimulation, pulse-labeled Tg exhibits primarily two kinetically distinct routes: ~80% is released in an apical secretory phase which is largely complete by 6-10 h, with most of the remaining Tg retained in intracellular storage from which delayed apical discharge is seen. The rapid export observed for most Tg is unlikely to be because of default secretion, since its apical polarity is preserved even during the period (<10 h) when p500 is released basolaterally by a constitutive pathway unresponsive to secretagogues. p500 also exhibits a second, kinetically distinct secretory route: at chase times >10 h, a residual fraction (~<8%) of p500 is secreted with an apical preponderance similar to that of Tg. It appears that this fraction of p500 has failed to be excluded from the regulated pathway, which has a predetermined apical polarity. From these data we hypothesize that a targeting hierarchy may exist in thyroid epithelial cells such that initial sorting to the regulated pathway may be a way of insuring apical surface delivery from one of two possible exocytotic routes originating in the immature storage compartment.

Epithelial cells have been reported to exhibit at least two kinds of exocytotic routing operations aimed at different targets: (a) polarized secretory pathways to the apical and basolateral surfaces (Kondor-Koch et al., 1985; Gottlieb et al., 1986; Caplan et al., 1987; Rindler and Traber, 1988); and (b) secretory pathways exhibiting different physiological regulation, including the segregation of "regulated secretory proteins" (Burgess and Kelly, 1987) in condensing vacuoles for intracellular storage (which, by the time they have become a fully mature storage compartment, may be out of the bulk flow of constitutive vesicular membrane traffic that also travels from the Golgi complex to the cell surface; Pfeffer and Rothman, 1987; Klausner, 1989).

Despite recent claims concerning the mechanism of molecular sorting into the regulated secretory pathway (Chung et al., 1989), we still do not know what, if any, relationship exists between the two kinds of sorting and targeting operations listed above, other than they are both initiated in the trans-Golgi or trans-Golgi network (TGN) (Griffiths and Simons, 1986; Orci et al., 1987; Tooze and Hutner, 1990) and can continue within condensing vacuoles/immature granules (Hashimoto et al., 1987; Sossin et al., 1990). Since recent evidence suggests that in addition to the pathways men-

1. Abbreviations used in this paper: 8Br-cAMP, 8 bromo cyclic adenosine monophosphate; Endo H, endoglycosidase H; NCS, newborn calf serum; Tg, thyroglobulin; TGN, trans-Golgi network; THS, thyroid stimulating hormone.
regulated pathway implies that a fraction of the newly synthesized secretory protein is retained in a Golgi/post-Golgi storage compartment (Palade, 1975; Salpeter and Farquhar, 1981; Kelly, 1985) from which it is delivered to the cell surface slowly (Mains et al., 1987; Arvan and Castle, 1987); secretagogue addition should accelerate release from this regulated compartment.

Herein we show the concurrent operation of stimulated and unstimulated exocytotic routes carrying Tg selectively to the apical cell surface, in which the unstimulated apical route may be a diversion pathway from immature granules. In contrast, another endogenous secretory protein of high molecular weight (p500), after a period of basolateral constitutive secretion, spontaneously reverses its secretory polarity to a preferentially apical discharge similar to that of Tg, and also exhibits a small secretagogue-induced apical secretion. Multiple sorting signals on p500 that confer differential secretory targeting are possible, but the data are most consistent with the hypothesis that a fraction of p500 fails to be excluded from the regulated storage pathway which has a predetermined apical polarity in thyroid epithelial cells.

Materials and Methods

Cell Culture

Primary thyroid follicular epithelial cells isolated from fresh porcine thyroid tissue by sequential trypsinization in the presence of EGTA according to published procedures (Mauchamp et al., 1979) were washed twice in 15 ml iced MEM containing 10% newborn calf serum (NCS) at the end of the digestion to quench residual trypsin. Cells were then seeded at high density (1 × 10⁶ cells/cm²) in DME containing 0.5% NCS onto Transwell-Col filters (containing penicillin, streptomycin, and amphotericin) without serum. Based on the presence of mitotic figures (not shown), it appears that cell division occurs on the filter surface. However, if a monolayer is not visibly confluent by day 4, it generally does not proceed to confluence and is not usable for experiments. Where indicated, thyroid stimulating hormone (TSH 0.1 mU/ml) was added on day 2 and removed on day 5 before experiments, which were performed routinely on day 7. This TSH treatment increased cellular and secreted Tg levels but had no effect on the fraction of Tg which was endoglycosidase H (Endo H) resistant, the relative secretory stimulation by secretagogue, or the secretory polarity of Tg. Epithelial monolayers remain stable, confluent, and viable for at least several weeks in culture.

Electrical Resistance and [¹⁴C]Inulin Permeability of Epithelial Monolayers

Transfer factor measurements were made (EVOM; World Precision Instruments Inc., New Haven, CT) on successive days of culture using identically treated filters without cells as a control. Minor fluctuation was observed for filter-grown monolayers (depending on the positioning of the electrodes); cells grown in TSH tended to have higher resistance values. To quantitate [¹⁴C]inulin permeability studies, ±1 μCi of [¹⁴C]inulin (New England Nuclear, Boston, MA) premixed in MEM was added to the apical chamber above monolayers or control filters at 37°C, and proportionate volume samplings of apical and basal media were removed for counting at different incubation times. A direct correlation was observed between the measured rise in transepithelial resistance, decrease in inulin permeability, and confluent appearance by phase microscopy.

Cell Labeling

For pulse-chase studies, filter-grown monolayers were starved either for methionine, cysteine, or both, in appropriately deficient DME for 30 min at 37°C. The cells were then labeled for 5–30 min in the same medium containing [³⁵S]methionine (New England Nuclear), by the addition of 1.0–0.5 mCi of radioactivity to the basolateral chamber. At the conclusion of the pulse, the cells were washed three times above and below the filter in a chase medium containing >100-fold excess of unlabeled amino acid, before returning the cells to the incubator in MEM. In certain experiments, 10 mM NH₄Cl was added to the DME for chase incubations. In kinetic studies, the apical and basal media were completely removed and replaced with identical fresh chase media (in those experiments using NaAcetate, the addition of fresh acetate by each medium change helped to minimize the possibility of acetate loss of cellular metabolism to cholesterol). To the removed media was added 1/20 volume of antiprotease cocktail to yield a final concentration of 0.02% BSA, 1 mM leupeptin, 10 mM pepstatin, 5 mM EDTA, 1 μg/ml aprotinin, 1 mM diisopropylfluorophosphate; in some experiments, iodoacetamide or dithiothreitol were added to a final concentration of 5 or 40 mM, respectively. In the experiment shown in Fig. 2 A, immediate TCA precipitation of the medium was employed so that protease inhibitors were not used. For steady-state labeling, filter-grown monolayers were continuously labeled for 2 days (>6 half lives for Tg) in complete MEM plus the addition of 0.25–0.5 mCi radiolabeled methionine, cysteine, or both to the basolateral chamber. Immediately before such experiments, the filters were rapidly washed above and below with PBS to remove unincorporated and secreted label. Pulse-chase and steady-state labeling experiments were terminated by adding to the monolayers an ice-cold solution containing 130 mM NaCl, 5 mM EDTA, 1% Na deoxycholate, 1% Triton X-100, 0.1% SDS, and the same mixture of protease inhibitors described above, for 20 min with agitation. Media and cell lysate samples were collected and spun for 5 min in a microfuge (Beckman Instruments, Palo Alto, CA) at 4°C to remove insoluble debris.

Tg Immunoprecipitation

Antisera were raised in rabbits to denatured monomeric porcine Tg purified by SDS-PAGE, or to rat Tg which was found to cross-react with porcine Tg (>100,000 dilution by ELISA). An excess of Tg antibody (5–10 μl serum) or an equal volume of pre-immune rabbit serum, was added to an aliquot of apical media, basal media, or cell lysate, and the sample was mixed overnight at 4°C. 50–100 μl of protein A agarose beads (diluted 1:5) either were added during the overnight incubation or mixed for a subsequent 1 h. The immunoprecipitates were then washed 4 times with 0.5 M NaCl in 25 mM Tris-HCl, pH 7.5, and once with H₂O.

Endo H Digestion

Steady-state labeled cells were washed twice in ice cold PBS and lysed as described above. To 10 μl of either Tg immunoprecipitate or cell lysate were added: 10 μl of 2 M NaSCN, 10 μl of 0.4 M NaCitrate pH 5.3, and 10 μl of either 5 μl Endo H (Boehringer Mannheim Biochemicals, Indianapolis, IN) in 50 mM NaPhosphate pH 7.0, or 10 μl of the buffer without Endo H. The samples were then digested or mock-digested at 37°C for 16 h with gentle mixing, the analyzed by SDS-PAGE and fluorography.

One-Dimensional Tryptic Mapping

Bands were excited from dried gels after exposure to x-ray film for identification. Excised bands were reswelled for 10 min in 0.1% SDS, 0.1 M Tris-HCl, pH 7.4, and placed in wells overlying a second 3–15% gradient SDS-PAGE. To these wells were added 50 μl of a 250 μg/ml toyosynthetic-alanine chloromethylketone-treated trypsin solution and a gel sample buffer which contained a reduced (0.1%) final concentration of SDS and 50 mM dithiothreitol. The samples were electrophoresed as usual through stacking and resolving gels.

SDS-PAGE and Fluorography

Unless otherwise stated, SDS-PAGE analysis of media, cell lysates, and immunoprecipitates, employed straight 4 or 3.75% gels according to Laemmili (1970). Gels were impregnated with Amplify (Amersham) or 1 M Na Phosphate, pH 7.0, or 1 M Na acetate or direct analysis by SDS-PAGE was comparable. Gels were transferred to nitrocellulose (Schleicher and Schuell) and probed with specific antisera, or, in preliminary studies, relative recovery of secreted Tg by either immunoprecipitation or direct analysis by SDS-PAGE was comparable.

Materials

Porcine TSH was obtained from Dr. A. F. Parlow via the National Hormone...
and Pituitary Program, NIDDK; bovine TSH and other stock chemicals were from Sigma Chemical Co. (St. Louis, MO). Translucent 24 mm, 0.4 μm pore filters were “Transwell-Col” from Costar, Data Packaging (Cambridge, MA). In preliminary studies specifically examining translucent filters from different suppliers, the use of the Costar product (as described in Materials and Methods) greatly facilitated our ability to generate stable filter-grown monolayers.

Results

Thyroid Epithelial Cells Form Polarized Monolayers on Porous Filters

Despite their origins as primary cultures, dispersed thyroid cells, enriched in epithelial cells relative to other cell types, are further enriched when seeded in low (<0.5%) concentrations of serum such that pure epithelial cell lines can be derived from these cultures (Ambesi-Impiombato and Coon, 1979; Fayet and Hovsepian, 1985). The low serum concentration appears to favor attachment and selective viability of the epithelial cells, resulting in minimal contamination by fibroblasts, C cells, macrophages, or endothelial cells (Ambesi-Impiombato and Coon, 1979; Fayet and Hovsepian, 1980; Fayet et al., 1982). We have seeded porcine primary thyroid cells in low serum on translucent filters that permit visualization of live (unfixed, unstained) cells by phase microscopy (Fig. 1). Although the quality of these phase images is variable because of the refractive properties of the filters, this method offers the advantage that the same cells can be used for experimentation or further culture after microscopic examination. Consequently, cultures can be monitored to follow the change from single refractile cells (day 0) to progressive attachment and spreading to confluence (day 2-4).

On day 1 postseeding, transfilter electrical resistance (see Materials and Methods) was similar to that of control filters without cells, whereas between days 2 and 4, a striking increase in transfilter resistance was observed. This increase varied between preparations, corresponding to a value of

![Figure 1](https://example.com/figure1)

Figure 1. Low-power phase-contrast microscopy of live thyroid epithelial cells cultured on transparent filters: progressive attachment, spreading, and growth to confluency. Primary porcine thyroid epithelial cells were prepared and seeded atop Transwell-Col filters as described in Materials and Methods. On the day of seeding (A), the cells are round and refractile before attachment to the filter. On day 1 (B), at a focal plane between the filter surface and the apical medium, some cells can be seen to have attached to the filter (faint streaking represents the fibers of the filter itself), while many refractile unattached cells still remain. By day 2 of culture (C), fewer unattached refractile cells remain and the filter is largely covered with a monolayer of follicular cells. At day 4 of culture (D), a confluent monolayer of polarized thyrocytes is evident across the filter surface with few residual unattached cells. At this time, transepithelial resistance is in the range of 200–1,000 Ω × cm² and transfilter permeability to labeled inulin is exceedingly low. These parameters establish the existence of epithelial tight junctions, a feature of thyroid epithelial cells which are not observed in parafollicular cells, endothelial cells, macrophages, or fibroblasts. Bar, 100 μm.
Figure 2. Thyroglobulin is secreted apically in a rapid unstimulated secretory phase during which release of a second protein (p500) is detected. Confluent filter-grown thyroid epithelial monolayers (cultured in the presence of 0.1 mU/ml TSH on days 2–5) were either continuously labeled for 24 h (A), or pulse labeled for 10 min with [35S]methionine/cysteine, washed three times in chase medium containing excess unlabeled amino acid, and then incubated for sequential 1-h-long chase periods in fresh apical and basolateral chase medium without secretory stimulators (B). At the conclusion of either incubation protocol, proportionate aliquots of apical and basolateral media were analyzed separately by nonreducing SDS-PAGE and fluorography. (A) After continuous labeling, the two most readily visible labeled protein bands in TCA precipitates are p500, which has a basolateral (Bas) secretory preference (see text), and Tg, which is apically (Ap) predominant. The position of the 200-kD standard is indicated; a 116-kD standard runs near the dye front. (B) After pulse chase, the secretion of labeled Tg and p500 (as quantitated by scanning densitometry) exhibited phasic release with a peak at 2–3 h of chase (note the scale difference between the two proteins). Although each protein exhibits kinetics of secretion which is similar on opposite sides of the filter, Tg secretion is predominantly apical, whereas p500 is secreted with mild basolateral preference. At the end of 10 h, ≈80% of newly synthesized Tg is released apically, ≈7% is released basolaterally, and ≈13% is retained in Golgi/post-Golgi storage (see text).
200–1,000 Ω-cm². In addition, asymmetric exposure of filter-grown monolayers to [¹⁴C]inulin revealed only trace permeation in confluent cultures. These results indicate the presence of functional tight junctions.

Polarized thyroid epithelial cells in vivo are known to secrete Tg, a homodimeric glycoprotein of ≈660 kD, predominantly to the apical extracellular space (Haddad et al., 1971). To establish that the filter-grown monolayers we used were properly polarized, we examined the directionality of Tg secreted from cells either continuously labeled for 24 h (Fig. 2 A), or pulse labeled with [³⁵S] amino acids and chased for 10 h, removing and replacing the chase medium at hourly intervals (Fig. 2 B). In either case, Tg was secreted with a high degree of fidelity to the apical extracellular space from the filter-grown cells. In contrast, when labeled Tg was added to control filters without cells, it appeared to equilibrate after overnight incubation (data not shown; and Chambard et al., 1987). In addition, a second band of high molecular weight (discussed below) was released preferentially at the basolateral surface under both labeling conditions.

Examination of Intracellular Tg in Filter-grown Thyroid Monolayers

In preliminary studies, SDS-PAGE analysis of cell lysates suggested the presence of more than one intracellular band of mobility similar but not identical to that of secreted Tg. Since Tg contains many N-linked oligosaccharide side chains of both core and complex types (Arima et al., 1972), we attempted to relate the mobility of Tg to the processing of its N-linked glycans. Tg in cell lysates of steady state-labeled monolayers were digested with Endo H (see Materials and Methods). In mock-digested samples, intracellular Tg migrated either as a broad band (Fig. 3) or as a doublet. Endo H treatment converted the broad band into sensitive (faster-migrating) and resistant (slower-migrating) species. To see which subregion of the undigested Tg band contained the Endo H–sensitive material, we divided the original broad band into two portions, and digested each portion separately.

with Endo H: material in the upper portion contained the Golgi-modified Endo H–resistant form, whereas material in the lower portion contained Tg which shifted substantially to the Endo H–sensitive position. The slight mobility shift of the Golgi/post-Golgi form upon digestion corresponds to a similar shift for Tg secreted into the culture medium (Fig. 3) and agrees with previous reports that a few N-linked glycans on mature Tg are never processed to complex sugars (Spiro and Spiro, 1985). Quantitation of Tg immunoprecipitated from steady state–labeled cells indicates that ≈50% of the intracellular Tg is Endo H resistant; this substantial

Table I. Quantitation of Tg Released by the Regulated Apical Secretory Pathway

| Treatment          | Secreted 0–30 min | Secreted 0–90 min |
|--------------------|-------------------|-------------------|
| Control (n = 4)    | 6.9 ± 5.9         | 13 ± 11           |
| 8Br-cAMP (n = 3)   | 25 ± 6.2          | 35 ± 9.6          |

Confluent filter-grown thyroid epithelial monolayers were labeled to steady state, washed, and then stimulated with 8Br-cAMP (1 mM) for 30 or 90 min, and Tg then immunoprecipitated from the apical medium and cell lysates as in Fig. 4. Quantitative data were obtained by densitometric scanning of the Tg band after electrophoresis of the immunoprecipitates. The total radiolabeled Tg in the apical media samples and cells was assigned 100% (i.e., basolateral secretion was not taken into account since these bands were negligible). The net above the unstimulated control at 90 min was 22%; note that most of the stimulated release occurred within the first 30 min after secretagogue addition.
fraction has reached or passed the medial Golgi complex. (Dunphy and Rothman, 1985).

**Thyroglobulin Is Released Apically by a Regulated as well as a "Constitutive" Secretory Pathway**

Although electron microscopic surveys of thyroid epithelial cells on porous filters do not reveal morphologically obvious secretory granules (Barriere et al., 1986; Penel et al., 1989; and data not shown) we searched for the presence of a regulated secretory pathway in two ways. First we examined the effect of secretagogues on cells in which Tg was labeled to (or near) steady state, to maximize detection of regulated secretion (Moore and Kelly, 1985; Sporn et al., 1986). In cells continuously radiolabeled for 2 d, after removal of the labeling medium, secretagogue addition (1 mM 8 bromo [8Br]-cAMP) resulted in a prompt increase in apical Tg release into fresh medium. Regulated discharge of Tg was specific and not because of cell injury, since high resolution (3.75% nonreducing) SDS-PAGE showed that stimulated apical release of Tg into the medium coincided with a decrease in the cellular content of Tg corresponding to the upper (Endo H-resistant, Golgi) band (Fig. 4). The same result was obtained whether the Tg was analyzed by direct immunoprecipitation or direct fluorography of SDS-gels (shown below). Quantitation of a series of such experiments revealed that Tg secreted in the presence of 8Br-cAMP was stimulated >threefold (Table I), with most of the stimulated secretion occurring promptly (within 30 min) after secretagogue addition. The Tg released by this pathway contained only (25-6.9%) 20% of total cellular Tg, but this represents 40% of the Tg found in Golgi/post-Golgi compartments, assuming half of the cellular Tg in the steady state is at or beyond the Golgi compartment (Fig. 3) and that the faster-migrating (Endo H-sensitive) form of Tg is little affected by secretagogue addition (Fig. 4). From these data it is clear that the storage capacity of Tg in the regulated secretory pathway is easily measurable, yet relatively small. Similar results were observed when the secretagogue was TSH (not shown). Basolateral Tg release was barely detectable during these 30-min intervals and could not be stimulated with 8Br-cAMP (not shown).

In a second approach we estimated the fraction of newly synthesized Tg that was retained in prolonged Golgi/post-Golgi storage in the absence of secretory stimulation, after constitutive pathways were largely depleted of label. For this purpose, the fractions of pulse-labeled Tg secreted apically, basolaterally, and that remaining intracellularly were analyzed in a 10-h pulse-chase experiment (Fig. 2 B). During this time newly synthesized Tg was released in a single kinetic phase that was largely complete by 6 h (Fig. 2 B). This unstimulated phase of apical secretion accounted for a majority (>80%) of the Tg synthesized during the pulse period. About 7% was secreted to the basolateral side of the filter and the remainder (>13%) was retained intracellularly. The latter fraction is in a Golgi/post-Golgi compartment, since by 8 h of chase, virtually all of the pulse-labeled Tg was resistant to the action of Endo H (P. Arvan, manuscript in preparation). Past 10 h, prolonged unstimulated collections of media showed continued apical discharge of Tg (described further below) which could be stimulated by secretagogue addition, suggesting the presence of labeled protein in regulated secretory vesicles. Although we did not establish a discrete chase time at which newly synthesized Tg becomes maximally stimulable, from these data it appears that most Tg is secreted relatively rapidly even in the absence of stimulation, while a smaller fraction can be secreted apically in a regulated manner.

**The Endogenous Protein p500 Is Released Constitutively at the Basolateral Surface**

The secretion of a second protein of high molecular weight (p500) became apparent primarily because it was resolved by low percentage SDS-PAGE of basolateral media (Fig. 2 A). p500 appeared to contain subunits, because reduction of sulfhydryl groups retarded the mobility of Tg. The secretion of a second protein of high molecular weight (p500) became apparent primarily because it was resolved by low percentage SDS-PAGE of basolateral media (Fig. 2 A). p500 appeared to contain subunits, because reduction of sulfhydryl groups retarded the mobility of Tg.

Unlike Tg, the constitutive release of p500 at times up to 10 h of chase exhibited a mild basolateral polarity preference (Fig. 2 B). Although there was slight variability between ex-
monolayers (Fig. 5; polarity (Caplan et al., 1987; Parczyk and Kondor-Koch, that perturbation of intraorganellar pH may affect secretory labeled protein secretion from filter-grown thyroid epithelial
tected basolaterally, as opposed to an 8-10-fold apical polar-

1989), we examined the effect of such a treatment on pulse-

netic delay in secretion of both proteins is evident in

Figure 6. Quantitation of the fractional release of p500 and Tg to

regarding the relative apical polarity observed for Tg (Fig. 5; late chase, 1 and 2). Densitometric quantitation of this experi-

netic delay of apical secretion was evident), whereas p500 secretion converted to a predominantly apical pattern. By contrast, addition of the weak acid Na acetate (10 mM) had no effect on apical Tg secretion, while p500 secretion was predominantly basolateral (Fig. 5; early chase, panel 4). These data seem to indicate that newly synthesized Tg and p500 initially take different exocytotic routes which are the result of mechanisms that are relatively pH insensitive and pH sensitive, respectively.

A Fraction of p500 Behaves as If It Is Missorted to The Regulated Apical Secretory Pathway

The above evidence taken as a whole suggested the presence of two possible apical secretory alternatives for Tg, as well as a basolaterally predominant secretory alternative exemplified by p500. We also tested to see if any exocytosis of p500 could be elicited from the Golgi/post-Golgi storage compartment. After a 15-min pulse labeling, apical and basolateral media were collected at 6-h-long chase intervals, followed by a single 4-h collection to optimally drain label from the secretory pathways not involved in protein storage; finally a 14-h chase period was collected, with the intention of obtaining roughly comparable amounts of radioactive secretory protein in the different blocks of time (Figs. 5 and 6). The apical/basolateral distributions of p500 and Tg secretion were essentially constant in each of the first 6 h as well as the 4-h interval from 6-10 h of chase, with a mild basolateral preference for p500 and strongly apical-polarized Tg release. However during the final collection which is increasingly enriched in secretory product deriving from the Golgi/post-Golgi storage compartment, p500 secretion was notably altered: 60-70% was now found apically, approaching the relative apical polarity observed for Tg (Fig. 5; late chase, 1 and 2). Densitometric quantitation of this experiment is shown in Fig. 6, indicating that ≈8% of secreted p500 is found apically in the delayed chase collection from control cells, and this was not blocked by weak bases or acids (Fig. 5; late chase, 3 and 4; and Fig. 6). The increase in relative apical secretion of p500 at late chase times was observed with every preparation of cells examined (n = 10).

To see if the change in secretory polarity was because of leakage or transcytosis of p500 during the final prolonged incubation, radioactive basolateral (or apical) medium removed early in the chase was placed into the basolateral (or apical) chamber contacting nonradioactive monolayers, and these samples were incubated in parallel with the usual 14 h collection. The basolateral p500 and Tg incubated in this manner were effectively impermeant with respect to the filter (Fig. 7). To establish that the p500 band was indeed the same protein on both sides of the filter as well as at early and late chase times (especially since minor bands of slightly faster mobility were also observed), we excised the apical and basolateral p500 bands from the gel, and examined their one-dimensional profiles after limited proteolysis with trypsin. As seen in Fig. 8 (lanes 1-3), these maps showed identity, and were different from that seen for Tg (Fig. 8, lane 4).

Since one way we could explain the reversal of secretory polarity for p500 was because of its inclusion in a regulated secretory pathway with predetermined apical polarity, we added secretagogue to steady state-labeled filters to exam-

experiments, on average 60–65% of secreted p500 was de-
tected basolaterally, as opposed to an 8-10-fold apical polarity

Figure 6. Quantitation of the fractional release of p500 and Tg to the apical and basolateral medium. Each of the chase collection intervals in the experiment displayed in Fig. 5 were analyzed by scanning densitometry. The total p500 and Tg secreted over the 24-h period was =8% in the control; =10% in the presence of NaAc. Also note the reversal of secretory polarity toward saturation of the x-ray film in the apical Tg bands. A

sodium acetate

Figure 6. Quantitation of the fractional release of p500 and Tg to the apical and basolateral medium. Each of the chase collection intervals in the experiment displayed in Fig. 5 were analyzed by scanning densitometry. The total p500 and Tg secreted over the 24-h period was assigned 100%; the data shown are fractions of this value. The apical polarity of Tg in control and NaAc-containing conditions are likely to be underestimations because of the tendency toward saturation of the x-ray film in the apical Tg bands. A significant kinetic delay in secretion of both proteins is evident in the presence of NH4Cl. Also note the reversal of secretory polarity for p500 in the last chase collection under control and NaAc-containing conditions. The fraction of p500 release apically during this period was ≈8% in the control; ≈10% in the presence of NaAc. A, apical; B, basolateral.
Limited tryptic digestion patterns of p500 collected on either side of the filter are identical. Confluent filter-polarized cells were cultured, pulse labeled, washed, chased, and apical and basolateral media analyzed by SDS-PAGE and fluorography as in Fig. 5. The labeled p500 bands from the apical and basolateral media from 2-3 h of chase (lanes 1 and 2, respectively), and the apical p500 band from 10-24 h of chase (lane J) were then excised from the gel and treated with trypsin as in Materials and Methods, and re-run on a second SDS-PAGE. As a control, the Tg band from the 2-3-h apical medium was treated in parallel (lane 4) and shows a different tryptic profile.

Figure 7. The polarity reversal of p500 at prolonged chase times is not because of transcytosis. Confluent filter-polarized cells were cultured, pulse labeled, and washed (as in Fig. 5), and six sequential 1-h-long chase collections made of apical and basolateral media. Radioactive apical medium (rich in labeled Tg, poor in p500) collected from the sixth chase hour was mixed with an equal volume of fresh chase medium and placed in the apical compartment above a fresh unlabeled confluent filter-grown monolayer for a 14-h incubation (left panel). Radioactive basolateral medium (more enriched in p500) was treated similarly and placed beneath another unlabeled filter-grown monolayer (right panel). At the conclusion of the 14-h incubation, proportionate volumes of apical (A) and basolateral (B) media from both filters were analyzed by SDS-PAGE and fluorography. In each case, the radiolabeled p500 and Tg did not cross the unlabeled cell layers to a measurable degree. However, some low molecular weight radioactive species (at the dye front) were seen on both sides of the filter after the 14-h incubation.

Discussion

In the last few years, considerable effort has been made to elucidate the different exocytotic pathways that secretory proteins may follow in exiting eukaryotic cells. In the case of polarized protein secretion, much of the classic work has concentrated on cell lines that have the advantage of possessing epithelial tight junctions but do not express a regulated secretory pathway (Kondor-Koch et al., 1985; Gottlieb et al., 1986; Caplan et al., 1987; Rindler and Traber, 1988). By contrast, much of the classic work describing the intracellular coexistence of regulated and constitutive routes (reviewed in Kelly, 1985) has focused on cell types in which spatial segregation of the regulated and constitutive secretory pathways may exist (Rivas and Moore, 1989; Sporn et al., 1989), but which lack epithelial tight junctions, making it more difficult to measure secretory polarity. We have sought a system that exhibits both of the above properties, to study secretory sorting and targeting operations in the same cells. Reestablishment of tight junctions on the surface of a porous filter permits the formation of a true polarized thyroid epithelium (Mauchamp et al., 1987; Chambard et al., 1987; and this report). Additionally, thyroid cells cultured in this manner continue to respond to secretagogues with stimulated Tg release. Thus to our knowledge, this system represents the first filter-grown epithelial monolayer with preserved apical/basal and regulated/constitutive secretory targeting ongoing concurrently. We have used this system to examine directly the exocytotic routes followed by two endogenous high molecular weight secretory proteins, Tg and p500.

Tg, a large (660 kD) homodimeric protein which is secreted as an exocrine product after heterogeneous processing during biosynthetic transport, is designed to serve in intrathyroidal storage of thyroxine in the apical lumen after its posttranslational iodination. It has been a long held view (Feeney and Wissig, 1972) that Tg secretion is a purely constitutive process. However, the data presented suggest that apically released Tg derives from both unstimulated and stimulated routes. These pools of Tg are unlikely to reside in different epithelial subpopulations, based on older autoradiographic studies which do not reveal heterogeneity in the kinetics of the Tg secretory process between epithelial cells (Nadler et al., 1964), and based on an analogous observations made in other systems (Moore and Kelly, 1985; Sporn et al., 1986; von Zastrow and Castle, 1987; Stoller and Shields, 1988). In the exocrine pancreas (a tissue whose structural organization is similar to that of the thyroid, except that the former contains a more developed regulated secretory pathway with numerous large storage granules),
unstimulated apical secretion of regulated secretory proteins can be divided into two kinetically distinct phases which derive from two different exocytotic routes: a quantitatively minor phase which exhibits relatively rapid release (complete by ~6 h of chase), and a quantitatively major, slower phase (half-life >2 d) that derives from exocytosis from the mature storage compartment (Arvan and Castle, 1987; Arvan and Chang, 1987). In the present case of cultured thyroid monolayers, it appears that without secretory stimulation, most Tg is delivered to the apical surface in an early phase within 6 h after synthesis (Fig. 2 B), whereas only a small fraction is found in prolonged intracellular storage and delayed secretion. Quantitative differences in the handling of secretory products by the different systems are likely to reflect tissue-specific differences, but we presume that qualitative handling of the secretory proteins is similar between the exocrine secretions of pancreas and thyroid.

In contrast to the many published reports concerning Tg biosynthesis and secretion (Malthiery et al., 1989), p500 is a newly described protein secreted constitutively from thyroid cells. We are confident that p500 is a product expressed by the epithelial cells because of the relative purity of the cell preparation, the apparent abundance of the protein in secretion and cell homogenates, and the ability to stimulate its secretion by thyroid epithelial cell secretagogues (Fig. 9; and data not shown). Based on SDS-PAGE mobility after treatment with reducing agents (Fig. 5), differences in secretory behavior (kinetics, polarity, and sensitivity to pH-perturbing drugs; Figs. 2 and 6) immunologic criteria (Fig. 9), and tryptic maps (Fig. 8), it seems unequivocal that p500 and Tg are distinct and unrelated proteins. The fact that these two proteins are initially secreted from the same cells with differing relative polarity can be taken as strong evidence for true molecular sorting, presumably at the level of the TGN. Our data further suggest that the initial targeting of Tg and p500 to the two different plasmalemmal surfaces involves mechanisms that are relatively pH insensitive and sensitive, respectively.

In contradistinction to the model of Moore (Chung et al., 1989) in which all constitutively secreted proteins are envisioned as lacking specific conserved signals present in regulated secretory proteins, the observation in exocrine (and many endocrine) systems that there is a substantial unstimulated release of regulated secretory proteins has led to the postulate that such a process is not due to inefficiency of sorting into the immature storage compartment, but rather to the extent to which vesicular traffic diverted from this compartment to the cell surface (Arvan and Castle, 1987) reduces the storage capacity of the regulated pathway in a tissue-specific manner. Storage capacity may be decreased or lost in many tissue culture conditions, and is a parameter likely to be under physiologic control (Scammell et al., 1986). The hypothesis of a vesicular diversion of regulated secretory proteins from immature granules (Arvan and Chang, 1987) that may be either tethered to, or separated from the TGN (von Zastrow and Castle, 1987), describes a somewhat different phenomenon than the sorting of regulated from constitutive molecules by a putative sorting receptor. It should be emphasized that models of these two different processes are not mutually exclusive and may now be distinguished better in polarized cell systems where constitutively secreted proteins are released with a secretory polarity different from that of the vesicular diversion from immature granules. Thus, lack of sorting does not appear to explain the large fraction of newly synthesized Tg which is secreted "constitutively," since it is still faithfully delivered with an apical predominance despite the fact that other proteins such as p500 are routed constitutively with basolateral preference. To demonstrate whether one of these two molecules (or both) is sorted away from the "default secretory pathway," it will be necessary to establish the volume of bulk flow of different routes going from the trans-Golgi to the cell.

**Figure 9.** Secretion of p500 can be stimulated by secretagogue. Confluent filter-grown monolayers were labeled to steady state, washed, and either stimulated with 5 mM 8 Br-cAMP or not stimulated, and the cells lysed as in Fig. 5. Four sequential 30-min chase collections were made. (A) The apical and basolateral media were analyzed by 4% SDS-PAGE and fluorography (CON, lanes 1-4; STIM, lanes 5-8). Only the apical media is shown. Two different lengths of exposure of the identical samples are shown: in the shorter exposure (left set of two panels) a substantial stimulation of labeled Tg release is seen and p500 can be detected only in the 30-min stimulated sample but not in the control. The basolateral media showed undetectable labeled protein only in long exposure, however, no secretagogue-stimulated effect was evident. In the long exposure, in which radioactivity in labeled apical proteins has saturated the film, it is evident that there is also unstimulated secretion of Tg and p500 in the control samples. (B) The same samples were analyzed for Tg secretion by immunoprecipitation; p500 is not immunoprecipitated by the polyclonal anti-Tg antisera.
surface (Burgess and Kelly, 1987; Pfeffer and Rothman, 1987; Klausner, 1989). Such studies in cultured thyroid epithelial cells are now in progress in our laboratory. Perhaps the most striking finding of all is the change in secretory polarity for p500 from basolateral to apical at prolonged chase times, unlike Tg which is always apically predominant. Control experiments demonstrated that such a change is due neither to leakage nor to transcytosis from the basolateral to apical sides (Fig. 7). In addition, we have no evidence to favor the notion of multiple or different sorting signals on p500 which could cause different targeting. By proteolytic fingerprints (Fig. 8), p500 collected apically is identical to p500 collected basolaterally. Thus, we hypothesize that a fraction of p500 fails to be excluded from the mature storage compartment and as such becomes a component of the regulated pathway, which must be a precommitted exocrine (apical) route in thyroid epithelial cells. Based on our experiments (Fig. 6), it appears that the net amount of newly synthesized p500 actually conveyed to the apical surface by the delayed pathway is small. However, it appears that proteins positively sorted, passively sorted, or missorted to the mature storage compartment become virtually indistinguishable in terms of secretory polarity. If true, this observation suggests the possibility of a targeting hierarchy in such cell types wherein routing to the immature storage compartment may be a way of insuring apical surface delivery, albeit by both early and delayed routes. Such a hierarchy would have important implications regarding the targeting signals directing regulated secretion and apical delivery, including the possibility that there could be overlap, redundancy, or even identity in such signals.

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