Immunohistochemical detection, regulation and antiproliferative function of G-protein-coupled receptor kinase 2 in thyroid carcinomas

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Abstract

TSH, via its G-protein-coupled receptor, activates cell growth of both benign and malignant thyroid tumors. G-protein-coupled receptors (GR) kinase 2 (GRK2) has been reported to regulate the TSH receptor but its role in cancer is unknown. To determine a possible function for GRK2 in the growth process of thyroid cancers, we analysed its expression in normal and tumoral thyroid tissues and studied thyroid cancer cell line proliferation after GRK2 overexpression. Thirty one thyroid tissues, including 16 non-medullary thyroid cancers and 15 adjacent normal tissues, were analysed by immunohistochemistry. Five paired tissues were also studied by western blotting for the GRK2 enzymatic activity. Immunohistochemical staining showed an increase in GRK2 in thyroid cancers including papillary, follicular, and anaplastic types, compared with their adjacent normal tissues. Immunoblot analysis and GRK2 enzymatic activity measurement confirmed

immunohistochemical study. TSH and TSH in association with insulin or IGF-I stimulated GRK2 protein accumulation in normal human thyroid cells in primary culture. The TSH effect on the GRK2 expression was mimicked by forskolin. After GRK2 overexpression in two poorly differentiated thyroid cell lines, all the clones showed a significant reduction in cell proliferation, ranging from 28 to 65% inhibition compared with vector alone after 96-h culture. In conclusion, thyroid mitogenic factor-stimulated GRK2 accumulation may explain, in part, high GRK2 levels in differentiated carcinoma, because TSH, insulin, or IGF-I is known to be involved in the thyroid cancer progression. Surprisingly, instead of stimulating, GRK2 reduced cell proliferation revealing a new role for this kinase in the growth of thyroid cancers.

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Introduction

G-protein-coupled receptor kinases (GRKs) are serine/threonine protein kinases that control the desensitization process of G-protein-coupled receptor (GPCR) family (Pitcher *et al.* 1998). The molecular events underlying a desensitization mechanism start generally with agonist-induced receptor phosphorylation by a GRK. The phosphorylated GPCR possesses an increased affinity for a cytosolic protein of the arrestin family (arrestin 1–4). This complex (phosphorylated receptor/arrestin) prevents the further coupling of that receptor to its G-protein, reducing over time the capacity of second messenger synthesis. Resensitization is triggered by internalization of an uncoupled receptor to endosomal compartments, allowing its dephosphorylation by a protein phosphatase and recycling back to the cell surface or degradation (Moore *et al.* 2007). Based on sequence and functional similarities, the GRK family has been divided in three subfamilies: 1) the rhodopsin kinases (GRK1 and GRK7), 2) the β -adrenergic receptor kinases (GRK2 and GRK3), and 3) the GRK4, GRK5, and GRK6 subfamily (Ribas *et al.* 2007).

The thyrotropin (TSH) receptor belongs to the GPCR family and is a major determinant of thyroid function (Vassart & Dumont 1992). Most of the TSH effects are mediated by cAMP via an adenylyl cyclase-activating G α protein, although at higher concentrations the TSH stimulates inositol 1,3,4-trisphosphate generation in human thyroid cells (Van Sande *et al.* 2006). A key role for GRK2, GRK5, and GRK6 was first demonstrated in the TSH receptor homologous desensitization using a rat thyroid cell line (FRTL-5; Iacovelli *et al.* 1996, 1999, Nagayama *et al.* 1996a). Subsequently, GRK3 was also shown to desensitize the TSH receptor in HEK 293 cells (Voigt *et al.* 2004). A predominant role for GRK5 (Metayé *et al.* 2002) and GRK3 (Voigt *et al.* 2004) has been suggested in differentiated thyroid carcinomas (DTC) and hyperfunctioning thyroid nodules respectively. Furthermore, these findings were reinforced by the detection of GRK proteins in human thyroid tissues (Voigt *et al.* 2004), with a major expression of GRK2 and GRK5 (Nagayama *et al.* 1996a, Metayé *et al.* 2002).

The mitogenic effect of cAMP on human thyrocytes has been well established *in vitro* and received convincing arguments *in vivo* (Leclerc *et al.* 1984, Roger *et al.* 1988,

Suarez *et al.* 1991, Parma *et al.* 1993, Russo *et al.* 1995). Indeed, the importance of TSH signaling in thyroid growth was demonstrated in DTC where the TSH suppression by thyroid hormone supplementation is used clinically to prevent the recurrence of thyroid cancer after surgery (Pujol *et al.* 1996). Therefore, understanding the TSH signaling regulation may help to better define new therapeutic targets in thyroid cancer progression. In DTC, we observed an increase in GRK2 activity, not confirmed at the protein level as measured on unpaired samples (Metaye *et al.* 2002). Thus, to bring a new insight into these findings, we decide to assess the GRK2 protein expression on paired samples using immunohistochemistry and to seek a role for GRK2 in thyroid cancers. We demonstrate that GRK2 protein is increased in non-medullary thyroid cancers including DTC and anaplastic carcinomas compared with their adjacent normal tissues. Moreover, the GRK2 protein expression is regulated by TSH, TSH+insulin or TSH+insulin-like growth factor-I (IGF-I). Finally, we show that GRK2 overexpression attenuates the proliferative process of two different thyroid cancer cell lines in culture.

Materials and Methods

Patient and sample characteristics

For the measurement of GRK2 expression, 16 thyroid carcinomas (eight papillary, four follicular, and four anaplastic types) and their normal surrounding tissues (except one) were used. All the samples were obtained by surgery and embedded in paraffin for immunochemistry or stored in liquid nitrogen for GRK2 extraction. The tissue samples were randomly selected after histological examination by two pathologists independently and classified according to the World Health Organization recommendations (DeLellis *et al.* 2004). A consensus diagnosis was reached after discussion among the pathologists when a disagreement occurred. This study was approved by the Poitiers Hospital Ethics Committee. The mean age of patients was 49.0 years (s.d. 16.2) and 76.2 years (s.d. 9.1) for DTC and anaplastic carcinomas respectively. Cancers occurred in 5 males and 11 females. The mean tumor size of DTC was 2.7 cm (s.d. 2.6). Six subjects exhibited positive lymph nodes and two had distant metastases preoperatively. Patients with thyroid cancers were biologically euthyroid and ^{99m}Tc scintigraphies showed cold lesions. All the patients were without treatment before operation. Seven normal thyroid tissues (NTT) adjacent to benign nodules were used immediately after operation for primary cell culture.

Immunohistochemistry

Paraffined thyroid biopsy sections (5 µm) were processed for immunocytochemistry using a commercially available kit (Dako REAL Detection system, Dako, Copenhagen,

Denmark). The sections were deparaffined, rehydrated, and heated to boiling point for 2 min in a pressure cooker containing citrate buffer at pH 6. Thereafter, the sections were cooled down, rinsed in PBS, and incubated for 30 min at room temperature using rabbit polyclonal anti-GRK2 at a 1/400 dilution (C-15, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and then they were rinsed in PBS and incubated with biotinylated anti-rabbit antibodies for 20 min at room temperature, rinsed again, and incubated with peroxidase-conjugated streptavidin. Peroxidase activity was revealed with 3,3'-diaminobenzidine in PBS. All the sections were counterstained with hematoxylin, examined under blind condition and photographed. The sections incubated without anti-GRK2 antibody were used as a negative control and a tissue clearly positive in a preliminary experiment was prepared subsequently as a positive control. The C-15 antibody was specific for GRK2 and there was no cross reaction with GRK5 by western blotting (not shown). GRK3 and GRK6 proteins were undetectable in thyroid extracts using antibodies from Santa Cruz Biotechnology (Metaye *et al.* 2002). For each case, the percentage of positive cells and intensity of the immunoreactivity were given for the tumorous zone, the surrounding and distant normal tissues. The intensity of staining of positive cells was measured using a scale from (1+) to (3+) by a pathologist; (1+) represents weak staining, (2+) moderate staining, and (3+) strong staining.

Sample preparation and GRK partial purification

Human thyroid tissues were cut into small pieces with a razor blade and mechanically pulverized at -180°C . The powders were then homogenized at 4°C in thrice its volume of HEPES buffer (20 mM HEPES, 2 mM EDTA, 250 mM NaCl, 1 mM dithiothreitol, 0.02% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, 20 µg/ml leupeptin, 5 µg/ml aprotinin, and 0.2 mg/ml benzamidine (pH 7.2)) using an Ultra-Turrax homogenizer (Janke and Kunkel, Staufen, Germany). The homogenates were incubated under agitation for 1 h at 4°C and centrifuged at 140 000 g to isolate cytosols. Aliquots of cytosols were stored at -70°C until they were used for GRK2 immunoblottings.

For measurements of the GRK2 enzymatic activity, the cytosols were further purified by cation exchange chromatography using SP-Sepharose (GE Healthcare Europe GmbH, Orsay, France), as described previously (Metaye *et al.* 2002), allowing to eliminate protein kinase C and thyroglobulin that interfere with phosphorylation and cellular protein assays respectively. The final extracts were stored at -70°C and used for phosphorylation assays.

Positive GRK2 controls were obtained from the cytosolic fraction of transfected COS-7 cells with expression vector containing GRK2 cDNA from human origin.

Protein concentrations were determined by the method of Bradford (1976) with a Bio-Rad protein assay reagent, using BSA as standard (Bio-Rad Labs).

Determination of GRK2 protein expression

The GRK2 protein expression was determined by electrophoresis and immunoblotting, as described previously (Metayé *et al.* 2002). SDS-PAGE was performed by the method of Laemmli (1970) with a 8.5% separating gel. After electrophoresis, the proteins were electrotransferred to a polyvinylidene difluoride (PVDF) membrane. The PVDF membranes were incubated for 1 h at room temperature with anti-GRK2/3 monoclonal antibody clone C5/1.1 (Upstate Biotechnology, Lake Placid, NY, USA) using a 10^{-4} dilution. After several washings, the membranes were incubated for another hour in a 6.7×10^{-5} dilution of peroxidase-conjugated second antibody. The immunoreactive bands were visualized with a commercial chemiluminescence system (ECL Plus, GE Healthcare Europe GmbH). The films were optically scanned with model GS 300 densitometer (Hoefer Scientific, San Francisco, CA, USA). The peak areas were analyzed with a GS365W program, version 2.22 from Hoefer. Results from different blots were normalized using the same positive GRK2 control and expressed in percentage relative to NTT values. All the results were confirmed in at least two separate experiments.

GRK2 enzymatic activity assay

The GRK2 enzymatic activity was assayed using light-dependent phosphorylation of rhodopsin, as described previously (Gagon & Kelly 1997, Metayé *et al.* 2002). Rod outer segment (ROS) membranes were prepared from dark-adapted bovine retinas and then treated with 5 M urea to deactivate the endogenous rhodopsin kinase (Bennett & Sitaramayya 1988). GRK2-dependent phosphorylation was measured by incubating 8 µg of protein extracts with a reaction mixture containing 250 pmol rhodopsin, 50 µM [γ - 32 P]ATP (2 Ci/mmol, GE Healthcare Europe GmbH), 20 mM Tris-HCl, 2 mM EDTA, and 5 mM MgCl₂ in the presence or absence of 2 µl anti-GRK2 antibodies (clone C5/1.1, Upstate Biotechnology and C-15 from Santa Cruz Biotechnology, Inc.) at pH 7.5 in a final volume of 40 µl. The minimal epitope recognized by the clone C5/1.1 is amino acids 483–485, whereas GRK2 antibody from Santa Cruz (C-15) was raised against a peptide corresponding to amino acids 675–689 mapping at the carboxy terminus of GRK2 from human origin. The reactions were carried out at 32 °C for 30 min in the presence or absence of light. The incubations were terminated by the addition of a sodium phosphate solution, followed by centrifugation. The ROS membrane pellets were resuspended in 20 µl SDS sample buffer and electrophoresed on 10% SDS-polyacrylamide gel. Rhodopsin bands were exposed for autoradiography and counted for 32 P radioactivity. Results, obtained by the difference between opsin phosphorylations in the presence and absence of light, were expressed as pmol of phosphate incorporated/min per mg protein. The GRK2 activity was the difference between data obtained in the absence and presence of antibodies. Anti-GRK2 antibodies brought

more specificity to this assay because the GRK2 activity resulted from the activity really inhibited by antibodies and excluded other kinases able to phosphorylate rhodopsin. The enzymatic reaction was linear over 30-min period for an activity no greater than 50 fmol phosphate incorporated/min. All the assays were performed with the same ROS membrane preparation and the results were confirmed in at least two separate experiments, tested in duplicate.

Normal human thyroid cell culture

Normal human thyroid tissues were obtained aseptically from patients who underwent thyroid surgery, usually for uninodular or multinodular goiter. Subsequent steps were performed as previously described by Roger *et al.* (1988). The isolated thyroid cells were cultured overnight in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (1:1) containing 5% foetal calf serum (FCS). Then, the cells were incubated for 24 h in serum-free medium containing 2.5 µg/ml human transferrin, 40 µg/ml vitamin C and bovine TSH, insulin, IGF-I or forskolin at the indicated concentrations. A cytological examination with May-Grundwald Giemsa coloration indicated that cultures contained a minimum of 95% of living thyrocytes, less than 3% of interstitial cells and 1–2% of dead cells.

After incubation, the thyroid cells were detached from the culture dishes by treatment with trypsin, washed and homogenized with a Dounce homogenizer in HEPES buffer containing protease inhibitors. The homogenates were incubated under agitation for 30 min at 4 °C and centrifuged at 25 000 g. The supernatants were diluted in SDS sample buffer, heated at 95 °C for 5 min and used for GRK2 immunoblottings.

Cell lines and stable transfection

FTC-238 is a cell line (ECACC, Salisbury, UK) derived from a lung metastasis of a follicular thyroid carcinoma from a 42-year-old man (Demeure *et al.* 1992). BRA-2 is a thyroid cell line established in our laboratory in 1995 and derived from a lymph node metastasis of a papillary thyroid carcinoma from a 74-year-old woman. The BRA-2 cells have lost their ability to respond to TSH as measured by cAMP synthesis but are still stimulated by forskolin, a direct activator of adenyl cyclase (Supplementary Figure 1, see Supplementary data in the online version of the Journal of Endocrinology at <http://joe.endocrinology-journals.org/content/vol198/issue1/>). Thyroglobulin was not detectable in BRA-2 cytosol. The BRA-2 cells express the cytokeratin 7 protein but not the thyroid transcription factor 1 (TTF1) protein, suggesting an epithelial nature for these cells but also a partial dedifferentiated phenotype. Colony formation in soft agar was obtained with BRA-2 cells, demonstrating an anchorage-independent growth and a malignant disposition for these cells. Both cell lines (FTC-238 and BRA-2) were cultured in DMEM/Ham's F-12 (1:1) containing L-glutamine and supplemented with 10% FCS at 37 °C in a 5% CO₂ incubator. The cells were plated 24 h before transfection at 60% confluency

on 35 mm in diameter plates. Stable transfections were performed using jetPEI reagent (Qbiogene, Montreal, Canada) for FTC-238 and Lipofectamine 2000 (Invitrogen) for BRA-2. The full-length cDNA of human GRK2 in pcDNA3.1 vector was obtained from GeneCopoeia (Germantown, MD, USA). The cell lines were transfected with 3 µg GRK2 plasmid or with the same amount of the pcDNA3.1 empty vector in serum-containing medium. The medium was aspired and transfected cells were then selected by inclusion of 400 µg/ml G418 in the fresh medium 24 h after transfection. Colonies of resistant cells were visible after about 15 days. These colonies were individually harvested with trypsin using cloning rings, placed into multiwell cell culture dishes and expanded. Clones were always grown under antibiotic selective pressure. The expression level of GRK2 in 12 different clones was determined by western blot analysis and cells expressing appropriated GRK2 levels were used.

Cell growth measurement

The cell lines were seeded in 75 cm² culture flasks and allowed to grow in DMEM/Ham's F-12 (1:1) containing L-glutamine and supplemented with 10% FCS at 37 °C in a 5% CO₂ incubator for the indicated times. Cells were harvested with trypsin, washed with medium and exposed to trypan blue, which stains damaged cells only. The number of viable cells, those that excluded the dye, was counted using a Malassez cell.

Statistical analysis

Groups were compared in pairs using Student's *t*-test. A value of *P* ≤ 0.05 was considered as a minimum level of significance.

Results

Immunohistochemical study of GRK2 expression in thyroid carcinomas

Sections from 16 thyroid carcinomas and 15 adjacent or distant NTT were subjected to immunohistochemical staining with antiserum to human GRK2, diluted at 1/400. The results are summarized in Table 1. GRK2 staining was obvious in neoplastic cells of papillary, follicular, and anaplastic types. Nearly, all malignant cells (80% at least) from 12 out of the 16 samples were stained, whereas a few normal cells in the same section were weakly stained. Moreover, the staining intensity was stronger in carcinomas than in adjacent NTT except for two patients (8 and 10) who had the same intensity in both tissues. No difference in percentage and intensity of NTT was observed between the surrounding and distant normal tissue from the tumorous zone. The sections of GRK2-positive tissues resulted in positive cytoplasmic staining with no nucleus localization of the antibody (Fig. 1). Specimens exhibited a rather homogenous staining pattern. The follicular epithelial cells were principally stained but some rare stromal cells, vascular endothelial cells, and lymphocytic infiltrates did also react with the GRK2 antibody. The strong GRK2 immunostaining in all cancer tissues, including anaplastic type, hindered any discrimination or relationship with tumor aggressivity parameters.

Western blotting and GRK2 activity in paired samples of normal and cancerous thyroid tissues

To bring further arguments to immunohistochemical data with different methods, we performed GRK2 protein immunodetection by Western blotting and GRK2 activity

Table 1 Comparative immunohistochemical staining for G-protein-coupled receptor kinase 2 (GRK2) in thyroid carcinoma and paired normal samples

Patient number	Histological group	Stage	Primary tumor		Normal thyroid	
			%	Intensity	%	Intensity
1	Papillary	pT1N0M0	100	++	<5	+
2	Follicular	pT2N1M0	100	+++	40	+
3	Papillary	pT3N1M0	80	+++	5	+
4	Papillary	pT3N0M0	90	+++	10	+
5	Papillary	pT1N1M0	60	++	<5	+
6	Papillary	pT1N0M0	70	++	5	+
7	Follicular	pT1N0M0	80	++	<5	+
8	Follicular	pT1N0M1	90	+	<5	+
9	Papillary	pT1N0M0	90	++	<5	+
10	Follicular	pT3N0M0	90	+	20	+
11	Papillary	pT1N1M0	95	+++	<5	+
12	Papillary	pT1N0M0	100	+++	40	+
13	Anaplastic	pT4bN1M1	100	+++	NP	NP
14	Anaplastic	pT4bN0M0	60	+++	20	+
15	Anaplastic	pT4bN1bM0	80	++	20	+
16	Anaplastic	pT4bN0M0	60	++	30	+

NP, normal tissue was not present.

Figure 1 Immunohistochemical staining of GRK2 in two thyroid carcinomas (B and D) and their adjacent NTT (A and C). Representative sections from a papillary (B) and a follicular (D) carcinoma are shown (magnification $\times 1000$). Nuclei appear in blue and GRK2 in brown. A and C note the presence of GRK2 in thyrocytes (arrow heads) and in endothelial or stromal cells (arrows). B and D note the presence of GRK2 in the cytoplasm of cancerous cells (arrows) with sometimes a staining more pronounced in the apical part of the cell (arrow heads). The great majority of cells are strongly immunostained in cancers compared with normal tissues.

by a phosphorylation assay using rhodopsin as substrate. Five patients, already tested by immunohistochemistry, had sufficient tissue samples to perform GRK2 extraction in cancer tissue and paired NTT. A 79 kDa immunoreactive band, comigrating with a recombinant GRK2 protein, was present in cytosols of NTT and thyroid carcinomas. Because GRK3 protein was not detected in NTT (Metayé *et al.* 2002), this suggest that GRK2 is the main β -adrenergic receptor kinase expressed in the human thyroid. Densitometric analysis of immunoblots (Fig. 2A) revealed a significant difference in relative expression of GRK2 protein between NTT (8.3 ± 1.7 , 14.9 ± 3.0 , and $9.6 \pm 0.7\%$) and cancers (100 ± 5.2 , 63.4 ± 5.8 , and $36.9 \pm 6.5\%$ respectively) in paired samples.

To measure the GRK2 activity, we used two anti-GRK2 antibodies, instead of heparin, to inhibit specifically rhodopsin phosphorylation by GRK2. Thus, the final results were the difference between total GRK activity minus activity obtained in the presence of anti-GRK2 antibodies (Supplementary Figure 2, see Supplementary data in the online version of the Journal of Endocrinology at <http://joe.endocrinology-journals.org/content/vol198/issue1/>). The whole protocol is extremely tissue consuming and only three paired samples of normal and cancerous thyroid tissues were available for treatment with 250 mM NaCl to extract GRKs. ROS were used in assay, resulting in phosphorylation

of a 38 kDa band that was consistent with the labeling of rhodopsin. The light-dependent phosphorylation of ROS by a recombinant GRK2 was completely inhibited by anti-GRK2 antibodies, showing that these antibodies could be used in phosphorylation assay to assess GRK2 activity with accuracy (Supplementary Figure 2). GRK2 activities in cancer samples (3.46 ± 0.30 , 3.01 ± 0.15 and 6.13 ± 0.61 pmol/min per mg protein) were significantly increased compared with activities in adjacent NTT (2.24 ± 0.16 , 1.03 ± 0.08 and 1.73 ± 0.17 pmol/min per mg protein respectively; Fig. 2B).

Altogether, these results confirm the immunohistochemical study demonstrating with other methods an increased GRK2 expression in thyroid cancer compared with normal tissue. Moreover, we can point out some quantitative variations from one cancer or one NTT to another, that are not always revealed by immunohistochemistry.

Regulation of GRK2 expression in human thyroid cell culture

To study the influence of thyroid mitogenic factors in GRK2 expression, the role of TSH, insulin, and IGF-I was investigated in normal human thyroid cell culture. Furthermore, to discriminate cAMP effect in TSH action, forskolin was also tested. Cellular GRK2 from a 24-h cell culture was analyzed by

Figure 2 Assessment of GRK2 protein expression (A) and GRK2 activity (B) in thyroid carcinomas and their adjacent NTT. (A) The lower panel represent an autoradiography of a Western blot depicting GRK2 immunoreactivity in three patients. Twenty micrograms of cytosol proteins were analyzed for GRK2 expression and protein transfer was controlled by staining the PVDF membrane with Coomassie brilliant blue R-250. The upper panel is a densitometric analysis of the lower panel and the data are expressed in percent of DTC value from patient 3. The migration of a recombinant GRK2 protein control is indicated by an arrow. The data represent the mean $\pm$ s.d. confirmed in at least three separate experiments. (B) GRK2 activity was measured using partially purified samples of thyroid cancers and NTT, as described in Materials and Methods. Patient's numbers are the same as in Table 1. The data are means $\pm$ range of duplicate determinations representative of two separate experiments. * $P < 0.01$; ** $P < 0.001$.

SDS-PAGE and immunoblotting (Fig. 3). A single band comigrating with a recombinant GRK2 was specifically detected by the anti-GRK2 antibody. A 24-h treatment of thyroid cells with TSH produced an increase (1.54-fold) in GRK2 expression level when compared with the control, whereas insulin or IGF-I had little or no effect. Furthermore, when TSH was tested in the presence of insulin or IGF-I, GRK2 protein expression increased by 2.0 and 2.09 respectively, compared with the control. Forskolin mimicked TSH effect (Fig. 3) suggesting that the GRK2 increase was secondary to cAMP accumulation. These results were confirmed using thyroid cells from three different patients, at least. The ability of

Figure 3 The regulation of GRK2 expression in normal human thyroid cell culture. Thyroid cells were cultured during 24 h in serum-free medium without (Ctl) or with 5 mU/ml TSH, 5 μ g/ml insulin (Ins), 100 ng/ml IGF-I, TSH (5 mU/ml) plus insulin (5 μ g/ml), TSH (5 mU/ml) plus IGF-I (100 ng/ml) and forskolin (Fk, 10 μ M). Then, cellular GRK2 was extracted as described in Materials and Methods. Similar amounts of protein (20 μ g) were analyzed by Western blotting and protein transfer was controlled by staining the PVDF membrane with Coomassie brilliant blue R-250. In the upper panel, the GRK2 lane indicated the migration of a recombinant GRK2 protein. The lower panel is a densitometric analysis of the upper panel and the data are expressed in percent of Ctl value representing the mean $\pm$ s.d. of four densitometric measurements. The results were confirmed in at least three separate experiments using different human thyroid cells. * $P < 0.001$ compared with Ctl; ‡ $P < 0.01$ compared with Ctl, and § $P < 0.01$ compared with TSH.

TSH plus insulin or IGF-I to increase GRK2 even exceeded levels induced by the addition of each growth factor alone, eliciting a synergistic effect. TSH action on GRK2 expression was reproduced in an heterologous cell system, CHO cells transfected with TSH receptor cDNA and stimulated during 24 h in the presence of serum (data not shown).

Effects of GRK2 overexpression on thyroid cancer cell proliferation

The augmented level of GRK2 by thyroid mitogenic factors suggests a role for this kinase in the proliferative process. Therefore, to determine whether GRK2 have a key role in thyroid cancer progression, we studied the effect of GRK2 overexpression on the growth of two thyroid cancer cell lines (FTC-238 and BRA-2) cultured in medium containing 10% serum. We generated different cellular clones by permanently transfecting cells with GRK2 cDNA and the empty vector pcDNA3.1 (vector) that was used as a control together with not transfected cells (NT). GRK2 overexpression resulted in 3.1- and 9.5-fold increase compared with NT in two different FTC-238 clones (Fig. 4Aa) and 21.7- and 38.9-fold increase compared with NT in two different BRA-2 clones (Fig. 4Ba). The growth rate was measured by counting cells using a hemacytometer. As shown in Fig. 4Ab and Bb, GRK2 expression limited the cell growth in all the clones tested in both cell lines, ranging from 28 to 65% inhibition compared

Figure 4 The effect of GRK2 expression on thyroid cancer cell growth in (A) FTC-238 and (B) BRA-2 cell lines. (a) Representative western blot and densitometric analysis, depicting GRK2 overexpression in FTC-238 or BRA-2 clones, permanently transfected with GRK2 cDNA (FT-C1, FT-C2 and BR-C1, BR-C2 respectively), empty vector (vector) or not transfected (NT). 20 μ g cytosol proteins were blotted and GRK2 immunoreactivity was detected as described in Materials and Methods. Data are representative of three independent experiments. (b) GRK2 overexpression attenuated serum-induced cell growth in FTC-238 and BRA-2 clones. Cells number was determined by counting viable cells four times using hemacytometer. Data are means $\pm$ S.D. representative of two independent experiments. * $P < 0.01$ compared with NT or vector; ** $P < 0.001$ compared with NT or vector.

with vector alone after 96-h culture. The best results were for FT-C1 (FTC-238 clone 1) and BR-C1 (BRA-2 clone 1), with 65 and 47% reduction ($P < 0.001$) in cell proliferation after 96-h culture respectively.

Discussion

This study clearly demonstrates, using immunohistochemistry, an increase in GRK2 protein expression in non-medullary thyroid cancers compared with their adjacent normal tissues. Immunoblot analysis and GRK2 enzymatic activity measurement confirmed immunohistochemical study, revealing a greater GRK2 content in carcinomas, but also some variations in the levels of GRK2 expression more evident with these techniques than with immunohistochemistry. Paired thyroid samples are advisable to measure GRK2 expression because both normal and cancerous tissues from a single patient have the same TSH impregnation. This may explain that the present study is not in perfect agreement with our previous findings on unpaired samples where a slight increase in GRK2 in cancers had not reached a significant statistical level (Metayé *et al.* 2002).

Although GRK2 has been studied in hormone-dependent tumors of thyroid, ovary (King *et al.* 2003) and prostate (Prowatke *et al.* 2007), the regulation of its expression and its role in cancer progression remain unanswered. Our experiments on GRK2 regulation showed that thyroid mitogenic factors such as TSH or TSH in association with insulin or IGF-I increased GRK2 expression in primary culture of human thyrocytes. Therefore, our *in vitro* system mimics, in part, the *in vivo* phenotype of GRK2 expression in thyroid cancers. These results suggest that GRK2 synthesis might be induced by stimulation of receptors present in thyroid cancers as demonstrated for TSH, insulin, and IGF-I. The synergistic effect of TSH + insulin/IGF-I supports the stimulation of two different and convergent pathways, leading to GRK2 expression in thyroid cells. Effectively, in the present study, we show that cAMP-mediated forskolin stimulation increases GRK2 expression in human thyrocytes. This result demonstrates tissue specificity, because no forskolin effect was observed in cells with other origins, such as astrocytoma or aortic smooth muscle (Ramos-Ruiz *et al.* 2000). As the human TSH receptor stimulates $G\alpha_s$ (Laugwitz *et al.* 1996), this suggests that TSH-stimulated cAMP accumulation might increase GRK2 synthesis. Furthermore, IGF-I by activating the PI3K/Akt pathway alters Mdm2-mediated GRK2

ubiquitination and decreases GRK degradation (Salcedo *et al.* 2006). Overall, the hypothesis of TSH action on GRK synthesis stimulation and the effects of IGF-I or insulin (Shepherd 2005) on GRK2 stability might explain a synergistic interaction on GRK2 expression. However, the absence of TSH receptor in anaplastic carcinoma also suggests that the increased GRK2 protein is not necessarily linked to TSH receptor signaling in this thyroid cancer subtype. The regulation of GRK2 by TSH in normal thyrocytes agrees with the up-regulation of genes (phosphodiesterases and regulator of G-protein signaling 2, RGS2) involved in negative feedback of TSH-stimulated cAMP accumulation (Van Staveren *et al.* 2006) and with the decreased GRK2 expression observed in pathological thyroid tissues showing a reduced cAMP stimulation (Voigt *et al.* 2005). Furthermore, alterations of GRK2 expression have been reported in other systems with an increase observed in mitogen-activated lymphocytes (De Blasi *et al.* 1995), mouse heart following treatment with β -adrenergic agonist (Iaccarino *et al.* 1998), angiotensin II-stimulated cardiomyocytes (Theilade *et al.* 2002), and α_1 -adrenergic agonist-activated aorta smooth muscle cell line (Ramos-Ruiz *et al.* 2000); while proinflammatory cytokines, such as interleukin 1, interleukin 6, tumor necrosis factor- α or interferon- γ , decreased GRK2 expression in human mononuclear leucocytes (Lombardi *et al.* 1999) and in rat smooth muscle cell line (Ramos-Ruiz *et al.* 2000). In quite contrast with the up-regulation of GRK2, TSH reduced GRK5 protein level in rat thyroid cells (Nagayama *et al.* 1996b).

Early studies performed in thyroid cell cultures have shown that the mitogenic effect of TSH is small in the absence of other growth factors, although it is greatly enhanced by the presence of insulin or IGF-I at physiological concentrations (Kimura *et al.* 2001). Thus, the synergistic response of TSH+insulin/IGF-I on GRK2 expression parallels hormone effects on thyroid cell proliferation, suggesting a role for GRK2 in the growth of thyroid cancer. Here, we show that GRK2 overexpression in two different human thyroid cancer cell lines significantly attenuates serum-induced cell proliferation. This unexpected finding indicates that GRK2 interacts with receptors or proteins that have an important mitogenic function in thyroid cells. TSH-stimulated cAMP synthesis as well as lysophosphatidic acid-stimulated proliferative response (Iacovelli *et al.* 1999, 2002) was negatively regulated by GRK2 in rat thyroid cells. As the cAMP cascade is involved in the control of human thyroid cell proliferation *in vivo*, it is tempting to speculate that in DTC, GRK2 would attenuate cAMP-dependent TSH signaling. However, FTC-238 and BRA-2 are poorly differentiated thyroid cancer cell lines where the TSH-Gs-cAMP system is not functional (Demeure *et al.* 1997), suggesting in these cells a GRK2 effect independent of TSH signaling.

It is usual to correlate an increased protein expression in cancers with their aggressiveness or their growth; however, our study demonstrates an inverse concept whereby the cell appears to use a physiological mechanism (GRK2 expression) to fight against cancer. Several recent findings have shown that GRK2 interacts with and phosphorylates proteins other than

the GPCRs, broadening the variety of its cellular functions (Metaye *et al.* 2005). Thus, several targets for GRK2 have been discovered such as the platelet-derived growth factor receptor- β (PDGFR- β), the protein kinase Akt and the mitogen-activated protein kinase kinase (MEK), with a potential role in cell growth attenuation. Seryl phosphorylation of the PDGFR- β by GRK2 increased receptor desensitization and diminished phosphoinositide hydrolysis, Akt activation and tyrosyl phosphorylation of the receptor itself (Hildreth *et al.* 2004). Furthermore, GRK2 overexpression reduced smooth muscle cells proliferation *in vitro* in response to endothelin-1 or PDGF and *in vivo* by studying neointimal hyperplasia in rabbit vein grafts (Peppel *et al.* 2002). On the other hand, a physical interaction of Akt with GRK2 was found in injured liver sinusoidal endothelial cells, inhibiting Akt activity (Liu *et al.* 2005). As Akt is a key mediator of several tyrosine kinase receptors, leading to cell survival or antiapoptosis (Shinohara *et al.* 2007), a link between Akt and GRK2 might exist in thyroid to reduce excessive growth of cancer. Finally, the GRK2 overexpression produced a significant attenuation of the extracellular signal-regulated kinase (ERK) response to the chemokine CC motif ligand 2 (CCL-2; Jiménez-Sainz *et al.* 2006) or to angiotensin II (Kim *et al.* 2005), involving a possible GRK2-MEK interaction or an arrestin-mediated system respectively. Given the role of MAPKs and chemokines in mitogenesis and motility of thyroid cancer cells, the regulation of these signaling cascades by GRK2 might represent fields of future research to better understand human thyroid cancer progression.

In summary, the present study showed an increased expression of GRK2 in non-medullary thyroid cancers using immunohistochemistry, western blotting and enzymatic activity measurement. Thyroid mitogenic factors, such as TSH or TSH+insulin/IGF-I, induced GRK2 accumulation in normal human thyroid cell culture. The contribution of GRK2 to attenuate cell growth revealed a new role for this kinase in the control of thyroid cancer progression. However, the mechanism by which GRK2 acts and the role of this kinase in human cancers other than thyroid remain to be explored.

Declaration of Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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