The expression of the sarco/endoplasmic reticulum Ca^{2+} -ATPases in thyroid and its down-regulation following neoplastic transformation

F Pacifico¹, L Ulianich², S De Micheli, S Treglia, A Leonardi², P Vito³,
S Formisano², E Consiglio^{1,2} and B Di Jeso

Dipartimento di Scienze e Tecnologie Biologiche ed Ambientali, Facoltà di Scienze Matematiche Fisiche e Naturali, Università degli Studi di Lecce,
Strada Provinciale Lecce-Monteroni, 73100 Lecce, Italy

¹Istituto di Endocrinologia ed Oncologia Sperimentale G Salvatore, CNR, Naples, Italy

²Dipartimento di Biologia e Patologia Cellulare e Molecolare L Califano, Naples, Italy

³BioGem Consortium, Naples, Italy

(Requests for offprints should be addressed to B Di Jeso; Email: bdijeso@ilenic.unile.it)

Abstract

Maintaining a high Ca^{2+} concentration in the lumen of the endoplasmic reticulum (ER), by the action of sarco/endoplasmic reticulum Ca^{2+} -ATPases (SERCAs), is important in many cellular processes, such as Ca^{2+} -mediated cytosolic signaling in response to extracellular stimuli, cell growth and proliferation, and synthesis, processing and folding of ER-translated proteins. In the thyroid gland, SERCAs have not been studied yet, and there is little information available on general problems such as the expression of SERCAs following neoplastic transformation.

In this study we investigated the expression of SERCA2b and SERCA3 in rat thyroid tissue and, in addition, in normal and transformed rat thyroid cell lines. RT-PCR and Northern blot assays showed that SERCA2b is the SERCA form preferentially expressed in the thyroid. In rat thyroid, SERCA2b mRNA was expressed at a higher level than that of other non-muscle tissues such as liver or spleen, but at much lower level than in brain. On the other hand, SERCA3 mRNA was not detected in thyroid by Northern blot analysis, or barely detected by RT-PCR assays. We also studied the SERCA2b expression pattern in PC Cl3 thyroid cells transformed by several oncogenes that induce different degrees of malignancy and dedifferentiation. RT-PCR and Northern blot assays showed that SERCA2b mRNA expression dramatically decreased in highly tumorigenic thyroid cells, while expression of glyceraldehyde-3-phosphate dehydrogenase mRNA, a housekeeping gene used as internal control, exhibited no variations. The dramatic down-regulation of SERCA2b expression in fully transformed thyroid cells was also evident by Western blot analysis. Also, following neoplastic transformation of thyroid cells, the enzymatic activity of SERCA2b was reduced in a measure which correlated with the mRNA and protein levels.

Therefore, rat thyrocytes expressed intermediate levels of SERCAs, mostly the SERCA2b isoform. This pattern of expression was basically reproduced in fully differentiated thyroid cells in culture and was sensitive to neoplastic transformation.

Journal of Molecular Endocrinology (2003) **30**, 399–409

Introduction

Cytoplasmic Ca^{2+} acts as a second messenger, controlling many different aspects of cellular physiology (Lytton *et al.* 1992). Intracellular organelles play a critical role in this regulation by acting both as sites of storage for subsequent release and as sites of accumulation for removal of Ca^{2+}

from cytoplasm (Lytton *et al.* 1992). The endoplasmic reticulum (ER) Ca^{2+} storage is crucial for a variety of Ca^{2+} -dependent processes, such as Ca^{2+} -mediated cytosolic signaling in response to extracellular stimuli (Berridge 1993), cell growth and proliferation (Ghosh *et al.* 1991, Berridge 1995), and synthesis, processing and folding of ER-translated proteins (Lodish & Kong 1990,

Lodish *et al.* 1992, Cooper *et al.* 1997). Ca^{2+} concentrations in ER are much higher than cytosolic ones because of the ability of three sarco/endoplasmic reticulum Ca^{2+} -ATPases (SERCA1, SERCA2 and SERCA3) to generate a Ca^{2+} gradient from cytoplasm to ER (Burk *et al.* 1989, Meldolesi & Pozzan 1998). Different transcripts from all three genes are produced as a consequence of mRNA alternative splicing mechanisms and expressed in a tissue-specific manner (Burk *et al.* 1989). The transcripts show a high grade of homology and give rise to a number of isozymes differing in their C-terminal regions (Burk *et al.* 1989). The splicing of the SERCA1 primary transcript produces two isozymes, SERCA1a and 1b (Brandl *et al.* 1987), both expressed only in fast-twitch skeletal muscle (Brandl *et al.* 1987). In particular, SERCA1a is expressed in adult skeletal muscle, whereas SERCA1b is the neonatal counterpart (Brandl *et al.* 1987). Similarly, SERCA2 gene encodes two isoforms, SERCA2a and 2b (Gunter-Hamblin *et al.* 1988, Lytton & MacLennan 1988), the former expressed in cardiac and slow-twitch skeletal muscle (Burk *et al.* 1989, Wu *et al.* 1995), the latter ubiquitously expressed in all non-muscle tissues at different levels (Burk *et al.* 1989, Wu *et al.* 1995). Three isozymes are produced from SERCA3 gene, SERCA3a, 3b and 3c (Burk *et al.* 1989, Dode *et al.* 1998, Martin *et al.* 2000), expressed in various non-muscle tissues, at variable levels, but always in the same tissues where SERCA2b is present (Wu *et al.* 1995, Launay *et al.* 1997, Lee *et al.* 1997). Transient expression of SERCAs in COS cells has proven to be a powerful system for addressing the specific function of different isozymes (Lytton *et al.* 1992). All isoforms display qualitatively similar enzymatic properties and are activated by Ca^{2+} in a cooperative manner (Lytton *et al.* 1992). The quantitative properties of SERCA1 isozymes and SERCA2a are identical, while SERCA2b appears to have a lower turnover rate for both Ca^{2+} transport and ATP hydrolysis (Lytton *et al.* 1992). SERCA3 isozymes display a reduced affinity for Ca^{2+} and an altered pH dependence when compared with the other SERCA isoforms (Lytton *et al.* 1992).

Recently, a new aspect of SERCA2b function has been proposed, given its ability to act as a member of the ER stress protein family whose expression is up-regulated in the ER stress-induced unfolded protein response (UPR) (Caspersen *et al.*

2000). In fact, an increase of both SERCA2b mRNA and protein levels, paralleled by an increase of mRNA levels for the ER stress marker proteins BiP/GRP78 and CHOP/GADD153, has been observed in PC12 cells after treatments with agents interfering with ER function, such as tunicamycin or dithiothreitol (Caspersen *et al.* 2000). On the contrary, SERCA3 mRNA levels did not change in the PC12 cells treated with the same stress agents (Caspersen *et al.* 2000).

While the tissue distribution and biochemical properties of SERCAs have been extensively studied in humans and rats, no information is available about thyroid expression of SERCA2b and SERCA3 isozymes (the non-muscle isoforms). Moreover, little is known about general problems such as the regulation of SERCA expression following neoplastic transformation. This latter issue is of considerable importance, and not only in the thyroid field. This stems from the multiplicity of cellular functions in which SERCAs are implicated (Lodish & Kong 1990, Ghosh *et al.* 1991, Lodish *et al.* 1992, Berridge 1995, Cooper *et al.* 1997, Caspersen *et al.* 2000) and which could participate in the establishment and maintenance of the neoplastic phenotype. Several of these SERCA-modulated cellular functions have been studied, in the thyroid, by our group (Zarrilli *et al.* 1990, Di Jeso *et al.* 1992, 1995, 1997, 1998, 1999, 2003, Pacifico *et al.* 1995, 1999, 2000, Leonardi *et al.* 2002).

To study the SERCA expression pattern in thyroid, we analyzed the expression of the non-muscle SERCA2b and SERCA3 mRNAs in rat thyroid. We found that SERCA2b was expressed in rat thyroid at intermediate levels between brain and spleen, while SERCA3 only at low levels.

To investigate the SERCA expression following neoplastic transformation, we employed normal and transformed PC Cl3 thyroid cells. PC Cl3 cells are fully differentiated thyroid cells (Fusco *et al.* 1987, Berlingieri *et al.* 1993), which express the typical markers of thyroid differentiation, such as thyroglobulin (Tg), thyroperoxidase (TPO), thyrotropin receptor (TSHR) and sodium iodide symporter (NIS) and are sensitive to thyrotropin (TSH) stimulation for their growth (Fusco *et al.* 1987, Berlingieri *et al.* 1993). These cells have been successfully employed to reproduce *in vitro* a multi-step model of cancerogenesis and to dissect

the sensitivity to neoplastic transformation of the differentiation program of thyroid cells (Fusco *et al.* 1987, Zarrilli *et al.* 1990, Berlingieri *et al.* 1993). We showed that only SERCA2b was expressed in normal PC Cl3 thyroid cells and its expression dramatically decreased in transformed PC Cl3 thyroid cells with a highly malignant phenotype.

Materials and methods

Cell lines

PC Cl3, a rat differentiated thyroid cell line, was grown in Coon's modified Ham's F-12 medium (Sigma, St Louis, MO, USA) supplemented with 5% calf serum (Sigma) and a mixture of hormones and growth factors (insulin 1 µg/ml; TSH 1 mIU/ml; glycyl-histidyl-L-lysine 10 ng/ml; human transferrin 5 µg/ml; cortisone 10 nM; somatostatin 10 ng/ml) (Sigma) (Fusco *et al.* 1987, Berlingieri *et al.* 1993). PC E1A, PC Raf, PC Py, PC E1A+Raf and PC E1A+Py are PC Cl3 cells transformed respectively by the adenovirus E1A gene, the *raf* oncogene, the polyoma virus middle T gene or by a combination of these oncogenes (Fusco *et al.* 1987, Berlingieri *et al.* 1993). They were grown in the same medium as PC Cl3 cells, but lacking the mixture of hormones and growth factors.

RNA extraction

Total RNA was extracted from rat winstar tissues and cell lines by the Chomczynski & Sacchi (1987) method. After checking the integrity of RNA by electrophoresis on agarose gel, an aliquot of total RNA was incubated for 15 min at 37 °C with 20 U of Rnase-free Dnase I (Promega, Madison, WI, USA) to digest contaminating genomic DNA.

RT-PCR assays

RT-PCR experiments were performed in accordance with standard methods (Innis *et al.* 1990). cDNA (0.5 µg) was amplified in 50 µl PCR buffer (Promega) containing 15 pmol SERCA2b or SERCA3 specific forward and reverse primers (see below). After a first step of denaturation for 2 min at 95 °C, 25 or 35 cycles of cDNA amplification from tissue and cell line extracts were performed as follows: 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C with a final extension of 5 min at 72 °C. The

last 20 cycles of amplification from both tissue and cell line extracts were carried out adding 15 pmol rat GAPDH forward and reverse specific primers (Pacifico *et al.* 1995) to the PCR mixture. The following SERCA primers were used: forward 5'-TCT ACC AGC TGA GTC ATT TC-3' (bp 2075–2094) and reverse 5'-TAA AGT TAG TGT CTG TGC TG-3' (bp 2589–2570) for rat SERCA2b (Franklin *et al.* 2001); forward 5'-CCT GCT CTC GGC TGC CGA CG-3' (bp 15–34) and reverse 5'-GTG GAC TTG ATC TCG ATG AG-3' (bp 483–464) for rat SERCA3 (Burk *et al.* 1989). The products of PCRs were analyzed by electrophoresis on 1% agarose gels and visualized under UV light after ethidium bromide staining. Bands were quantitated by densitometry with a desk scanner (Pharmacia Discovery system; Pharmacia, Uppsala, Sweden) and RFLPrint software (PDI, Huntington Station, NY, USA). Only gel exposures within the linear range of the film and the scanner were measured. Longer exposures were usually used for photography. Measurements of SERCA2b and SERCA3 mRNA levels in rat tissues and cell lines were performed at at least two cDNA concentrations and normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA content.

Northern blot experiments

Twenty micrograms of total RNA from rat tissues and cell lines were analyzed by electrophoresis on a 1.2% formaldehyde agarose gel and blotted onto nitrocellulose membranes (BioRad, Hercules, CA, USA), according to Maniatis *et al.* (1989). Filters were washed twice in 10 ml 2 × SSPE (0.3 M NaCl, 0.02 M NaH₂PO₄, 0.002 M EDTA), incubated for 5 h at 42 °C in 10 ml pre-hybridization buffer (50% formamide, 5 × SSPE, 2 × Denhardt's solution, 0.2% SDS, 100 µg/ml sonicated denatured salmon sperm DNA) and, then, for 16 h in 10 ml of the same buffer with 1 × 10⁷ c.p.m./ml of [³²P]dATP-labeled specific probe encompassing bp 2074–2590 of rat SERCA2b cDNA (Franklin *et al.* 2001). After the hybridization step, filters were washed twice at room temperature in 10 ml 2 × SSPE, 0.2% SDS and once at 42 °C in the same washing buffer. Detection was by autoradiography. Bands were quantitated by densitometry with a desk scanner (Pharmacia Discovery system) and RFLPrint software. Only gel exposures within

the linear range of the film and the scanner were measured. Longer exposures were usually used for photography.

Western blot assay

Isolation of a microsomal fraction from rat tissues and cell lines was carried out as previously described (Maruyama & MacLennan 1988). After evaluation of protein content, 100 µg total proteins from microsomal fractions of tissues and cell lines were boiled in loading buffer containing 2% SDS and 5% β-mercaptoethanol and analyzed by SDS-PAGE. Then, proteins were electroblotted onto polyvinylidene difluoride (PVDF) sheet (Millipore, Bedford, MA, USA). The filter was blocked for 15 h at 4 °C with Tris-buffered saline Tween 20 (TBST) buffer (10 mM Tris-HCl pH 8.0, 0.1% Tween 20, 150 mM NaCl) containing 10% non-fat dry milk and then incubated in TBST buffer for 2 h at room temperature with a 1:100 dilution of the goat polyclonal Ab-SERCA2 (N-19) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After TBST washing, the blot was incubated for 1 h at room temperature with horseradish peroxidase-conjugated rabbit anti-goat antibody (BioRad, Segrate, Italy) diluted 1:3000 in TBST buffer; detection was by enhanced chemiluminescence (ECL) (Amersham, Little Chalfont, Bucks, UK).

Assay of Ca²⁺-dependent ATPase activity of SERCAs

Total cell lysates and microsomal fractions of normal and transformed thyroid cell lines were also used to measure Ca²⁺-dependent ATPase activity of SERCAs. Isolation of a microsomal fraction from cell lines was carried out as previously described (Maruyama & MacLennan 1988). To prepare total cellular lysates, cells were washed twice with PBS, then swollen by incubation with 5 mM KMOPS, pH 7.0, for 15 min on ice. After addition of 0.1 mM phenylmethylsulfonyl fluoride and 100 units/ml trasylol, cells were homogenized with 50 strokes in a glass Dounce homogenizer. ATPase activities were determined at 37 °C (Ottolenghi 1975) by measuring inorganic phosphorus (Pi) production by a modification of the method of Fiske and Subbarow (Higgins 1987). This method is based on the reaction between

phosphate and molybdate to give the yellow molybdate phosphoric acid, which contains molybdate (Mo(VI)), which is then reduced to Mo(V) present in a blue colored heteropolyacid compound. This blue compound is directly measured by reading the absorbance at 700 nm. Typically, 10 µg microsomes or 100 µg total cell lysates were incubated in 25 mM KMOPS, 100 mM KCl, 5 mM MgCl₂, 4 mM ATP, 0.11 mM EGTA, 107 µM CaCl₂ pH 7.0, [Ca]^{free} 5 µM, for 10 min at 37 °C. The free Ca²⁺ concentration was calculated using the CHELATOR program (implemented in Turbo Pascal 5.5 for IBM PC) (Schoenmakers *et al.* 1992). The phosphate produced was determined directly by reading the absorbance at 700 nm of a standard curve. Activity either in the presence of EGTA alone or in the presence of 25 nM thapsigargin (Lytton *et al.* 1991) (which gave equivalent results) was subtracted. Inclusion in the buffer of sodium azide or *p*-trifluoromethoxyphenylhydrazide, potent inhibitors of mitochondrial Ca²⁺ uptake, did not modify the results. Also, the results were not modified whether PC E1A+Raf or PC E1A+Py cells were cultured in the absence or in the presence of the six hormone and growth factor mixture. Reactions were linear with respect to time and protein concentrations.

Statistical analysis

Data were expressed as means ± S.E.M. The statistical significance of differences between cell types was determined using a one-tailed Student's *t*-test of the STAT VIEW program (Abacus Concepts Inc., Berkeley, CA, USA). *P* < 0.05 was considered statistically significant.

Results

Analysis of SERCA2b and SERCA3 mRNA expression in rat thyroid

Expression of SERCA2b and SERCA3 mRNAs has been reported in a wide range of rat tissues (Burk *et al.* 1989), but no information is available about their expression in the thyroid gland. Therefore, we analyzed first SERCA2b and SERCA3 mRNA expression by semi-quantitative RT-PCR on total RNA from rat thyroid. Figure 1

Figure 1 Analysis of SERCA2b and SERCA3 mRNA expression in rat thyroid. (A) RT-PCR assays with specific SERCA2b (25 cycles), SERCA3 (25 and 35 cycles) or GAPDH (20 cycles) primers were performed on total RNA from rat thyroid, liver and spleen extracts. Amplification products were analyzed by 1% agarose gel electrophoresis and visualized under UV light after ethidium bromide staining. (B) Northern blot assays with specific SERCA2b (upper panel) or GAPDH (lower panel) 32 P-labeled cDNA probes were performed on 20 μ g total RNA from rat brain, thyroid and liver. After the hybridization step, filters were washed twice at room temperature in 10 ml 2 $\times$ SSPE, 0.2% SDS and once at 42 $^{\circ}$ C in the same washing buffer. Detection was by autoradiography. SERCA2b transcripts sizes and ribosomal 28S and 18S RNA are indicated. Bands corresponding to 6.2 and 4.6 kb transcripts were quantitated by densitometry with a desk scanner (Pharmacia Discovery system) and RFLPrint software. Only gel exposure within the linear range of the film and the scanner were measured. Longer exposures were usually used for photography.

shows that, after 25 cycles of PCR amplification, a band of the expected size (514 bp), corresponding to SERCA2b, was detected in thyroid and its intensity was much higher than that from liver and spleen RNA samples (Fig. 1A, panel 1). On the other hand, under the same experimental conditions, no expression of SERCA3 mRNA was detected in either thyroid or liver, while a SERCA3 cDNA fragment of the expected size (468 bp) was amplified in spleen (Fig. 1A, panel 2). By increasing the number of PCR cycles (35 cycles instead of 25), a faint band appeared in thyroid, with a size corresponding to that amplified from spleen RNA (Fig. 1A, panel 3). Even at high PCR amplification cycles, SERCA3 mRNA was not amplified from liver RT samples (Fig. 1A, panel 3), confirming its absence previously shown on poly (A) $^{+}$ RNA from rat liver (Burk *et al.* 1989). RNA levels of GAPDH, the housekeeping gene used as internal control to normalize SERCA2b and 3 mRNA content, were identical in all samples (Fig. 1A, panel 4). The

specificity of the cDNA bands amplified by PCR was evaluated by the inability of Taq polymerase to amplify any product from a sample containing the PCR reaction mixture minus cDNA (Fig. 1A, 'Control').

To further support these data, we analyzed SERCA2b mRNA expression by Northern blot. Figure 1B shows that SERCA2b mRNA was detected in rat thyroid and, at much higher levels, in brain, but not in liver. Only at longer filter exposure was it very barely seen in liver (data not shown). In particular, in thyroid were clearly expressed two (6.2 and 4.6 kb) of the three spliced isoforms of SERCA2b mRNA (Burk *et al.* 1989), as indicated by transcript sizes (Fig. 1B, upper panel), while the third transcript (7.7 kb) was detected only at longer filter exposure. An additional band slightly slower than 6.2 kb transcript was detected in brain (Fig. 1B, upper panel). The mRNA isoforms arise from an alternative splicing of the 3'-UTR region of SERCA2b mRNA (Burk *et al.*

Table 1 Normal and transformed rat thyroid cell lines. The cell lines were assigned to different groups on the basis of their ability to induce tumors in nude mice assayed by injecting 1×10⁶ cells into 4- to 6-week-old mice. The PC C13 rat thyroid cells were derived from 18-month-old normal Fisher rats (Fusco *et al.* 1987, Berlingieri *et al.* 1993)

	Oncogene	TSH dependence	Tg production
Cell type			
Parental differentiated cells, PC C13	—	+	+
Non-tumorigenic cells			
PC E1A	E1A	+/-	-
PC Raf	v-raf	-	-
Low tumorigenic cells, PC Py	Py middle T	+/-	+
High tumorigenic cells			
PC E1A+Py	E1A+Py middle T	-	-
PC E1A+Raf	E1A+v-raf	-	-

1989). They were evident by Northern blot, but not by RT-PCR assays on tissue as well as on cell line RNA extracts (see below), since in this latter assay we used primers able to amplify a portion of the coding region of SERCA2b mRNA. These results were confirmed by using SERCA2b-labeled probes encompassing different regions of SERCA2b full-length cDNA (data not shown), to exclude any possible cross-hybridization with SERCA3 isoforms, given the high grade of homology between SERCA2b and SERCA3 cDNA sequences (Burk *et al.* 1989). The same RNA content from rat brain, thyroid and liver preparations was analyzed by Northern blot, as normalized to GAPDH mRNA levels (Fig. 1B, lower panel).

Analysis of SERCA2b mRNA expression in normal and transformed PC C13 thyroid cells

To study the expression of SERCA2b mRNA in normal and neoplastic cells, we used PC C13 thyroid cells, normal and transformed by several oncogenes (Fusco *et al.* 1987, Zarrilli *et al.* 1990, Berlingieri *et al.* 1993). Properties of normal and transformed rat PC C13 thyroid cells are shown in Table 1. Besides the parental, fully differentiated PC C13 cell line, the various oncogene-carrying cell lines have been divided in three groups according to their degree of malignancy. The first group includes cell lines that are not tumorigenic when injected into athymic mice, the second includes cell lines that give rise to small, non-aggressive tumors

after 3–4 weeks of latency, while the third group includes the most malignant cell lines that induce very aggressive tumors in 7–10 days, causing the death of the animals (Fusco *et al.* 1987, Berlingieri *et al.* 1993, Pacifico *et al.* 2000). SERCA2b mRNA expression in normal and transformed PC C13 cells was initially evaluated by semi-quantitative RT-PCR assays (Fig. 2A). A SERCA2b cDNA fragment of 514 bp was amplified from RT samples of all cell lines analyzed, while no PCR amplification was seen in the negative control (Fig. 2A). In particular, SERCA2b mRNA was detectable in all PC C13 cell lines at as early as 25 cycles of amplification, albeit at different levels. In fact, PC C13 cells transformed by a single oncogene (PC E1A, PC Raf and PC Py), that are non- or low-tumorigenic cells (Table 1), expressed an amount of SERCA2b mRNA comparable with that of normal PC C13 cells (Fig. 2A and B), whereas PC C13 cells transformed by two oncogenes (PC E1A+Raf and PC E1A+Py), that display features of highly tumorigenic cells (Table 1), showed a significant decrease of SERCA2b mRNA expression with respect to normal PC C13 cells (Fig. 2A and B). GAPDH mRNA levels were not affected by neoplastic transformation of PC C13 cells (Fig. 2A). Measurements of SERCA2b mRNA expression were performed at at least two cDNA concentrations and normalized to the GAPDH mRNA content (Fig. 2B).

Since semi-quantitative RT-PCR showed a reduced expression of SERCA2b mRNA in highly tumorigenic PC E1A+Py and PC E1A+Raf cells,

Figure 2 Analysis of SERCA2b mRNA expression in normal and transformed thyroid cell lines. (A) RT-PCR assays with specific SERCA2b (25 cycles) or GAPDH (20 cycles) primers were performed on total RNA from normal and transformed PC Cl3 thyroid cell extracts. Amplification products were analyzed by 1% agarose gel electrophoresis and visualized under UV light after ethidium bromide staining. (B) Levels of SERCA2b mRNA (normalized to GAPDH) in normal and transformed PC Cl3 thyroid cells expressed as a percentage of the PC Cl3 cells value. Results are means $\pm$ S.E.M. of at least three separate experiments. * $P < 0.05$, significantly different from PC Cl3 cells. (C) Northern blot assay with a specific SERCA2b 32 P-labeled cDNA probe was performed on 20 μ g total RNA from PC Cl3, PC E1A+Py and PC E1A+Raf thyroid cell line extracts. After the hybridization step, the filter was washed twice at room temperature in 10 ml 2 $\times$ SSPE, 0.2% SDS and once at 42 $^{\circ}$ C in the same washing buffer. Detection was by autoradiography. SERCA2b transcripts sizes and ribosomal 28S and 18S RNA are indicated. Bands corresponding to 6.2 and 4.6 kb transcripts were quantitated by densitometry with a desk scanner (Pharmacia Discovery system) and RFLPrint software. Only gel exposure within the linear range of the film and the scanner were measured. Longer exposures were usually used for photography.

we further analyzed these data by Northern blot on total RNA from normal PC Cl3 cells and highly tumorigenic PC E1A+Py and PC E1A+Raf cells (Fig. 2C). By using the same SERCA2b cDNA probe of Fig. 1B, we clearly detected all three

SERCA2b transcripts in both normal and transformed PC Cl3 cells (Fig. 2C, upper panel). More importantly, Northern blot analysis showed that the expression of all SERCA2b mRNA isoforms was strongly decreased in PC E1A+Py cells and even

Figure 3 Western blot analysis of SERCA2b expression in rat thyroid tissue and in normal and transformed PC Cl3 thyroid cells. (A) One hundred micrograms of total protein from rat thyroid, brain and liver as well as from PC Cl3, PC E1A+Py and PC E1A+Raf microsomal fractions were electrophoresed on 10% SDS polyacrylamide gels, blotted on PVDF membrane and incubated with the Ab-SERCA 2 (N-19). SERCA2b reactivity was revealed by incubation with horseradish peroxidase-conjugated antibodies and ECL. (B) Relative levels of SERCA protein in rat tissues and cell lines, expressed as percentage of the brain value. Results are means $\pm$ S.E.M. of at least three separate experiments.

further in PC E1A+Raf cells (Fig. 2C, upper panel), in which SERCA2b mRNAs were detectable at longer exposure of the filter (data not shown). Also in this case, we probed the filter with different SERCA2b cDNA fragments and we obtained identical results (data not shown). The

Figure 4 Ca²⁺-ATPase activity of the SERCA2b pump in normal and transformed thyroid cells. Ca²⁺-ATPase activity of the SERCA2b pump in the microsomal fraction of normal (PC Cl3, FRTL-5) and transformed (PC E1A+Py and PC E1A+Raf) thyroid cell lines was determined at 37 °C (Ottolenghi 1975) by measuring Pi production by a modification of the method of Fiske and Subbarow (Higgins 1987). Results are means $\pm$ S.E.M. of at least three separate experiments.

same RNA content from normal and transformed cells was analyzed, as normalized to 28S and 18S ribosomal RNA levels (Fig. 2C, lower panel).

Western blot analysis of SERCA2b expression in thyroid tissue and in normal and transformed PC Cl3 thyroid cells

The expression of SERCA2b in thyroid tissue as well as in normal and transformed PC Cl3 thyroid cells was also performed by Western blot analysis (Fig. 3A). As shown in the Figure, the Ab-SERCA2 (N-19) was able to recognize a band of the expected molecular mass for SERCA2b (115 kDa) in thyroid and, at higher levels, in brain, but not in liver (Fig. 3A and B), mimicking the data obtained by Northern blot analysis (Fig. 1B). The Ab-SERCA2 (N-19) also showed SERCA2b expression in normal PC Cl3 cells and, at lower levels, in highly tumorigenic PC E1A+Py cells, while in highly tumorigenic PC E1A+Raf cells the SERCA2b protein was reduced even further (Fig. 3A). As in the case of the Northern blot, a longer exposure of the filter showed a clear expression of SERCA2b protein in PC E1A+Raf cells (data not shown). Therefore, the expression pattern of SERCA2b protein reflected that observed at the mRNA level: a strong decrease of the protein content was evident in highly malignant PC E1A+Py and PC E1A+Raf

cells with respect to normal PC Cl3 cells (Fig. 3A and B). The SERCA2b protein content was normalized to the total protein content and expressed as a percentage of brain value (Fig. 3B).

Ca²⁺-ATPase activity of SERCA2b pump in normal and transformed PC Cl3 thyroid cells

The method used here to measure the Ca²⁺-ATPase activity was sensitive and reproducible. Ca²⁺-ATPase activity increased linearly with increasing amounts of microsomes and total cell lysates from 5 to 60 µg and from 5 to 400 µg, respectively, and with increased incubation times from 5 to 30 min. Ca²⁺-ATPase activity in microsomes of PC Cl3 cells was 482·30 nmol Pi/min per mg protein (Fig. 4). A very similar value (431·15 nmol Pi/min per mg protein) was obtained in microsomes prepared from FRTL-5 cells (Fig. 4), another fully differentiated thyroid cell line (Ambesi-Impimbatto & Villone 1987). Strikingly, the activity measured in microsomes of dedifferentiated, fully transformed thyroid cells, PC E1A+Py (158·78 nmol Pi/min per mg protein) decreased about 3-fold, and that measured in microsomes of PC E1A+Raf cells (73·87 nmol Pi/min per mg protein) decreased about 7-fold (Fig. 4). Furthermore, to verify that this dramatic decrease of Ca²⁺-ATPase activity in fully transformed thyroid cells was present also before subcellular fractionation, we measured this activity in whole cellular lysates of PC Cl3 cells and PC E1A+Raf cells. We obtained values (69·12 nmol Pi/min per mg protein and 8·23 nmol Pi/min per mg protein respectively) that accurately reflected the differences found with the microsomal preparations. Therefore, the decreased expression of SERCA2b in PC E1A+Py and in PC E1A+Raf cells, at mRNA and protein levels, correlated with a decreased Ca²⁺-ATPase activity of the pump.

Discussion

The results of the present study demonstrate that the ubiquitously expressed isoforms of SERCA enzymes, SERCA2b and SERCA3, are differentially expressed in rat thyroid. In fact, while SERCA2b mRNA is detectable at high level in rat thyroid, by both semi-quantitative RT-PCR and Northern blot assays, SERCA3 mRNA is not

detected by Northern blot analysis on total rat thyroid RNA and it is barely amplified after a high number of PCR cycles from RT samples. The expression analysis of the two isozymes also shows that SERCA2b mRNA is lower in thyroid than in brain, but much higher than in liver or spleen, while SERCA3 mRNA levels are about 20-fold lower in thyroid than in spleen. Therefore, while both enzymes are generally co-expressed in a number of tissues, with a similar degree of expression (Launay *et al.* 1997, Lee *et al.* 1997), in the thyroid SERCA2b mRNA is expressed at much higher levels than that of SERCA3 mRNA, which is almost undetectable. The different expression levels of the two enzymes and, more importantly, the high levels of SERCA2b mRNA, suggest an important role of SERCA2b in the normal thyroid. SERCA2b catalyzes the active transport of Ca²⁺ from cytosol into ER, a fundamental function for a number of cell activities (Lodish & Kong 1990, Ghosh *et al.* 1991, Lodish *et al.* 1992, Berridge 1993, 1995, Cooper *et al.* 1997). The most important and abundant product of the thyrocyte is Tg, the precursor of thyroid hormones, a very complex glycoprotein with a molecular mass of 330 kDa (Werner & Ingbar 1991). Tg may contribute up to 50% of protein synthesis in the gland (Werner & Ingbar 1991). We have demonstrated that post-translational modifications as well as correct ER exit of Tg require Ca²⁺ (Di Jeso *et al.* 1992, 1997, 1999). In fact, treatment of thyroid cells with thapsigargin, a well known inhibitor of SERCA pumps acting as an ER Ca²⁺-depleting agent (Lytton *et al.* 1991), inhibits the terminal glycosylation of Tg or, if performed in low Ca²⁺ medium, blocks its export to the Golgi complex (Di Jeso *et al.* 1998). This latter effect was caused by a perturbation of the Ca²⁺-dependent interactions of Tg with ER chaperones (BiP/GRP78, GRP94, calnexin, calreticulin) involved in Tg folding (Di Jeso *et al.* 2003). Thus, when the Ca²⁺ concentration in the ER lumen falls, the folding of Tg in the ER does not occur correctly. As a consequence, unfolded Tg accumulates in ER and is not exocytosed, thereby blocking thyroid hormone synthesis. Although not proven, a similar Ca²⁺ requirement for folding could be shown by TPO, an apical enzyme catalyzing iodide organification and iodotyrosine coupling (Werner & Ingbar 1991), and by the Na⁺/I⁻ symporter, a basolateral iodine pump, also involved in thyroid hormone synthesis

(Werner & Ingbar 1991). Thus, the high level expression of the SERCA2b pump could be fundamental for the physiological functions of the thyroid gland, given its role in the generation and maintenance of the high Ca^{2+} concentration in the ER lumen necessary for the correct folding of Tg and other proteins involved in hormonogenesis.

In this paper we also show that the fully transformed phenotype of thyroid cells negatively regulates the expression and function of SERCA2b enzyme. RT-PCR, Northern blot, Western blot and enzymatic assays all show that the expression of SERCA2b dramatically decreases in highly tumorigenic thyroid cells (PC E1A+Py and PC E1A+Raf). To the best of our knowledge, this is one of the first reports of a dramatic down-regulation of SERCA enzymes following neoplastic transformation. In fact, very recently it has been reported that the expression of SERCA3 is lost in human colon carcinomas and dramatically reduced in colonic and gastric carcinoma cell lines, while the expression of SERCA2 is not altered (Gelebart *et al.* 2002). These results could appear at variance with ours reported here. In fact, in colon and gastric carcinomas, SERCA3 is down-regulated, while SERCA2b is unchanged. The data of Gelebart *et al.* are in line with the general concept that SERCA3 expression is linked to differentiation while SERCA2b serves general housekeeping duties.

This view has been recently questioned, as it has become clear that SERCA2b could be the exclusive SERCA isoform expressed by highly differentiated cells (Franklin *et al.* 2001). In this respect, our results support this new concept: the thyrocyte appears to behave similarly to enamel cells in that it is a highly differentiated cell expressing almost exclusively SERCA2b. Moreover, as stressed above, SERCA2b activity could be necessary to the central function of the thyrocyte, the synthesis of triiodothyronine and thyroxine. Therefore, our results suggest that in cells expressing exclusively or mostly SERCA2b, neoplastic transformation interferes with the expression of this form of SERCA. In this respect we are verifying whether these observations are confirmed at the level of natural thyroid tumors. We do not know, at present, if these findings have a functional significance in the biology of a neoplastic cell. However, it is known that SERCA2b acts as a member of the ER stress protein family whose expression is up-regulated in the ER stress-induced

UPR (Caspersen *et al.* 2000). The UPR is a cell response that makes cells able to survive stress or to die by apoptosis (Kaufman 1999). It is possible that neoplastic cells with a decreased expression of the SERCAs have an altered UPR following various stresses, usual in the tumor microenvironment. The altered UPR, in turn, could play a role in the specific behavior of neoplastic cells. This hypothesis is currently under investigation in our laboratory.

Acknowledgements

We thank Dr Domenico Liguoro for valuable contributions, Mr Fortunato Moscato for excellent technical assistance and Dr Santo Marsigliante for helpful discussions. This work was supported in part by a grant from Ministero dell'Università e Ricerca Scientifica Grant No. 2001065217 (to S F).

References

- Ambesi-Impiombato FS & Villone G 1987 The FRTL-5 thyroid cell strain as a model for studies on thyroid cell growth. *Acta Endocrinologica* **281** (Suppl) 242–245.
- Berlingieri MT, Santoro M, Battaglia C, Grieco M & Fusco A 1993 The adenovirus E1A gene blocks the differentiation of a thyroid epithelial cell line, however the neoplastic phenotype is achieved only after cooperation with other oncogenes. *Oncogene* **8** 249–255.
- Berridge MJ 1993 Cell signalling. A tale of two messengers. *Nature* **365** 388–389.
- Berridge MJ 1995 Calcium signalling and cell proliferation. *BioEssays* **17** 491–500.
- Brandl CJ, deLeon S, Martin DR & MacLennan DH 1987 Adult forms of the Ca^{2+} ATPase of sarcoplasmic reticulum. Expression in developing skeletal muscle. *Journal of Biological Chemistry* **262** 3768–3774.
- Burk SE, Lytton J, MacLennan DH & Shull GE 1989 cDNA cloning, functional expression, and mRNA tissue distribution of a third organellar Ca^{2+} pump. *Journal of Biological Chemistry* **264** 18561–18568.
- Caspersen C, Pedersen PS & Treiman M 2000 The sarco/endoplasmic reticulum calcium ATPase 2b is an endoplasmic reticulum stress-inducible protein. *Journal of Biological Chemistry* **275** 22363–22372.
- Chomczynski P & Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Analytical Biochemistry* **162** 156–159.
- Cooper GR, Brostrom CO & Brostrom MA 1997 Analysis of the endoplasmic reticular Ca^{2+} requirement for alpha1-antitrypsin processing and transport competence. *Biochemical Journal* **325** 601–608.
- Di Jeso B, Liguoro D, Ferranti P, Marinaccio M, Acquaviva R, Formisano S & Consiglio E 1992 Modulation of the carbohydrate moiety of thyroglobulin by thyrotropin and calcium in Fisher rat thyroid line-5 cells. *Journal of Biological Chemistry* **267** 1938–1944.
- Di Jeso B, Feliciello A, Pacifico F, Iannelli A, D'Armiento F, Liguoro D, Giuliano P, Ferulano G & Avvedimento VE 1995 Inhibition of c-Ras reactivates thyroglobulin synthesis in middle-T transformed thyroid cells. *Cell Growth and Differentiation* **6** 1315–1319.

- Di Jeso B, Formisano S & Ulianich L 1997 Perturbation of cellular calcium delays the secretion and alters the glycosylation of thyroglobulin in FRTL-5 cells. *Biochemical and Biophysical Research Communications* **234** 133–136.
- Di Jeso B, Pereira R, Formisano S, Consiglio E & Satrustegui J 1998 Demonstration of a calcium requirement for thyroglobulin dimerization and export to the Golgi complex. *European Journal of Biochemistry* **252** 583–590.
- Di Jeso B, Formisano S & Consiglio E 1999 Depletion of divalent cations within the secretory pathway inhibits the terminal glycosylation of complex carbohydrates of thyroglobulin. *Biochimie* **81** 497–504.
- Di Jeso B, Ulianich L, Pacifico F, Leonardi A, Vito P, Consiglio E, Formisano S & Arvan P 2003 The folding of thyroglobulin in the calnexin/calreticulin pathway and its alteration by a loss of Ca^{2+} from the endoplasmic reticulum. *Biochemical Journal* **370** 449–458.
- Dode L, De Greef C, Mountian I, Attard M, Town MM, Casteels R & Wuytack F 1998 Structure of the human sarco/endoplasmic reticulum Ca^{2+} -ATPase 3 gene. Promoter analysis and alternative splicing of the SERCA3 pre-mRNA. *Journal of Biological Chemistry* **273** 13982–13994.
- Franklin IK, Winz RA & Hubbard MJ 2001 Endoplasmic reticulum Ca^{2+} -ATPase pump is up regulated in calcium-transporting dental enamel cells: a non-housekeeping role for SERCA 2b. *Biochemical Journal* **358** 217–224.
- Fusco A, Berlingieri MT, Portella G, Di Fiore PP, Grieco M & Vecchio G 1987 One-and two-step transformation of rat thyroid epithelial cells by retroviral oncogenes. *Molecular and Cellular Biology* **7** 3365–3370.
- Gelebart P, Kovacs T, Brouland JP, Van Gorp R, Grossmann J, Rivard N, Panis Y, Martin V, Bredoux R, Enouf J & Papp B 2002 Expression of endomembrane calcium pumps in colon and gastric cancer cells. Induction of SERCA3 expression during differentiation. *Journal of Biological Chemistry* **277** 26310–26320.
- Ghosh TK, Bian JH, Short AD, Rybak SL & Gill DL 1991 Persistent intracellular calcium pool depletion by thapsigargin and its influence on cell growth. *Journal of Biological Chemistry* **266** 24690–24697.
- Gunter-Hamblin AM, Greb J & Shull GE 1988 A novel Ca^{2+} pump expressed in brain, kidney, and stomach is encoded by an alternative transcript of the slow-twitch muscle sarcoplasmic reticulum Ca -ATPase gene. Identification of cDNAs encoding Ca^{2+} and other cation transporting ATPases using an oligonucleotide probe derived from the ATP-binding site. *Journal of Biological Chemistry* **263** 15032–15040.
- Higgins A 1987 Separation and analysis of membrane lipid components. In *Biological Membranes, a Practical Approach*, pp 103–137. Eds JBC Findlay & WH Evans. Oxford, Washington DC: IRL Press.
- Innis MA, Gelfand DH, Sninsky JJ & White TJ 1990 *PCR Protocols, a Guide to Methods and Applications*. San Diego, California: Academic Press.
- Kaufman RJ 1999 Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. *Genes and Development* **13** 1211–1233.
- Launay S, Bobe R, Lacabartz-Porret C, Bredoux R, Kovacs T, Enouf J & Papp B 1997 Modulation of endoplasmic reticulum calcium pump expression during T lymphocyte activation. *Journal of Biological Chemistry* **272** 10746–10750.
- Lee MG, Xu X, Zeng W, Diaz J, Kuo TH, Wuytack F, Racymackers L & Muallem S 1997 Polarized expression of Ca^{2+} pumps in pancreatic and salivary gland cells. Role in initiation and propagation of $[\text{Ca}^{2+}]_i$ waves. *Journal of Biological Chemistry* **272** 15771–15776.
- Leonardi A, Vito P, Mauro Ci, Pacifico F, Ulianich L, Consiglio E, Formisano S & Di Jeso B 2002 Endoplasmic reticulum stress causes thyroglobulin retention in this organelle and triggers activation of nuclear factor-kappa B via tumor necrosis factor receptor-associated factor 2. *Endocrinology* **143** 2169–2177.
- Lodish HF & Kong N 1990 Perturbation of cellular calcium blocks exit of secretory proteins from the rough endoplasmic reticulum. *Journal of Biological Chemistry* **265** 10893–10899.
- Lodish HF, Kong N & Wikstrom L 1992 Calcium is required for folding of newly made subunits of the asialoglycoprotein receptor within the endoplasmic reticulum. *Journal of Biological Chemistry* **267** 12753–12760.
- Lytton J & MacLennan DH 1988 Molecular cloning of cDNAs from human kidney coding for two alternatively spliced products of the cardiac Ca^{2+} -ATPase gene. *Journal of Biological Chemistry* **263** 15024–15031.
- Lytton J, Westlin M & Hanley MR 1991 Thapsigargin inhibits the sarcoplasmic and endoplasmic reticulum Ca -ATPase family of calcium pumps. *Journal of Biological Chemistry* **266** 17067–17071.
- Lytton J, Westlin M, Burk SE, Shull GE & MacLennan DH 1992 Functional comparisons between isoforms of the sarcoplasmic or endoplasmic reticulum family of calcium pumps. *Journal of Biological Chemistry* **267** 14483–14489.
- Maniatis T, Fritsch E & Sambrook J 1989 *Molecular cloning: a laboratory manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Martin V, Bredoux R, Corvazier E, Papp B & Enouf J 2000 Platelet Ca^{2+} -ATPases: a plural, species-specific, and multiple hypertension-regulated expression system. *Hypertension* **35** 91–102.
- Maruyama K & MacLennan DH 1988 Mutation of aspartic acid-351, lysine-352, lysine-515 alters the Ca^{2+} transport activity of the Ca^{2+} -ATPase expressed in COS-1 cells. *PNAS* **85** 3314–3318.
- Meldolesi J & Pozzan T 1998 The endoplasmic reticulum Ca^{2+} store: a view from the lumen. *Trends in Biochemical Sciences* **23** 10–14.
- Ottolenghi P 1975 The reversible delipidation of a solubilized sodium-plus-potassium-ion-dependent adenosine triphosphatase from the salt gland of the spiny dogfish. *Biochemical Journal* **151** 61–66.
- Pacifico F, Laviola L, Ulianich L, Porcellini A, Ventra C, Consiglio E & Avvedimento VE 1995 Differential expression of the asialoglycoprotein receptor in discrete brain areas, in kidney and thyroid. *Biochemical and Biophysical Research Communications* **210** 138–144.
- Pacifico F, Liguoro D, Acquaviva R, Formisano S & Consiglio E 1999 Thyroglobulin binding and TSH regulation of the RHL-1 subunit of the asialoglycoprotein receptor in rat thyroid. *Biochimie* **81** 493–496.
- Pacifico F, Liguoro D, Montuori N, Mellone S, Macchia V, Formisano S, Consiglio E & Di Jeso B 2000 The rat hepatic lectin-1 subunit of the asialoglycoprotein receptor is up-regulated by thyrotropin and down-regulated by neoplastic transformation of thyroid cells. *Thyroid* **10** 201–209.
- Schoenmakers TJ, Visser GJ, Flik G & Theuvsen AP 1992 Chelator: an improved method for computing metal ion concentrations in physiological solutions. *BioTechniques* **12** 870–879.
- Werner & Ingbar 1991 *The Thyroid: a Fundamental and Clinical Text*, edn 6, pp 51–110. Eds LE Braverman & RD Utiger Philadelphia, PA: JB Lippencott Company.
- Wu KD, Lee WS, Wey J, Bungard D & Lytton J 1995 Localization and quantification of endoplasmic reticulum Ca^{2+} -ATPase isoform transcripts. *American Journal of Physiology* **269** C775–C784.
- Zarrilli R, Formisano S & Di Jeso B 1990 Hormonal regulation of thyroperoxidase in normal and transformed thyroid cells. *Molecular Endocrinology* **4** 39–45.

Received in final form 21 January 2003

Accepted 21 February 2003