CLINICAL STUDY

Mutation analysis of the Epac–Rap1 signaling pathway in cold thyroid follicular adenomas

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Abstract

Objective: The cyclic AMP (cAMP) cascade is the main regulatory pathway in thyrocytes. Whilst activating mutations in the TSH receptor or in the Gs α-subunit, which increase cAMP levels, have been shown to be responsible for 80% of the autonomous adenomas, no such mutations have been observed in the other types of thyroid tumors, suggesting that other mechanisms exist. The discovery of Epac (‘exchange nucleotide protein directly activated by cAMP’), a novel cAMP-binding protein, which is strongly expressed in the thyroid, raised the possibility of a role for this protein in the generation of the unexplained cold thyroid follicular adenomas. Thus, we investigated whether activating mutations in either Epac or Rap (the downstream target of Epac) could be responsible for the generation of these thyroid nodules.

Design: Epac and Rap1 (Rap1A and Rap1B) cDNAs were sequenced in 10 patients. The sequencing of the cDNAs was realized on both strands in the cold nodule and the juxtanodular tissue of each patient.

Results: No mutations in either Epac or Rap1 cDNAs were found. For five patients, a polymorphism in Epac at codon 332 (Gly–Ser) was observed.

Conclusions: In this report, we show that the cAMP–Epac–Rap1 signaling pathway in the thyroid gland does not play a major role in the generation of cold thyroid follicular adenomas, since no mutations in either Epac or Rap1 could be observed in the 10 nodules studied.

European Journal of Endocrinology 144 605–610

Introduction

Encapsulated benign thyroid tumors are defined, using pathological criteria, as follicular adenomas. They may be solitary in an otherwise normal thyroid or may present as multinodular goiter. Encapsulated and non-encapsulated tumors are clinically defined as nodules. When they take up iodide with avidity and secrete thyroid hormones independently of thyroid-stimulating hormone (TSH), they progressively repress TSH secretion and thus progressively lead the rest of the thyroid to quiescence. Such lesions are called either ‘hot’ or ‘autonomous’. Whether encapsulated or not, 80% have been shown to be caused by mutations conferring constitutive activity on the TSH receptor or on the Gs α-subunit (Gs), its downstream GTP-binding protein, thus leading to chronic stimulation of adenylyl cyclase and enhancement of cyclic AMP (cAMP) accumulation (1, 2). Nodules that do not take up radio-iodide in the presence of normal serum TSH levels are called ‘cold’. In general, the expression of the iodide transporter (NIS) is very low and, as expected, no activating mutations of the TSH receptor or Gs have been found in them (3–7). Their cause is unknown. Activating mutations of the various Ras isotypes have been found in some of them (8).

Although most cAMP-mediated effects in eukaryotes have been ascribed to the binding of cAMP to cAMP-dependent protein kinases (PKAs), it is now clear that other cAMP-target proteins exist. The search for new cAMP-effector molecules resulted from the observations that, in certain systems, some effects of cAMP could not be reproduced by the active catalytic subunit of protein kinase A. Among these systems is the brain, in which some neuronal functions, such as neuroplasticity and cognition (9–11), have been shown to be modulated by cAMP (though no involvement of PKAs could be demonstrated). Another example of such a system is the thyroid gland. Indeed, in a recent study of dog thyroid cells, our group showed that PKA stimulation alone could not reproduce the effects of cAMP on cell proliferation and thyroglobulin gene expression (12). These data led us to suggest that another cAMP-dependent – but PKA-independent –
pathway could exist and account for the mitogenic action of TSH. To date, in addition to PKAs, two types of cAMP-target proteins have been identified: cyclic-nucleotide-regulated channels (13), and the protein Epac ('exchange nucleotide protein directly activated by cAMP') (14, 15). The latter, which is a specific guanine nucleotide exchange factor (GEF) for the small G protein Rap1, was recently identified in the database (accession number EMBL U78168) as a genomic sequence with homology to cAMP-binding sites as well as to GEF domains for Ras-like proteins. Although this protein is directly activated by cAMP, its stimulation has been shown to be independent of the activation of PKAs (14). The Epac family consists of two highly homologous members, Epac1 and Epac2, as well as a new related GEF also acting on Rap1 (16). Whereas Epac1 is broadly expressed, nonetheless showing preferential expression in the thyroid, ovary, kidney and specific areas of the brain (the cortex, the amygdala, the corpus colossum and the caudate nucleus), Epac2 is restricted to the brain and the adrenal glands. The Rap1 proteins, which are small GTPase proteins activated by Epac, have been reported to be involved in various cellular processes such as cell differentiation (17, see comments), T-cell anergy (18), platelet activation (19) and also proliferation (20–22). Rap1, for example, has been described as exerting positive control over the proliferation of Swiss 3T3 cells, in which (as in thyrocytes) cAMP exerts a stimulatory effect on cell growth (20). Moreover, alteration of the Rap1 signaling pathway has also been shown in human gliomas (23).

In dog thyroid cells, Rap1 activation is a common feature of several cascades stimulated by mitogenic agents such as TSH, forskolin, epidermal growth factor, insulin, and phorbol esters (24).

The functional properties of Epac and its thyroidal tissue expression (Epac1 isoform) raised the possibility of a role for this protein in the generation of thyroid adenomas and nodules, and, in particular, in the generation of the unexplained cold thyroid nodules. An effect of Epac on mitogenesis but not on the functional effects of cyclic AMP, which are mediated by protein kinase A, could indeed account for this disease (Fig. 1). Rooij et al. have shown that the deletion of the cAMP-binding domain of the protein activates it, even in the absence of cAMP.

The presence of activating mutations in Epac1 was investigated in 10 cold thyroid nodules. For each patient, Epac1 was entirely sequenced in the nodule, but also in the normal adjacent tissue (to demonstrate an eventual somatic mutation). To clarify the cAMP–Epac signaling pathway in cold thyroid nodules, we completed our analysis by investigating whether somatic activating mutations in Rap1 could be found in these thyroid tumors. As the Rap1 family consists of two highly homologous members, Rap1A and Rap 1B (15), the search for eventual activating mutations was undertaken for both isoforms. In this report, we show that the cAMP–Epac–Rap1 signaling pathway in the thyroid gland does not play a major role in the generation of cold thyroid tumors, as no mutations in either Epac or Rap1 could be observed.

Materials and methods

Patients

Thyroid tissues were obtained from 10 patients (six females, mean age, 42 ± 9 years; four males, mean age, 53 ± 17 years) undergoing surgery for clinical reasons. The cold-nodule diagnosis was confirmed by scintiscanning, and thyroid functions were normal. The histological analysis identified nine follicular adenomas and one colloid nodule. The study was approved by the local ethical committee, and informed consent was obtained from all participants before testing.

Sequencing of Epac and Rap

The RNA was extracted from frozen nodular and juxtanodular tissue obtained from 10 patients, by
PCR amplification, the resultant PCR products were
8
for 1 min, 72
8
for 1 min, 56
8
57
8
were started by a denaturation step at 93
RT). The cDNA synthesis was carried out in a 100
m
leukemia virus (MMLV) reverse transcriptase (MMLV-
cDNA synthesis using hexamer and murine Moloney
Netherlands). Total RNAs were used as templates for
using the SNAP kit (Invitrogen, Groningen, The
Netherlands). Total RNAs were used as templates for
Table 1 Summary of the primers used for the PCR amplification of Epac (accession number EMBL U78168), Rap1A (accession number EMBL M22995) and Rap1B (accession number EMBL X08004).

<table>
<thead>
<tr>
<th>Pair</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epac 1</td>
<td>85 5’-GAAACATGAAGTGCTTGGCTG-3’ 104</td>
<td>503 3’-CTGAGACCCGGGACCCTGAA-5’ 522</td>
<td>437</td>
</tr>
<tr>
<td>Epac 2</td>
<td>424 5’-GCCCAACCTCTACCTCGGAG-3’ 443</td>
<td>804 3’-CTCGAGAGCTTGATTTCCG-5’ 823</td>
<td>399</td>
</tr>
<tr>
<td>Epac 3</td>
<td>734 5’-CTCTGCTACGTGTCGGACTC-3’ 783</td>
<td>1094 3’-GGCAACCTTTGTCCTGCTG-5’ 1112</td>
<td>378</td>
</tr>
<tr>
<td>Epac 4</td>
<td>1055 5’-AGCCACCATCTACCTCGGAG-3’ 1074</td>
<td>1478 3’-CTGCTAGACCCGGACACTG-5’ 1497</td>
<td>442</td>
</tr>
<tr>
<td>Epac 5</td>
<td>1334 5’-AATCTCCATAGCGACTTC-3’ 1354</td>
<td>1677 3’-GCTCTATCCTGGCCTTGA-5’ 1695</td>
<td>361</td>
</tr>
<tr>
<td>Epac 6</td>
<td>1555 5’-TCCCTCAAACAAACTCTAGAC-3’ 1575</td>
<td>1901 3’-GTTAGACCTCGTACCTGG-5’ 1919</td>
<td>364</td>
</tr>
<tr>
<td>Epac 7</td>
<td>1882 5’-GAGCGGCTTGGTGAAAGGTC-3’ 1901</td>
<td>2342 3’-GTTAAGGAAAGACCGACGTAC-5’ 2363</td>
<td>481</td>
</tr>
<tr>
<td>Epac 8</td>
<td>2232 5’-TCAGGAGCAGAAGAAATGCAA-3’ 2344</td>
<td>2459 3’-GCACCTAGGGATACCTTG-5’ 2477</td>
<td>154</td>
</tr>
<tr>
<td>Epac 9</td>
<td>2468 5’-CTCATGGAACACCCGGGTAT-3’ 2487</td>
<td>2921 3’-CCCGGTTCTAAGTGATCG-5’ 2940</td>
<td>472</td>
</tr>
<tr>
<td>Rap1A 1</td>
<td>302 5’-CACATACATCATCTACGTCG-3’ 319</td>
<td>594 3’-CCTGACCTTCCTGCTCAA-5’ 614</td>
<td>312</td>
</tr>
<tr>
<td>Rap1A 2</td>
<td>535 5’-GGCCAGGTTTGGTCTAGT-3’ 554</td>
<td>914 3’-GACACAGGATTCCTCTTCGCA-5’ 935</td>
<td>400</td>
</tr>
<tr>
<td>Rap1B 1</td>
<td>11 5’-CGTGAGAGGTTGTCAGGAGTACT-3’ 32</td>
<td>665 3’-CAGGGTCGTAAGGTTGAAAC-3’ 684</td>
<td>673</td>
</tr>
</tbody>
</table>

using the SNAP kit (Invitrogen, Groningen, The
Netherlands). Total RNAs were used as templates for
cDNA synthesis using hexamer and murine Moloney leukemia virus (MMLV) reverse transcriptase (MMLV-
RT). The cDNA synthesis was carried out in a 100 µl reaction volume containing 5 µg total RNA. 5 µl hexamer (0.48 µg/µl), 2 µl each deoxynucleoside triphosphate (dNTP) (10 mM), 10 µl dithiothreitol (0.1 mol/l), 3 µl RNase inhibitor (40 U/µl), 25 µl Tp x 5 (BRL, Invitrogen) and 5 µl MMLV-RT (200 U/µl). After heating of the samples at 65 °C for 15 min to remove secondary structures, the transcription was carried out at 42 °C for 1 h. The reaction was stopped by denaturing the MMLV-RT for 5 min at 95 °C. The samples were stored at −20 °C. Table 1 summarizes the pairs of primers chosen to amplify Epac1, Rap1A and Rap1B. Each PCR was performed in a final volume of 50 µl containing 10 µl cDNA, 10 pmol each primer, 2.5 U Taq DNA polymerase (BRL),10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 1.5 mmol/l MgCl2, 0.01% gelatin and 0.2 mmol/l dNTPs. All of the reactions were started by a denaturation step at 93 °C for 2 min 30 s, followed by 35 cycles as follows: 93 °C for 1 min, 60 °C for 1 min, 72 °C for 30 s (for Epac 1); 93 °C for 1 min, 57 °C for 1 min, 72 °C for 1 min (for Epac 2); 93 °C for 1 min, 60 °C for 30 s, 72 °C for 1 min (for Epac 3); 93 °C for 1 min, 64 °C for 1 min, 72 °C for 1 min (for Epac 4); 93 °C for 1 min, 57 °C for 1 min, 72 °C for 1 min (for Epac 5); 93 °C for 1 min, 57 °C for 1 min, 72 °C for 1 min (for Epac 6); 93 °C for 1 min, 56 °C for 30 s, 72 °C for 30 s (for Epac 7); 93 °C for 1 min, 54 °C for 30 s, 72 °C for 30 s (for Epac 8); 93 °C for 1 min, 57 °C for 1 min, 72 °C for 1 min (for Epac 9); 93 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min (for Rap1A 1); 93 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min (for Rap1A 2); 93 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min 30 s (for Rap1B); a final elongation step of 6 min at 72 °C follows. After the PCR amplification, the resultant PCR products were purified using the QIA quick PCR purification kit (Westburg, Leusden, The Netherlands) and sequenced on both strands. Samples were loaded onto a 373 or 377 Stretch Sequencing Instrument (Perkin-Elmer, Applied Biosystems, Norwalk, CT, USA) and analyzed using factura and sequence navigator software (Perkin-Elmer Applied Biosystems).

**Gly–Ser polymorphism searches in control patients**

Restriction reactions with the HpaII enzyme were realized on DNA fragments (1689 bp) amplified by PCR with the following pair of primers: forward primer, 5’-AGCCACCATCATCTGGAG-3’; reverse primer, 5’-TGACCAGCGACATCTGG-3’. The DNAs were obtained from control patients from the hospital’s Genetics Department. PCRs were performed in a final volume of 50 µl containing 2.5 µg DNA, 10 pmol each

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**Figure 2** Sequencing results. (a) Direct sequencing of PCR products from a patient in which no substitution was found. (b) Direct sequencing of PCR products from a patient possessing a G-to-A transition leading to a substitution of a glycine for a serine (GGC --> AGC) at position 332. Sequencing was performed on both strands.
primer, 2.5 U Taq DNA polymerase (BRL), 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 1.5 mmol/l MgCl₂, 0.01% gelatin and 0.2 mmol/l dNTPs. All of the reactions were started by a denaturation step at 93 °C for 2 min 30 s, followed by 35 cycles comprising 93 °C for 1 min, 64 °C for 1 min and 72 °C for 1 min.

The restriction reaction was realized in a final volume of 20 µl, and was carried out at 37 °C for 2 h. A 10 µl sample of the PCR product and a 1 µl sample of the enzyme (10 U/µl) was used for each reaction. The products of the restriction were separated on a 3% agarose gel.

**Figure 3** Schematic representation of the results expected after the restriction reaction with the HpaII enzyme on heterozygotic patients, harboring the Gly → Ser mutation (X), and homozygotic patients, in which no mutation is present.

**Figure 4** Analysis of the incidence of the Gly → Ser mutation in the Epac genes of 20 control patients. The DNA was first amplified by PCR and then restricted with the HpaII enzyme for 2 h at 37 °C (see Materials and methods).
Results and discussion

We have searched for somatic mutations in Epac in cold thyroid nodules. Our results indicate that Epac1 is not mutated in these lesions. Indeed, none of the 10 solitary ‘cold’ thyroid nodules analyzed contained somatic mutations in the cDNA coding for Epac1. Five of the patients investigated, however, had a polymorphism at codon 332 (Gly–Ser) (Fig. 2). As 50% of the patients tested showed the mutation, we investigated whether this polymorphism could be associated with the development of cold thyroid adenoma. There is, indeed, increasing evidence showing that polymorphisms in specific genes can be associated with diseases, e.g. lung cancer (25) or cardiovascular disease (26). Thus, we investigated 20 healthy control patients for the presence (and incidence) of the Gly-to-Ser mutation. As this mutation suppresses an HpaII enzyme cleavage site (Fig. 3), an easy way to check whether this mutation was present or not (in these control patients) was to amplify the desired region by PCR and to subject the PCR products to a restriction reaction with the HpaII enzyme. In homozygotic patients, in which no mutations are found, three bands are expected (one at 613 bp, another at 1044 bp, and a third at 32 bp); in heterozygotic patients (possessing the polymorphism), however, an additional band is expected at 645 bp, as both alleles are present. Thus the 645 bp band indicates the presence of the mutation in the patient. The results (Fig. 4) show that 55% of the patients possessed the mutation. These data, therefore, suggest that there is no association between the polymorphism in the Epac gene and the generation of a thyroid follicular adenoma.

To complete our analysis of the cAMP–Epac–Rap1 cascade in the cold thyroid nodules, we searched, in the 10 patients, for mutations of the Rap1 protein. However, as for Epac, no mutations could be found for either Rap1A or Rap1B (isoforms of the Rap1 protein).

Taken together, our results do not support the hypothesis that somatic mutations in the Epac–Rap1 pathway can account for the generation of thyroid cold nodules, at least not as a frequent cause. Thus the pathogenic mechanisms involved in these lesions remains an enigma, and will require further investigation.

Acknowledgements

A Allgeier is ‘Chargé de Recherches’ of the Belgian Fonds National de la Recherche Scientifique. V Vanvooren is a Fellow of the Fonds Pour la Formation à la Recherche dans l’Industrie et dans l’Agriculture. This work was supported by the Ministère de la Politique Scientifique (PAI), the Fonds National de la Recherche Scientifique, the Fonds de la Recherche Scientifique Médicale, the Fonds Cancérologiques de la CGER, the Association Contre le Cancer, the Association Sportive Contre le Cancer, and Télévie.

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Received 28 November 2000
Accepted 2 March 2001