

Differential regulation of apical and basal iodide transporters in the thyroid by thyroglobulin

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Abstract

We have shown that thyroglobulin (Tg) is a potent autocrine regulator of thyroid-specific gene expression, and proposed that the accumulated follicular Tg within the colloid is a major factor in determining follicular function. In the present report, we examined the effect of Tg on the action of TSH/cAMP and iodine with special focus on the regulation of basolateral and apical iodide transporters; the sodium/iodide symporter (NIS) and the

pendred syndrome gene (PDS) by Tg. We show that expression of NIS and PDS are differentially regulated by Tg concentration and exposure time. In addition, we found that PDS gene was induced by TSH/cAMP and iodide in the presence of Tg. Based on these results, we propose a model for the physiological turnover of follicular function that is dynamically regulated by Tg.

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Introduction

Follicular structures are the minimal functional units of the thyroid, and are the sites of thyroid hormone biosynthesis. However, the function of each follicle is heterogeneous and not synchronized. Indeed, significant follicular variability exists, not only in shape, size and colloid density, but also in the ability to accumulate iodide, diffusion of iodinated thyroglobulin (Tg) within the follicular lumen (Gerber *et al.* 1985, 1986, Suzuki *et al.* 1999*c*), levels of Tg and thyroid hormones within the follicles (Suzuki *et al.* 1999*a*, 1999*c*), and even in expression of thyroid transcription factor (TTF-1), an essential transcription factor (Suzuki *et al.* 1999*a*, 1999*b*). In addition, heterogeneity in the expression of other proteins and cell growth has been noted under various experimental conditions (Yamamoto *et al.* 1988, Kawaoi *et al.* 1991, Suzuki *et al.* 1991, 1992) and in human goiters (Peter *et al.* 1985, Studer *et al.* 1989).

Biosynthesis of thyroid hormones depends on the expression and function of several thyroid-specific genes, including Tg, thyroperoxidase (TPO), sodium/iodide symporter (NIS), pendred syndrome gene (PDS), and thyrotropin receptor (TSHR). Dietary iodide is taken up through NIS expressed on the basolateral membrane of the thyroid (Dai *et al.* 1996, Caillou *et al.* 1998, Jhiang *et al.* 1998), and secreted into the follicular lumen via the apical iodide transporter pendrin (Everett *et al.* 1997, Royaux *et al.* 2000, Yoshida *et al.* 2002, Gillam *et al.* 2004,

Yoshida *et al.* 2004) and an iodide channel (Golstein *et al.* 1992, Nilsson *et al.* 1992, Yoshida *et al.* 1997, 1998).

We have shown that Tg, the major product of the thyroid follicular cell, is a potent suppressor of thyroid-specific gene expression, and that this action of Tg overcomes TSH-mediated effects. Thus, it appears that Tg is a feedback suppressor of its own expression, as well as of TPO, NIS and TSHR, through suppression of specific transcription factors, such as TTF-1, TTF-2 and Pax-8 (Suzuki *et al.* 1998*b*, 1999*b*). Suppression of NIS expression by Tg decreases iodide uptake *in vitro*, while the accumulation of Tg in the follicular lumen correlates with low iodide uptake *in vivo* (Suzuki *et al.* 1999*c*). Tg not only exhibits suppressive activity, but increases mRNA levels and promoter activity of major histocompatibility complex class I genes (Suzuki *et al.* 1998*b*), and significantly induces PDS expression (Royaux *et al.* 2000). Final Tg concentrations of 1 to 10 mg/ml in cell culture medium were effective in experiments using rat thyroid FRTL-5 cells *in vitro* (Suzuki *et al.* 1998*b*, 1999*b*, 1999*c*). This is thought to represent the lower range of Tg concentration within the normal thyroid follicle, which varies from 0.1 mg/ml up to 250 mg/ml depending on the method used for measurement (i.e. aspiration biopsy or micropuncture of a single follicle). The relatively large range in estimates of physiological Tg concentration is most probably the result of the significant functional heterogeneity between follicles (Hayden *et al.* 1970, Smeds 1972, Salabe *et al.* 1996).

It is interesting to note that Tg suppresses the basolateral iodide transporter NIS, but induces the apical transporter, pendrin (Suzuki *et al.* 1999c, Royaux *et al.* 2000), suggesting differential regulation of the basal and apical iodide transporters by Tg. Furthermore, although TSH/cAMP is the strongest inducer of NIS (Kogai *et al.* 1997), there was no detectable effect of TSH on PDS gene expression (Royaux *et al.* 2000). In addition, iodide is known to down-regulate iodide uptake and NIS mRNA levels even in cultured thyroid cells (Eng *et al.* 1999), while the effect of iodide on PDS gene expression is not known. Therefore, in the present study, we tried to clarify the differential effects of Tg on NIS and PDS expression, as well as the role of Tg on the action of TSH/cAMP and iodide to regulate the expression of these genes.

Materials and Methods

Cells

An F-1 subclone of rat FRTL-5 thyroid cells (ATCC CRL8305) was provided by the Interthyr Research Foundation, Baltimore, MD, USA. Cells were grown in Coon's modified F-12 medium (Sigma Chemical Co) containing 5% heat-treated, mycoplasma-free calf serum (GIBCO Laboratories Life Technologies), 1 mM non-essential amino acids (GIBCO), and supplemented with six hormones or growth factors (termed 6H) that included bovine TSH (1×10^{-10} M), insulin (10 µg/ml), cortisol (0.4 ng/ml), transferrin (5 µg/ml), glycyl-L-histidyl-L-lysine acetate (10 ng/ml), and somatostatin (10 ng/ml). Bovine Tg was added directly to the culture medium as described previously (Royaux *et al.* 2000, Suzuki *et al.* 1998c, 1999c).

RNA isolation and Northern blot analysis

Total RNA isolation and Northern blot analysis were performed as previously described (Suzuki *et al.* 1998b, 1998c). RNA was prepared using an RNeasy Mini Kit (Qiagen). Cells were cultured in 10 cm dishes, washed with PBS, recovered with 600 µl lysis solution, and passed through a QIAshredder (Qiagen). After the addition of 600 µl 70% ethanol, the mixture was passed through a spin column, washed with 600 µl RW1 wash solution, and twice with 500 µl RPE wash solution. RNA was eluted with 30 µl of diethylpyrocarbonate-treated water. RNA samples were electrophoresed on denaturing agarose gels and capillary blotted onto Nytran nylon membranes (11 × 14 cm, Schleicher & Schuell, Keene, NH, USA) using a Turboblotter (Schleicher & Schuell). Hybridization was performed after u.v. cross-linking. Probes were labeled with 32 P-dCTP using a Ladderman Labeling Kit (PanVera, Madison, WI, USA) in the presence of random primer, dNTP, and *Bca* DNA polymerase. Radiolabeled

probe (1×10^7 c.p.m.) was premixed with 100 µl sonicated salmon sperm DNA (Stratagene, La Jolla, CA, USA), heated at 94 °C for 5 min, chilled on ice, and added to membranes prehybridized with 10 ml QuickHyb Hybridization Solution (Stratagene) for 1 h at 68 °C. After hybridization for 3 h, membranes were washed in 4 × SSPE, 0.5% SDS for 30 min at 37 °C; 2 × SSPE, 0.1% SDS for 20 min at 65 °C; and 1 × SSPE, 0.1% SDS for 20 min at 65 °C. Membranes were reprobed after incubation in 50% formamide, 50 mM Tris-Cl, pH 8.0, and 10% SDS, for 1 to 2 h at 65 °C.

Immunohistochemistry

Rat thyroid tissues were fixed with neutral-buffered formalin and immunostained as previously described (Suzuki *et al.* 1991, 1992, 1998a, 1998c). Briefly, deparaffinized and rehydrated sections were treated with 3% H₂O₂ for 10 min at room temperature, washed in PBS, incubated with 10% normal goat serum, and incubated with rabbit anti-rat Tg antibody (a gift from Dr A Kawaoi, Yamanashi Medical University, Yamanashi, Japan) diluted 1:1000. After three 5-min washes in PBS, sections were exposed to horseradish peroxidase-labeled swine anti-rabbit IgG antibody (1:100 dilution; DAKO) for 1 h. Sections were then rinsed in PBS for 5 min three times, and peroxidase activity detected with 3,3'-diaminobenzidine solution containing 0.003% H₂O₂. Cell nuclei were lightly counter-stained with hematoxylin.

Others

Highly purified bovine TSH was obtained from the hormone distribution program of the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health (NIDDK-bTSH; 30 U/mg). Bovine Tg, 8-bromo cAMP and forskolin were obtained from the Sigma Chemical Co. Bovine follicular 19S Tg prepared by salt extraction and agarose chromatography were used in some experiments and showed essentially the same results (Ulianich *et al.* 1999). All experiments were repeated at least three times using different batches of cells.

Results

Synergistic effect of TSH/cAMP and follicular Tg on induction of PDS gene expression

In order to study the effects of Tg on TSH action, we treated FRTL-5 cells with TSH (1×10^{-10} M) in the presence or absence of 1 mg/ml Tg for 24 h. As previously reported (Suzuki *et al.* 1999c, Royaux *et al.* 2000), TSH alone induced NIS expression, but had no effect on PDS expression (Fig. 1A, lanes 2). Although Tg alone induces PDS expression, it was further enhanced when both TSH

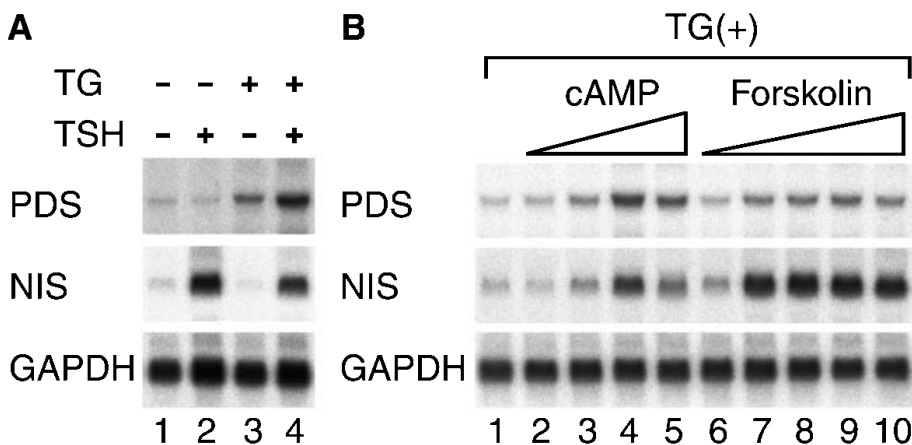


Figure 1 Coordinate regulation of PDS and NIS mRNA levels by TSH/cAMP and Tg. Either TSH (1×10^{-10} M) or Tg (1 mg/ml) alone or both together were added to cultures of FRTL-5 cells maintained without TSH for 5 days (A). Cyclic AMP (8-bromo cAMP) at final concentrations of 0.01, 0.1, 1 and 10 mM, and forskolin at final concentrations of 0.05, 0.5, 5, 10 and 50 μ M were added to the TSH-depleted FRTL-5 cells with 1 mg/ml of Tg (B). In both cases, cells were further maintained for 24 h and Northern blot analysis performed as described in the Materials and Methods section.

and Tg were added (Fig. 1A, lane 4), suggesting that TSH and Tg acted synergistically on PDS expression.

Such a synergistic effect of TSH was reproduced by cAMP or forskolin. Thus, when cAMP or forskolin were added in the presence of 1 mg/ml of Tg, PDS expression was significantly enhanced (Fig. 1B). These results suggest that the ability of low Tg concentrations to induce PDS expression was amplified in the presence of a TSH/cAMP signal. The same concentrations of cAMP or forskolin alone induced NIS, but not PDS expression (partly shown in Fig. 2B). This is relevant to the absence of PDS induction by TSH as shown in Fig. 1A.

Expression of PDS and NIS are differently regulated by Tg and the TSH/cAMP signal

In order to study the kinetics of PDS and NIS gene expression, FRTL-5 cells were maintained without TSH for five days, then treated with Tg, forskolin or both Tg and forskolin. Northern blot analysis was performed at various time points after treatment. In the absence of TSH/cAMP stimulation, Tg induced PDS mRNA in 6 h and suppressed NIS mRNA in 3 h (Fig. 2A). PDS expression was only transient and had decreased by 12 h, while NIS expression was suppressed between 3 and 12 h (Fig. 2A). Forskolin alone significantly increased NIS expression at 12 h, but had no effect on PDS expression (Fig. 2B), which is similar to that observed for TSH (Fig. 1A, lane 2). Interestingly, when both forskolin and Tg were added, PDS expression was prolonged beyond 72 h and the time of maximum expression shifted to 48 h post-stimulation (Fig. 2C). Induction of NIS mRNA was weaker than observed using forskolin alone, suggesting

that NIS mRNA levels represented the sum of forskolin-induction and Tg suppression (Fig. 2C). These results suggest that TSH/cAMP significantly modulated the ability of follicular Tg to induce PDS expression.

Since TSH constantly exists *in vivo* situation, we then studied the effect of low Tg concentrations on cells chronically treated with TSH. FRTL-5 cells maintained with TSH were treated with 1 mg/ml Tg, and RNA isolated at various time points. PDS mRNA was induced from 3 h after Tg stimulation, peaked at 24 h, and then decreased (Fig. 3A). In contrast, NIS mRNA, maximally induced by chronic TSH stimulation, was gradually decreased by Tg treatment (Fig. 3A) as previously reported (Suzuki *et al.* 1999c). When Tg was removed from the culture medium, PDS mRNA levels significantly decreased in 12 h (Fig. 3B), while NIS expression was increased by 24 h (Fig. 3B).

These results suggest that the expression of NIS and PDS, two major transporters responsible for the influx and efflux of iodide, respectively, are differentially regulated by Tg and TSH/cAMP. Significant changes in PDS expression compared with NIS expression under constant TSH stimulation further suggests that regulation of PDS expression may be a more important factor than NIS expression in determining the rate of iodine transport into follicular space.

Iodide induction of PDS and suppression of NIS expression

Differential regulation of NIS and PDS by changes in follicular Tg concentration further suggests that iodide concentration within follicular cells may be considerably different in these situations. Thus, in a follicle where Tg

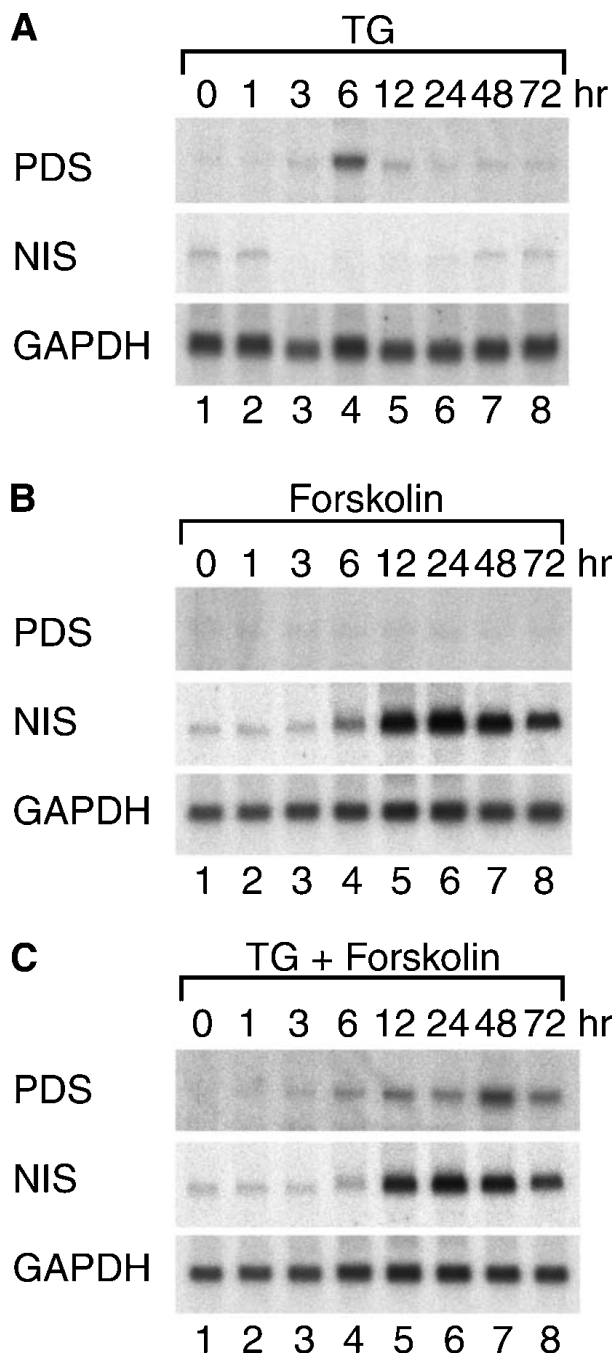


Figure 2 Time-dependent regulation of PDS and NIS mRNA levels by cAMP signaling and Tg. TSH was depleted from the culture medium of FRTL-5 cells at 40–50% confluency, maintained for 5 days, then 1 mg/ml Tg (A), 10 μ M forskolin (B) or both Tg and forskolin (C) added. Cells were further maintained for 1 to 72 h and Northern blot analysis performed.

has not accumulated but is exposed to constant TSH stimulation (as shown in Fig. 3A, lane 1), iodide may accumulate within the thyrocyte by maximum uptake

through NIS, but minimal efflux due to the lack of PDS expression. As a result, iodine concentrations within the follicular cell may become much higher than extracellular concentration. In fact, we have shown that NIS can still transport iodide even when intracellular iodide concentration reaches 2 mM (Yoshida *et al.* 2002). Therefore, although relatively low concentrations of iodide had no direct effect on PDS expression (Royaux *et al.* 2000), the effect of higher concentration of iodide was tested in the presence of Tg to evaluate possible effects on NIS and PDS expression.

When NaI was added (5 mM) to the culture medium of FRTL-5 cells for 24 h in the presence of TSH (1×10^{-10} M) but not Tg (1.0 mg/ml), no change was observed in PDS expression (Fig. 4A, lane 2 vs lane 1). However, when NaI was added with Tg, follicular Tg-induced PDS expression was further enhanced from 6 to 12 h, whereas NIS expression was suppressed in 12 h (Fig. 4B). These results suggest that accumulation of iodide also regulates PDS and NIS expression in the presence of TSH and Tg.

Tg-mediated regulation of gene expression and Tg synthesis in vivo

As previously reported, there was an inverse correlation between the accumulation of follicular Tg and uptake of radio-iodine *in vivo* (Suzuki *et al.* 1999c). This evidence prompted us to investigate the relationship between follicular Tg accumulation and new Tg synthesis within follicular epithelium. Tg immunostaining showed variable intensity within the colloid as well as in the cytoplasm of follicular cells (Fig. 5A). Colloid staining represented accumulated Tg, while cytoplasmic staining reflected newly synthesized Tg in the Golgi apparatus and rER rather than reabsorbed Tg in the lysosomes, as confirmed by immunoelectron microscopy on various aspects of follicular function (Yamamoto *et al.* 1997, Yi *et al.* 1997, Suzuki *et al.* 1999a). Fig. 5A clearly shows that in a follicle with poor Tg accumulation (marked as I), active Tg synthesis occurs within the cytoplasm (as arrowed). In contrast, in a follicle where a large amount of Tg has accumulated within the follicular lumen (marked as III), Tg staining within the cytoplasm was significantly reduced (as indicated by arrowheads). In a follicle showing intermediate Tg accumulation (marked as II), the intensity of cytoplasmic staining of Tg was between I and III. Thus, there was an inverse correlation between the accumulation of follicular Tg and new synthesis of Tg within follicular cells.

These three follicle types may represent the differential effects of accumulated follicular Tg on thyroid hormone synthesis. This is demonstrated in differentially regulated gene expression by follicular Tg. Thus, in the absence of Tg, gene expression of NIS, Tg itself, TPO and vascular endothelial growth factor/vascular permeability (VEGF/VPF) is maximal (Fig. 5B, i). With low concentrations of

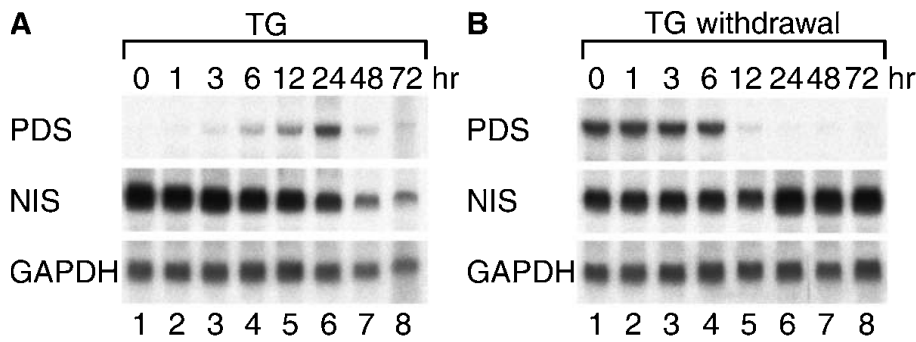


Figure 3 Effect of adding or withdrawing Tg under chronic TSH/cAMP stimulation. FRTL-5 cells at 40–50% confluency were maintained with 1×10^{-10} M TSH, then 1 mg/ml Tg was added and incubated for a further 1 to 72 h (A). In a different experiment, cells were maintained under the same conditions for 24 h to induce maximal PDS expression, after which Tg was washed out from the culture medium and grown in normal medium with TSH for 1 to 72 h.

follicular Tg, PDS gene expression is weakly induced, but expression of the other genes is still maintained (Fig. 5B, ii). However, under higher concentrations of Tg, the expression of genes necessary for iodide transport and hormone synthesis is suppressed (Fig. 5B, iii). These results support the *in vivo* evidence shown in Fig. 5A.

Discussion

TSH, iodide and Tg are the essential regulators of the thyroid

We have shown in previous studies that follicular Tg is a potent regulator of follicular function and mediates the

transcriptional suppression of genes essential for iodide transport and hormone synthesis (Suzuki *et al.* 1998b, 1999b, 1999c). We have also shown that follicular Tg is a major inducer of PDS expression in rat FRTL-5 thyroid cells (Royaux *et al.* 2000). In the present study, we demonstrated that PDS expression is additionally regulated by TSH and iodide in the presence of Tg. These findings support a concept that TSH, iodide and Tg are the major regulators of follicular function. However, in the *in vivo* situation, where serum TSH levels are relatively constant, differences in follicular Tg content may act as the dynamic regulator of follicular function. Furthermore, although serum iodide concentration appear not to be significant to regulate follicular function and thyroid gene expression,

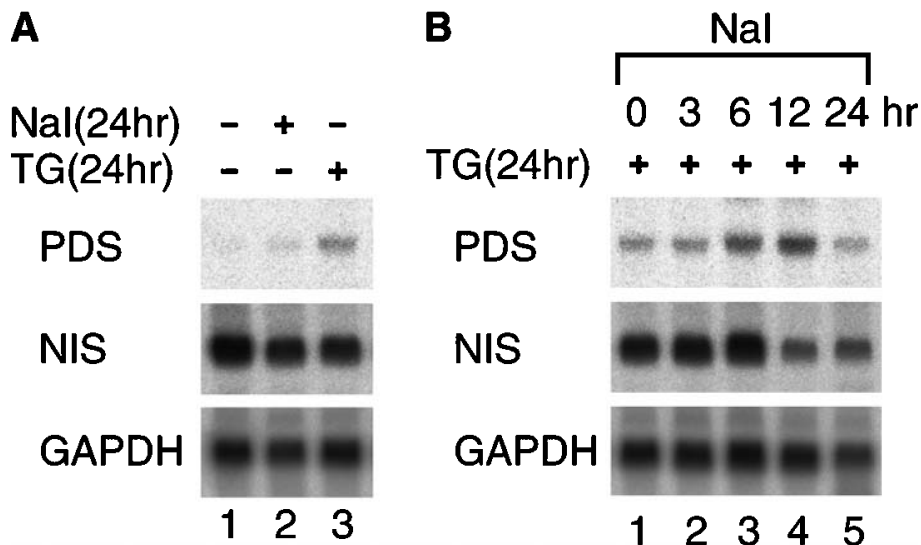


Figure 4 Additive effect of iodide and Tg in the induction of PDS mRNA. FRTL-5 cells were treated with either Nal (5 mM) or Tg (1.0 mg/ml) for 24 h (A), or Nal plus Tg added to cells pre-treated with Tg for 24 h and further maintained for 3 to 24 h (B). Both experiments were performed in the presence of TSH (1×10^{-10} M).

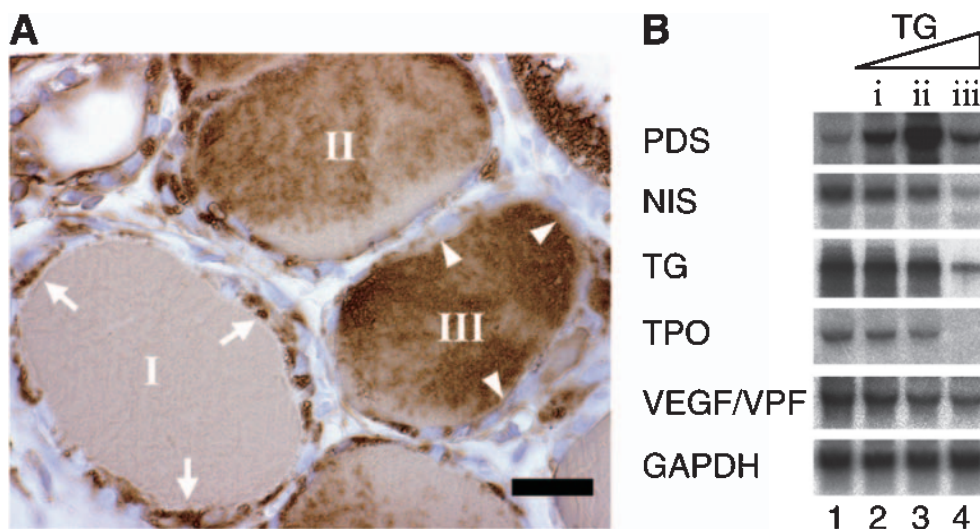


Figure 5 Immunohistochemical demonstration of Tg with three representative follicles shown as I, II and III (A). A follicle with weak follicular Tg staining, but strong cytoplasmic Tg staining marked as I; a follicle with strong follicular Tg staining, but weak cytoplasmic staining marked as III; and an intermediate follicle marked as II. Perinuclear Tg staining in the follicular cells indicated by arrows represents newly synthesized Tg situated in the Golgi apparatus and rER rather than reabsorbed Tg in lysosomes, as demonstrated by immunoelectron microscopy (Suzuki *et al.* 1999a, Yamamoto *et al.* 1997, Yi *et al.* 1997). In B, changes in mRNA levels of thyroid genes in FRTL-5 cells by gradient of Tg concentration (i, ii and iii) are shown. Increasing amounts of Tg (0·1, 1 and 10 mg/ml) were added to the culture medium of FRTL-5 cells maintained with TSH, and incubated for a further 24 h.

high expression of NIS, but not PDS in the presence of low Tg concentration, will result in high levels of iodide accumulation within the thyrocyte that is enough to affect gene expression. Our results also showed differential regulation of PDS expression compared with other important thyroid genes, e.g. NIS, Tg, TPO, TSHR and VEGF/VPF. Thus, while all the other genes were down regulated by Tg, PDS was induced. Therefore, changes in PDS expression may have a unique role in the regulation of follicular function.

Heterogeneous follicles represent different functional phases

Even though the role of follicular Tg content on regulation of gene expression is well established, the mechanism by which follicular heterogeneity is generated remains unknown. There are several possibilities that may explain the nature of follicular heterogeneity. Some evidence suggests that individual thyrocytes within the follicle are not functionally uniform. Using chimeric mice, it has been shown that epithelial cells surrounding a follicle do not originate from a single cell, but are mosaic (Feder 1976, Thomas *et al.* 1989). It is known that cloned thyroid cells show significant variability in growth and function in culture (Huber *et al.* 1990). These findings suggest that the function of each follicular cell is originally heterogeneous. Furthermore, the processes of biosynthesis and secretion have their own kinetics, resulting in changes in the

functional states of follicles over time. Thus, the functional difference among follicles can be explained by differences in its phases of the active cycle. This possibility is supported by the following observations. The first step in the biosynthesis of thyroid hormones involves iodide transport across the basolateral membrane. In contrast, the secretion is a process from the follicular lumen to basal membrane of follicular cells, and involves resorption, hydrolysis and secretion processes. Thus, these two pathways have opposing vectorial transport pathways through follicular cells and quite different kinetics. Wollman & Loewenstein (1973) estimated the turnover of Tg during secretion and resorption, and found that the replacement of absorbed colloid requires 460 subapical vesicles per cell per minute. Using immunogold-labeled antibodies and immunoelectron microscopy, Tg content was differentially evaluated in each subcellular organelle in rat follicular cells at various time points after TSH injection (Yamamoto *et al.* 1997, Yi *et al.* 1997, Suzuki *et al.* 1999a). Compared with the significant Tg content in colloid droplets and lysosomes after short time periods, the increased Tg content in the rER, Golgi and subapical vesicles was insignificant even after TSH injection. These differences indicated that the new synthesis and accumulation of Tg within the follicular lumen were slow processes compared with the rapid removal of Tg from the follicular colloid. Therefore, follicular heterogeneity may be the result of the different kinetics of these processes.

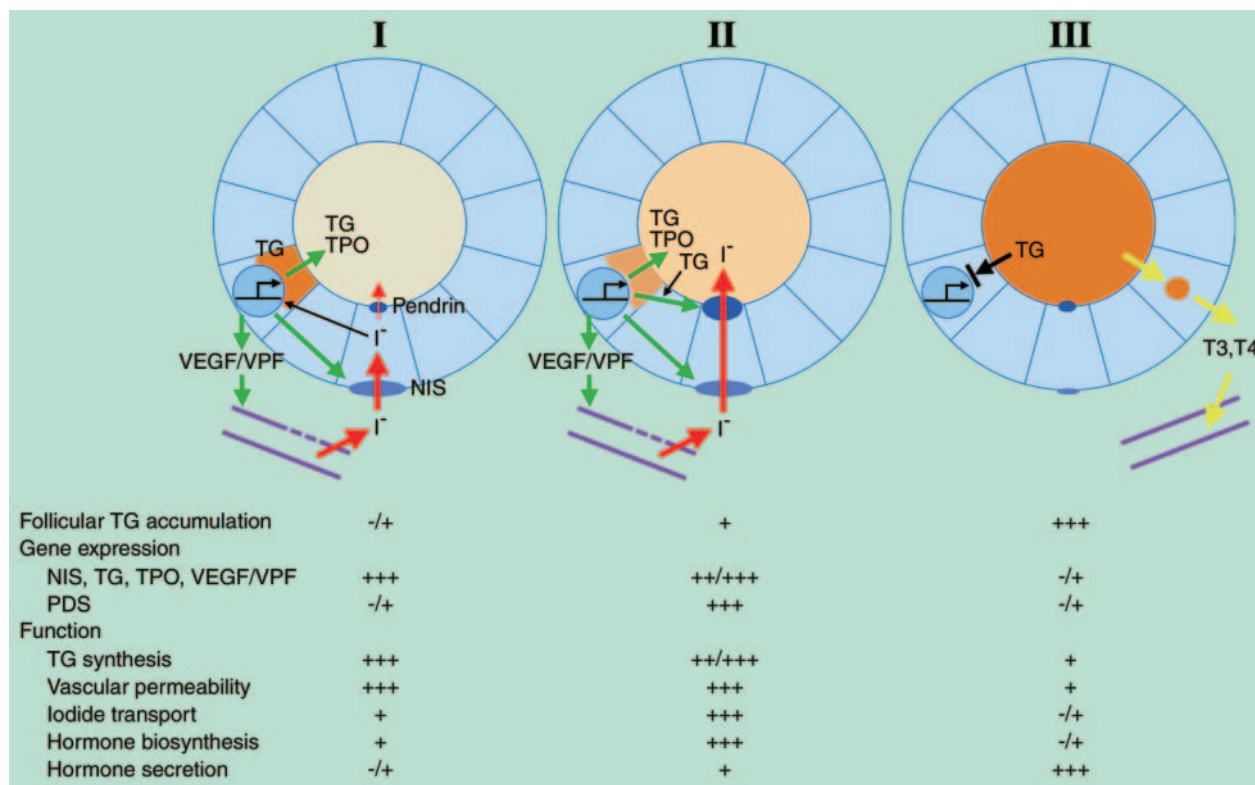


Figure 6 A modified model for the physiological regulation of follicular function. Three representative follicles (I, II and III) showing different follicular Tg accumulation and gene expression as indicated. Tg accumulation within the follicular lumen and cytoplasmic Tg staining in follicles I, II and III represent those shown in Fig. 5A, and levels of gene expression represent mRNA levels shown in Fig. 5B. We propose that iodinated Tg accumulates within the follicular lumen over time, from I to II, and then to III, after which reabsorption of colloid decreases Tg storage, and the cycle repeats. The effect of follicular Tg or iodide on resorption of colloid remains to be demonstrated.

A model for the physiological cycle of follicular function

Fig. 6 is the schematic representation of a concept based on the present data and the above discussion, and serves as a model for the physiological regulation of follicular function. In a follicle where Tg accumulation is minimal, gene expressions of NIS, Tg, TPO and VEGF/VPF are at their maximum under constant TSH supply (phase I). Maximum VEGF/VPF expression will increase the vascular permeability and iodide efflux into the perivascular space (Suzuki *et al.* 1999c). Iodide is transported into the thyrocytes via NIS, but efflux is limited due to minimal pendrin expression. Under these conditions, follicular Tg starts to accumulate within the follicle and iodination takes place only gradually. When a low concentration of Tg accumulates in a follicle, maximal PDS gene expression is induced, and iodide efflux reaches maximal (phase II). In addition to Tg, accumulated iodide within the thyrocytes also contribute to the induction of PDS shown in Fig. 4B. Expression of the other essential genes is maintained, therefore maximal hormone synthesis is achieved during this phase. When sufficient Tg accumulates

within a follicle (phase III), all gene expression necessary for iodide transport and hormone biosynthesis is suppressed. The biosynthesis phase is completed, and such a follicle is ready to supply thyroid hormones by a process of resorption, degradation and secretion. Resorption of colloid occurs in greater amounts and in a shorter time than synthesis of Tg (Wollman & Loewenstein 1973, Yamamoto *et al.* 1997, Yi *et al.* 1997, Suzuki *et al.* 1999a), so that the follicular Tg content in such a follicle decreases rapidly and the whole process of phases I, II and III is repeated. Since the kinetics of biosynthesis and resorption are different, many follicles in the same thyroid do not synchronize. Such follicles in different phases may reflect as significant heterogeneity on a tissue section. We should note that this concept applies to conditions with a normal TSH and iodide intake, and that the regulation of the studied genes may be different under conditions of a high TSH and/or iodide deficiency.

As it is not possible to observe a single follicle over time, it will be difficult to confirm this model *in vivo*. One of the questions that remains is the effect of Tg on resorption and colloid degradation. Although TSH is known to regulate

these processes, it is unlikely that the resorption/hydrolysis activity of a follicle with low levels of Tg is the same as in a follicle with high Tg concentration. The effect of follicular Tg on hormone secretion remains to be determined.

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