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Identification and characterization of a novel activated RhoB binding protein containing a PDZ domain whose expression is specifically modulated in thyroid cells by cAMP

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In a search for genes regulated in response to cAMP we have identified a new protein, p76^{RBE}, whose mRNA and protein expression is enhanced in thyrocytes following thyrotropin stimulation of the cAMP transduction cascade. This protein presents important similarities with Rhophilin and contains different protein–protein interaction motifs. The presence of HR1 and PDZ motifs as well as a potential PDZ binding domain motif suggests that p76^{RBE} could be implicated in targeting or scaffolding processes. By yeast two-hybrid screenings and coimmunoprecipitation, we show here that p76^{RBE} is a specific binding protein of RhoB and binds selectively to the GTP-bound form of this small GTPase. p76^{RBE} also binds *in vitro* to components of the cytoskeleton, including cytokeratin 18. p76^{RBE} is essentially cytoplasmic in transfected COS-7 mammalian cells and seems to be recruited to an endosomal compartment when coexpressed with the activated form of RhoB. p76^{RBE} was shown to be mainly expressed in tissues with high secretion activity. Our data suggest that p76^{RBE} could play a key role between RhoB and potential downstream elements needed under stimulation of the thyrotropin/cAMP pathway in thyrocytes and responsible for intracellular motile phenomena such as the endocytosis involved in the thyroid secretory process.

Keywords: rhophilin-like; activated RhoB; scaffold; endocytosis; PDZ.

The major known function for most Rho GTPases is to regulate the assembly and organization of the actin cytoskeleton [1]. The requirement of Rho GTPases as key components in cellular processes that are dependent on the actin cytoskeleton is now well described. A role for Rho family members has been shown in cell adhesion, cell movement, endo- or exocytosis processes, and membrane and vesicle trafficking [2]. The molecular mechanism by which the small GTPases Rho-link extracellular signals to transduction pathways are of particular interest for understanding these biological processes. In addition, Rho GTPases are also able to influence biochemical pathways, the generation of lipid secondary messengers, cell cycle progression and cell transformation in some cell types [2].

RhoA, which has been most studied, causes the formation of stress fibers and focal adhesion plaques [3] and has been shown to activate the transcription factor SRF [4]. RhoB is closely related to RhoA in sequence but is differently localized, regulated and prenylated. RhoB is short-lived and is an immediate early gene induced in response to v-Src, epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) [5]. RhoB is localized in endosomes [6] where it could be implicated in receptor-mediated endocytosis events [7] and where it targets PRK-1 (protein kinase C-related kinase 1) [8]. RhoB also has cell cycle inhibitory effects suggested by its up-regulation by UV radiation and DNA damaging and by its ability to regulate NFκB dependent transcription [9,10].

Many efforts have been focused on elucidation of Rho signaling events and recent studies have reported the identification of several Rho effectors. Based on their different Rho-binding motifs, several proteins can be proposed as Rho target molecules: PRK-1 and PRK-2 [11,12], Rhophilin [13] and Rhotekin [14] all contain a Rho-binding motif of type I (HR-1). Both the coil-coiled kinases ROCK-I [15] and ROCK-II [16–18] contain a Rho-binding motif of type II; citron [19] and p140mDIA [20] are two other Rho-binding proteins which have low similarity with the previous ones. Different regions of Rho determine Rho-selective binding of different classes of Rho target molecules [21]. Various data suggest that they could be potential links between the extracellular signal and the actin cytoskeleton [22]. Nevertheless, how each of these different target proteins regulates the cell response to different stimuli and the real specificity of the interactions between the various forms of Rho and the different effectors remains to be determined.

To better understand differentiated epithelial growth regulation, we initiated a study aimed at identifying genes

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Abbreviations: EGF, epidermal growth factor; EGFP, enhanced green fluorescence protein; GSt, glutathione *S*-transferase; HGF, hepatocyte growth factor; IPTG, isopropyl thio-β-D-galactoside; MAPK, mitogen-activated protein kinase; PDGF, platelet-derived growth factor; PKC, protein kinase C; PMA, 4β-phorbol 12-myristate 13-acetate; PRK-1, protein kinase C-related kinase 1; wt, wild type.

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that are regulated by the thyrotropin-activated pathways in dog thyroid cells. By differential screening of a chronically stimulated dog thyroid cDNA library, we identified several new differentially expressed genes [23]. Among these, we identified a novel Rho target protein, 76 kDa RhoB effector protein ($p76^{RBE}$) (reported as clone 45 [23]), which contains a PDZ domain and presents a high similarity with Rhophilin. $p76^{RBE}$ interacts only with the GTP-bound form of RhoB and is targeted to endosomes upon stimulation of the small GTPase. The expression of $p76^{RBE}$ is up-regulated by the stimulation of the thyrotropin/cAMP cascade in thyrocytes.

MATERIALS AND METHODS

Plasmids and antibodies

The dog $p76^{RBE}$ coding sequence was amplified by PCR and cloned into pGEX (Amersham Biosciences, Roosendaal, Netherlands), pcDNA3.HA and pEGFP-C3 (Clontech, Erembodegem, Belgium). Likewise, we fused full-length keratin cDNA to the His-tag of pcDNA3 (Invitrogen, Merelbeke, Belgium). The pcDNA3.myc–RhoB wild type (wt), pcDNA3.myc–RhoBT19N dominant negative and pcDNA3.myc–RhoBQ63L constitutively active were created [8]. RhoA and Rac1 wt, dominant negative and constitutively active cDNAs were kindly provided by M. Spaargaren (Utrecht University, the Netherlands). The Rho C wt cDNA was a gift from J. Camonis (Curie Institute, Paris). The RhoCT19N and RhoCQ14L cDNA were obtained by quickchange punctual mutations of the wt cDNA of RhoC in pPC86 (kind gift of P. Chevray of the University of Texas, Houston, TX, USA and D. Nathans from the Howard Hughes Medical Institute, Baltimore, MD, USA). All the full-length Rho GTPases cDNAs were cloned in pPC86 by PCR. All constructions were verified by DNA sequencing.

The mouse anti-HA and anti-MYC (9E10) mAbs were purchased from Roche and the mouse anti-HIS mAb was purchased from Clontech. Polyclonal antibodies against $p76^{RBE}$ were generated by immunizing rabbits with a synthetic peptide (QPLEKESDGYFRKGC) corresponding to amino acids 11–25 of the dog $p76^{RBE}$ sequence and a second peptide (LPTPFSLLNSDSSLY) (amino acids 672–686) located in the C terminus. The N-terminal antibody was further purified using peptide affinity chromatography.

Two-hybrid screenings and constructs

The N-terminal domain ($p76^{RBE}$–HR1) (amino acids 1–127) or the complete sequence of $p76^{RBE}$ was cloned by PCR downstream of the Gal4 DNA-binding domain in the yeast two-hybrid vector pPC97 (kind gift of P. Chevray and D. Nathans). Both constructions were verified by DNA sequencing. The cDNAs of the different Rho proteins described above or the cDNA library synthesized from dog thyroid poly(A)+ RNAs (Superscript plasmid system, Gibco BRL) were fused to the Gal4 transcription activating domain in the yeast two-hybrid vector pPC86. The yeast host strain used for the screening and the reconstruction steps was the pJ69–4A (MAT a, ade 2 trp 1-Δ901 leu 2–3,112 ura 3–52 his 3–200 gal-4Δ gal-80Δ LYS2::GAL1-HIS3 ADE2::GAL2-ADE2 met1::GAL7-LACZ) [24]. For the interactions with small G proteins, the pJ69–4A harboring pPC97–$p76^{RBE}$–HR1 or the complete $p76^{RBE}$ were transformed with the different Rho constructs in pPC86. For the screening, pJ69–4A harboring pPC97–$p76^{RBE}$–HR1 was transformed with the dog thyroid library in pPC86 described previously [25]. The transformants were first selected on αHIS medium, then on αADE and finally reconstructed for specificity.

Coimmunoprecipitation

COS cells were cotransfected using Superfect (Invitrogen) with the complete HA-tagged $p76^{RBE}$ in pcDNA3 and with expression vectors containing various myc epitope-tagged RhoB protein constructs. Cells were harvested 48 h after transfection, in cell lysis buffer [50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 1% (v/v) TritonX-100, 20 mM NaF, 1 mM dithiothreitol, 100 μM sodium vanadate, 100 nM okadaic acid, a half tablet Complete protease inhibitor cocktail (Roche Applied Science, Bruxelles, Belgium)] and precleared with 20 μL packed volume of protein G-sepharose, at 4 °C for 1 h. The extracts were centrifuged at 12 000 ***g*** for 5 min at 4 °C and the supernatants were incubated with 4 μg of 9E10 for 1 h tumbling at 4 °C, with a further 2 h after the addition of 20 μL packed volume of protein G–sepharose. The beads were collected by centrifugation at 12 000 ***g*** for 5 min at 4 °C, washed and the bound proteins were solubilized in SDS/PAGE sample buffer and analyzed by SDS/PAGE and Western blotting.

Localization of p76 in cells by fluorescence

COS cells were cotransfected with the full-length fluorescently tagged $p76^{RBE}$ in pEGFP-C3 and with expression vectors containing various myc epitope-tagged RhoB protein constructs. Forty-eight h after transfection, cells were prepared for visualization by confocal microscopy with the Slow Fade Light Antifade Kit (Molecular Probes, Oregon).

GSt-pulldown assay

Freshly plated *Escherichia coli* BL-21 (Amersham Biosciences, the Netherlands) transformed with glutathione *S*-transferase (GSt) or with GSt-$p76^{RBE}$ expressing plasmids were grown on LB agar in the presence of ampicillin overnight. The following day, two colonies diluted in 50 mL YTA (yeast tryptone alkaline) were grown to $D_{600} \approx 0.5$ and induced by isopropyl thio-β-D-galactoside (IPTG) 0.1 mM for 75 min.

Cells were pelleted, and proteins were extracted and affinity purified on glutathione agarose beads (Sigma, Bornem, Belgium) by the method previously described by Frangioni [26]. Purity and integrity of GSt-fused proteins were assessed by SDS/PAGE and Coomassie blue staining. Keratin was produced and labeled with [^{35}S]Met by an *in vitro* transcription/translation kit TnT (Promega, Leiden, the Netherlands) under the control of T7 promoter using pcDNA3.HIS, and the quality of synthesis was verified by SDS/PAGE and exposure of the dried gel. GSt or GSt-$p76^{RBE}$ proteins bound to glutathione agarose beads were incubated with 5 μL of TnT product in binding buffer [50 mM potassium phosphate, pH 7.5, 150 mM KCl, 1 mM

$MgCl_2$, 10% (v/v) glycerol, 1% (v/v) Triton X-100] overnight at 4 °C. Beads were washed with binding buffer and the proteins boiled for 10 min in sample buffer and analysed by SDS/PAGE. The gel was stained with Coomassie blue, dried and exposed to an X-ray film for 2 days.

Primary culture of dog thyroid cells

Thyroid follicles, obtained by collagenase (127 $U \cdot mL^{-1}$, Sigma) digestion of dog thyroid tissue (as detailed previously) [27] were seeded in 100-mm dishes in control medium [DMEM plus Ham's F12 medium plus MCDB 104 medium (all Gibco; 2 : 1 : 1 v/v/v)], supplemented with 1 mM sodium pyruvate, 5 $\mu g \cdot mL^{-1}$ bovine insulin (Sigma), 40 $\mu g \cdot mL^{-1}$ ascorbic acid, 100 $U \cdot mL^{-1}$ penicillin, 100 $\mu g \cdot mL^{-1}$ streptomycin and 2.5 $\mu g \cdot mL^{-1}$ amphotericin B. The medium was changed on days 1 and 3. On day 4, either 1 $mU \cdot mL^{-1}$ bovine thyrotropin (Sigma), 10^{-5} μ forskolin (Calbiochem-Bering, LaJolla, CA), 25 $ng \cdot mL^{-1}$ murine EGF (Sigma), 50 $ng \cdot mL^{-1}$ hepatocyte growth factor (HGF) (Sigma), 10 $ng \cdot mL^{-1}$ phorbol myristate acetate (PMA) (Sigma), 5 $\mu g \cdot mL^{-1}$ actinomycin D (Pharmacia), 10 $\mu g \cdot mL^{-1}$cycloheximide or 10 $\mu g \cdot mL^{-1}$ puromycin were added directly to quiescent cells in the culture medium for different lengths of time. Cell monolayers (3.4×10^4 $cells \cdot cm^{-2}$) consisted of more than 99% thyrocytes [28,29].

Northern blotting and hybridization

At the time of harvest, the cells, in subconfluent monolayers, were rapidly scraped from the dishes in 4 M guanidinium monothiocyanate. Separation and purification of total RNA was performed by ultracentrifugation (Beckman L7, rotor SW55, 35 000 rpm) on a CsCl cushion [30]. After spectrophotometric quantification, equal amounts of total RNA were denatured with glyoxal according to the procedure of MacMaster and Carmichael [31] and separated by electrophoresis. Because several housekeeping genes are modulated by the agents used in our study [32], acridine orange staining was performed to ensure that equal amounts of RNA were loaded in each lane. Transfer of RNA to nylon membranes was performed using 20× NaCl/Cit (1× NaCl/Cit, 0.15 M NaCl, 0.015 M sodium citrate) [33]. Commercial Northern blots were purchased from Clontech. Prehybridization (4 h at 42 °C) and hybridization (overnight at 42°) were carried out in 50% (v/v) formamide, 5 × Denhardt's [0.1% (w/v) Ficoll, 0.1% (v/v) poly(vinylpyrrolidone), 5 × SSPE (0.9 M NaCl, 0.05 M sodium phosphate, pH 8.3, 5 mM EDTA), 0.3% (w/v) SDS, 250 $\mu g \cdot mL^{-1}$ denatured salmon testis DNA and 200 $\mu g \cdot mL^{-1}$ BSA. Dextran sulfate (10%, w/v) was added to the hybridization solution along with the denatured probe as described previously [34]. The probe was a 2 kb PCR fragment corresponding to nucleotides 23–2081 and was ^{32}P-labeled using the random primer technique (Amersham Multiprime Kit). Filters were washed four times for 10 min in 2× NaCl/Cit, 0.1% (w/v) SDS at room temperature and four times for 20 min in 0.1× NaCl/Cit, 0.1% (w/v) SDS at 65 °C. They were then autoradiographed at −70 °C using hyperfilm MP (Amersham). All our results were reproduced in at least two independent cell cultures.

Thyroid protein extracts and Western blotting

Stimulation with mitogens was performed on day 4 of culture. After the appropriate incubation period, cells were washed with $NaCl/P_i$ and lysed on ice by addition of Laemmli buffer supplemented with protease inhibitors [60 $\mu g \cdot mL^{-1}$ Pefabloc (Pentapharm, Basel, Switzerland), 1 $\mu g \cdot mL^{-1}$ aprotinin and 1 $\mu g \cdot mL^{-1}$ leupeptin]. Protein quantification was performed as described previously [35]. Protein lysates were resolved by electrophoresis on 7.5% SDS-polyacrylamide gels and subsequently transferred to poly(vinylidene difluoride) membranes (Amersham) overnight at 26 V at 4 °C. The membranes were blocked with Tris/NaCl/Tween buffer [100 mM NaCl, 10 mM Tris/HCl, 0.1% (v/v) Tween-20] containing 5% (w/v) BSA for 1 h. They were then incubated with the primary antibody at a concentration of 1 $\mu g \cdot mL^{-1}$ for 2 h at room temperature, and with protein A peroxidase (Sigma) at a 1 : 10 000 dilution for 1 h. Detection was performed using the ECL reagents from Amersham.

Antibody specificity studies

COS cells were transfected with a pcDNA3-$p76^{RBE}$ construct using Fugene (Roche). Cells were lysed in Laemmli buffer 48 h after transfection, denatured by boiling and analysed by Western blotting. The $p76^{RBE}$ insert was the same 2 kb PCR fragment that was used for the Northern blot probes.

RESULTS

Isolation and sequence of dog and human $p76^{RBE}$ cDNA

Dog $p76^{RBE}$ cDNA was isolated in a search for genes whose expression is regulated after mitogenic stimulation, by differential screening of a cDNA library prepared from a dog thyroid chronically stimulated *in vivo* by thyrotropin [23]. Nucleotide sequence analysis yielded a 3231 bp sequence, having a single open reading frame encoding 686 amino acid residues. The size of the cDNA sequence was in agreement with the 3.2 kb size of the mRNA estimated by Northern analysis. The full-length human cDNA encoding $p76^{RBE}$ has been cloned by PCR-based methods. As shown in Fig. 1, the human protein has 87% identity with the dog protein. Between amino acids 35 and 122, $p76^{RBE}$ contains an HR1-Rho-binding domain, and between amino acids 522 and 579, the PROFILE SCAN program identifies a PDZ domain showing 30% identity with the PDZ domains existing in a wide variety of proteins. The protein ends by a potential PDZ binding domain motif (SSWY) and contains at least two potential phosphorylation sites (indicated by arrows). The nucleotide sequence data reported here are accessible in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers AJ347749 for the dog sequence and AJ347750 for the human sequence.

A Blast search [36] revealed that $p76^{RBE}$ is 44% identical and 51% similar to rhophilin (U43194), a RhoA binding protein [13] (Fig. 1). Both $p76^{RBE}$ and rhophilin present significant homologies to the N-terminal parts of the budding yeast Bro1 (P48582) [37], *Xenopus* Xp95 (AF115497) [38], filamentus fungus *Aspergillus nidulans*

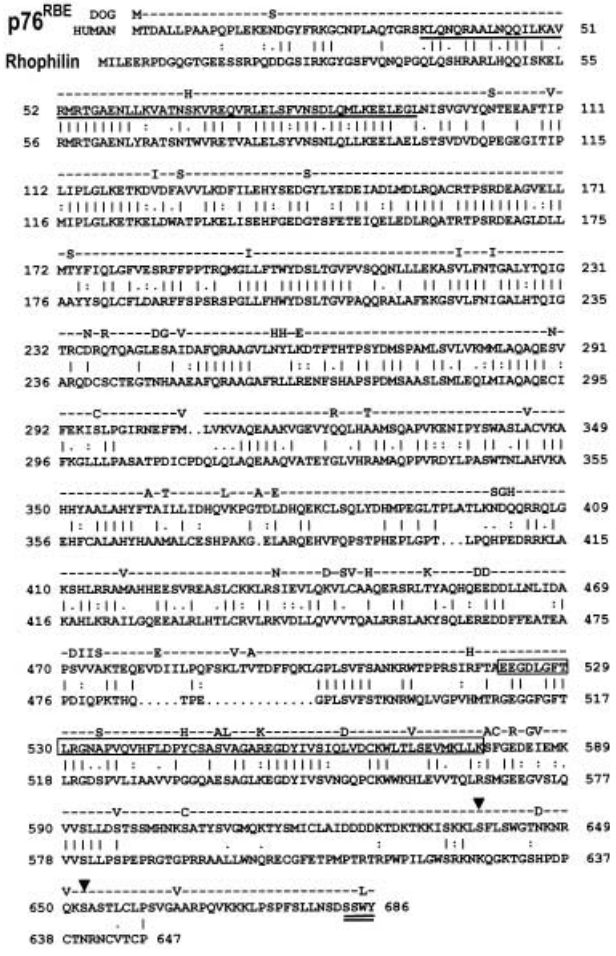

Fig. 1. Sequence comparison between human p76^{RBE} and mouse Rhophilin [13]. The percentage identity (|) is 49% and percentage similarity (:) is 57% between both proteins. Numbers indicate the respective position in the amino acid sequence. Dog amino acid differences are written above. The HR-1 domain is underlined and PDZ domain is boxed. Potential PDZ binding C-terminus consensus is double-underlined. Potential phosphorylation sites are indicated with arrowheads.

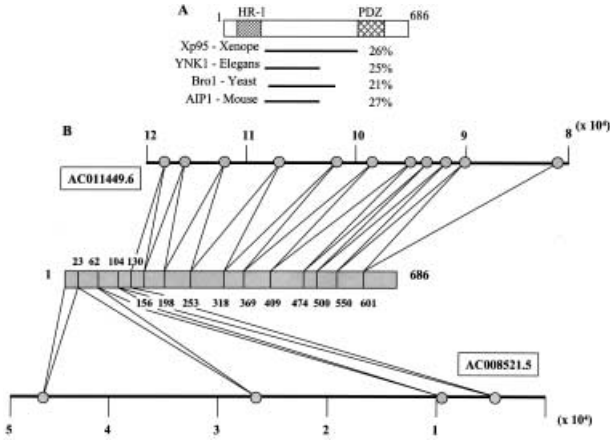

Fig. 2. Schematic representation of (A) the p76^{RBE} protein and (B) the human genomic structure of p76^{RBE} gene. (A) Schematic representation of the 686 amino acid p76^{RBE} protein with delimitation of both HR-1 and PDZ domains. Regions of homology with proteins of other species are underlined and the percentage of amino acid identity is indicated. (B) Schematic representation of the human genomic structure of p76^{RBE} gene. Exons are positioned on two BACs containing the p76^{RBE} coding sequence. Position of the introns in the cDNA are indicated by lines and positions in amino acids.

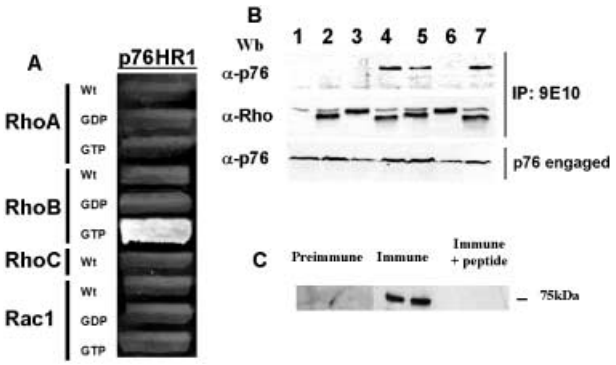

Fig. 3. Interaction between p76^{RBE} and Rho proteins. (A) Using the two-hybrid system, the pJ69–4A strain was transformed successively with pPC97-p76^{RBE}-HR1 and with various pPC86-Rho constructs. Three different mutants were tested: wild-type (wt), dominant negative (GDP) or constitutively active (GTP) forms. The transformants were plated as patches on the appropriate selective media. (B) Myc epitope-tagged RhoB protein constructs and p76^{RBE} were coexpressed in COS cells. Proteins were extracted 48 h after transfection and the amounts of p76^{RBE} present in the total cell lysates shown by immunodetection using C-terminal p76^{RBE} antibody (1 : 500) (lower panel). The extracts were immunoprecipitated with 9E10 MYC mAb as described under Experimental procedures. The proteins were analyzed by Western blotting for the presence of p76^{RBE} (α-p76) using C-terminal antibody (1 : 500) and RhoB (α-Rho) using 9E10 mAb (1 : 5000). p76^{RBE} was expressed alone (lane 1) or with RhoB wt (lanes 2 and 5), RhoB-GDP (lanes 3 and 6) or RhoB-GTP (lanes 4 and 7), stimulated (lanes 5, 6 and 7) or not (lanes 2, 3 and 4) by 10 ng·mL^{-1} EGF for 30 min. In the α-Rho Western blot, there is a band derived from the 9E10 IgG, which migrates close to Rho. (C) COS cells transfected with pcDNA3/p76^{RBE} were analysed by Western blotting using preimmune sera (1 : 500), C-terminal antibody (1 : 500), and C-terminal antibody (1 : 500) preincubated for 2 h with the peptide used for immunization.

Pal A (Z83333) [39], mouse AIP1/Alix (AC007591) [40] and nematode *Caenorhabditis elegans* YNK1 (U73679) [41]. In that region the residue Y174 is very well conserved between the different proteins (Fig. 2A).

The results of the Blast search localize the gene coding for protein p76^{RBE} on human chromosome 19 (clone CTC-263F14 and 461H2). Analysis of 19q genomic sequence revealed that p76^{RBE} consists of 15 exons (Fig. 2B) and maps to 19q13.11 between PDCD5 and FLJ110206 genes (UCSC Genome Browser).

Specific association of p76^{RBE} with GTP-bound form of RhoB

The presence of an HR1 domain and the high degree of homology with Rhophilin in the NH_2 part of the protein suggested that p76^{RBE} could be able to interact with a small G protein of the Rho family. On this basis, we used the two-hybrid system (Fig. 3A) and showed that p76^{RBE}-HR1

strongly interacts with full-length RhoB in its constitutively active form (RhoB-GTP), while no interaction could be detected with wt or dominant negative RhoB. No interaction could either be detected with other members of the Rho family (RhoA, RhoC or Rac1) in their full-length wt, dominant negative or constitutively active forms. The same results were obtained with the complete $p76^{RBE}$ protein. The Rho constructs were all transformed in pJ69–4A expressing an unrelated bait fusion with Gal4-DBD as negative control (data not shown).

We further examined the cellular interaction of RhoB and $p76^{RBE}$ using wt and mutated Rho proteins. The MYC-tagged RhoB proteins were coexpressed in COS cells with HA-tagged $p76^{RBE}$ and then isolated by immunoprecipitation using anti-Myc 9E10 mAb. The presence of associated $p76^{RBE}$ was detected by Western blotting with the anti-$p76^{RBE}$ and anti-HA Igs. Among the RhoB proteins, only the constitutively mutated GTP-bound form was seen to form an association with $p76^{RBE}$ that was stable to extraction (Fig. 3B). An association with the overexpressed wild-type RhoB was also revealed after stimulation of the cells by EGF (10 ng·mL^{-1}) for 30 min, resulting in the activation of RhoB in these cells. $p76^{RBE}$ fulfilled the criteria required of a RhoB effector in that it formed a stable association with the activated RhoB (RhoB-QL), but not with the dominant negative RhoB (RhoB-TN), nor with the nonactivated wt form.

The specificity of the $p76^{RBE}$ polyclonal antibody directed against the C-terminal peptide has been tested previously in transfected COS cells, with a band of 76 kDa revealed by Western blotting. This band was not present when immunodetection was performed with preimmune sera or when the antibody was preincubated with the corresponding peptide (Fig. 3C).

Cellular localization of $p76^{RBE}$ in transfected COS cells

We expressed EGFP-$p76^{RBE}$ in mammalian COS cells and 36 h after transfection obtained a light diffuse cytoplasmic staining with a clear perinuclear accumulation (Fig. 4B). Moreover $p76^{RBE}$ is, at least partially, located in the cell plasma membrane as indicated by arrows (Fig. 4B). The same pattern was observed when $p76^{RBE}$ was coexpressed with RhoB-TN (Fig. 4C). This localization is not due to enhanced green fluorescence protein (EGFP) alone as it was not found when EGFP was cotransfected with RhoB-QL (Fig. 4A). When RhoB-QL was coexpressed, we observed a drastic change in the $p76^{RBE}$ localization. $p76^{RBE}$ gave then mainly a punctate staining pattern in most cells, suggestive of a translocation of $p76^{RBE}$ protein to a vesicular compartment due to the presence of activated RhoB (Fig. 4D).

Regulation of $p76^{RBE}$ mRNA *in vitro* in thyroid cells

As $p76^{RBE}$ was initially isolated from a dog thyroid cDNA library and its mRNA was induced *in vivo* by thyrotropin [23], we investigated this modulation by Northern blotting in response to three main signal transduction pathways in dog thyrocytes in primary culture [thyrotropin/cAMP, EGF–HGF/MAPK (mitogen-activated protein kinase) and PMA/PKC (protein kinase C)] (Fig. 5). This experimental model has been studied extensively in our laboratory and closely reflects human thyrocyte physiology *in vivo* [42].

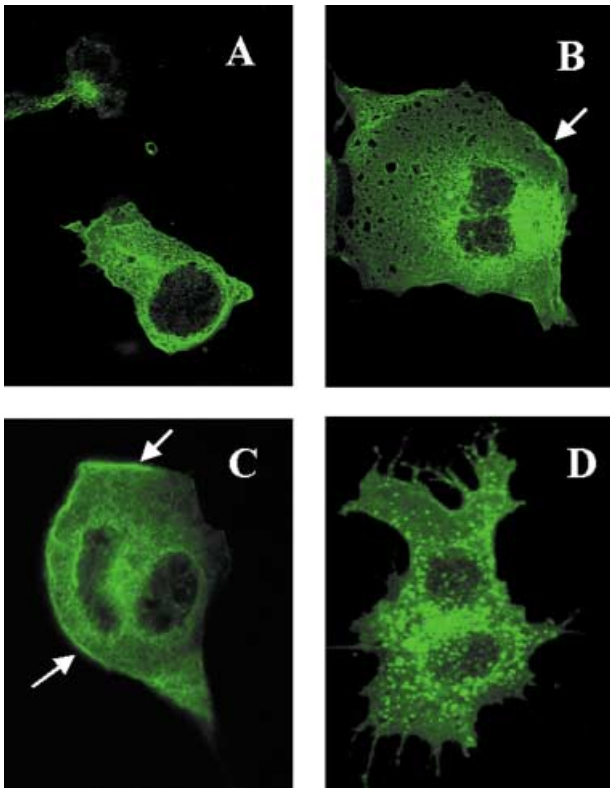

Fig. 4. Localization studies of $p76^{RBE}$ by fluorescence microscopy. COS cells were transfected with empty pEGFP and RhoB-QL (A), pEGFP-$p76^{RBE}$ (B), pEGFP-$p76^{RBE}$ and RhoB-TN (C), and pEGFP-$p76^{RBE}$ and RhoB-QL (D). Arrows indicate plasma membrane labeling.

In response to thyrotropin stimulation, mRNA levels increased after 4–6 h and declined thereafter (Fig. 5). To confirm that this increase of mRNA levels was secondary to the activation of the adenylyl cyclase/cAMP pathway, the same experiments were performed using 10^{-5} M forskolin, an adenylate cyclase activator. A pattern similar to the thyrotropin stimulation was observed in these experimental conditions.

Activation of the tyrosine kinase/MAP kinase pathway by EGF (25 ng·mL^{-1}) or HGF (50 ng·mL^{-1}) did not induce $p76^{RBE}$ mRNA accumulation, on the contrary, these growth factors decreased $p76^{RBE}$ mRNA levels as early as 2–4 h after treatment. Activation of the phorbol ester/PKC pathway, by 4β-phorbol 12-myristate 13-acetate (PMA) (10 ng·mL^{-1}), also resulted in a slight decrease of $p76^{RBE}$ mRNA levels.

In order to assess the stability of the mRNA, quiescent thyrocytes were exposed to the transcription inhibitor actinomycin D (5 μg·mL^{-1}) for different time periods. No changes in the mRNA levels were observed with short incubation periods of up to 8 h. Thereafter, there was a progressive decline, suggesting a half-life of approximately 12 h (Fig. 5).

Two protein synthesis inhibitors, cycloheximide (10 μg·mL^{-1}) and puromycin (10 μg·mL^{-1}), were used to evaluate whether the increase in mRNA levels following thyrotropin administration required new protein synthesis. No decrease in the mRNA levels was observed in response to these agents (Fig. 5). On the contrary, an increased level

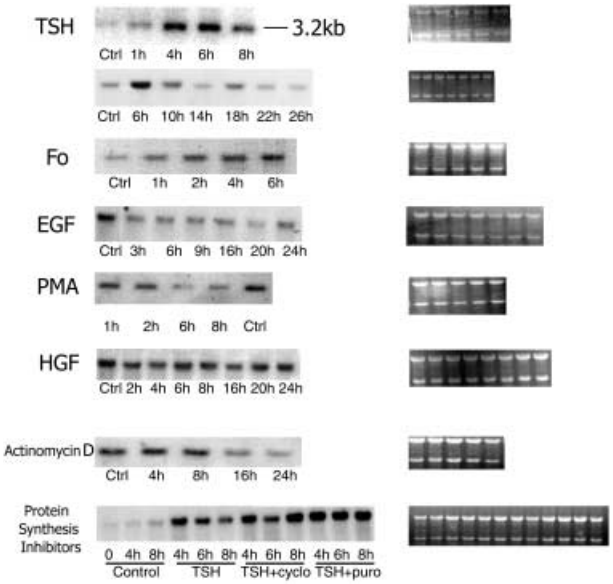

Fig. 5. p76^{RBE} mRNA modulation in thyroid cells. mRNA levels in response to stimulation of the cAMP pathway by thyrotropin (1 mU·mL^{-1}) and forskolin (10^{-5} M), the tyrosine kinase/MAPK pathway by EGF (25 ng·mL^{-1}) and HGF (50 ng·mL^{-1}) and the phorbol ester/PKC pathway by PMA (10 ng·mL^{-1}) for various time periods were analysed by Northern blotting. The stimulations were performed on quiescent primary culture thyrocytes on the fourth day of culture. mRNA stability was assessed using actinomycin D (5 μg·mL^{-1}) and effect of protein synthesis inhibitors, cycloheximide (10 μg·mL^{-1}) and puromycin (10 μg·mL^{-1}), on p76^{RBE} mRNA levels following thyrotropin (1 mU·mL^{-1}) stimulation. The right panels show the acridine orange staining of the mRNA gels, performed to ascertain homogenous loading of all lanes. p76^{RBE} is expressed as a 3.2 kb transcript.

of mRNA was present in comparison to the thyrotropin stimulus alone. This suggests that no new protein synthesis is required for mRNA expression after thyrotropin stimulation. The different intensities observed for the control levels reflect both the fact that we used primary culture of thyrocytes and the different exposure times are used in order to optimize detection and to demonstrate weak modulation. All these results were reproduced in at least two independent cultures.

P76^{RBE} protein expression in response to thyrotropin

To assess whether the response of p76^{RBE} mRNA to thyrotropin stimulation also occurred at the protein level, we performed Western analysis of thyrocytes samples lysed in Laemmli buffer using both N-terminal and C-terminal p76^{RBE} antibodies. Despite a lower expression of p76^{RBE} protein compared to its mRNA, we observed, in response to thyrotropin stimulation, that protein levels of p76^{RBE} increased after an incubation period of 6–8 h, which is closely correlated to the time of induction observed for the mRNA (Fig. 6).

P76^{RBE} binds the epithelial cytokeratin 18 *in vitro*

With the aim of identifying other proteins interacting with p76^{RBE}, p76^{RBE}-HR1 was used to screen a dog thyrocyte cDNA library cloned into the GAL4 activation domain

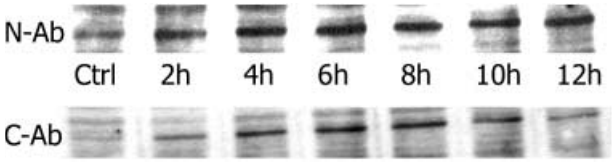

Fig. 6. Western analysis of thyrocyte lysates in response to thyrotropin stimulation. Quiescent thyrocytes on the fourth day of primary culture were exposed to thyrotropin (1 mU·mL^{-1}) for different lengths of time. Equal amounts of total cell lysates in Laemmli buffer were analyzed by standard Western blotting using p76^{RBE} N- and C-terminal antibodies.

vector pPC86. Screening of 5×10^5 transformants with the bait was carried out. Of 60 His+ clones, 35 were Ade + and 23 were specific of p76^{RBE}-NH and also interacted with the complete p76^{RBE} protein. Among the positive clones, seven encoded cytokeratin 18 (K18) polypeptides. On the seven clones, five were full-length while two of them corresponded to 5′ deleted proteins (Fig. 7A). To verify whether the cytokeratin interaction with p76^{RBE} obtained in the two-hybrid was specific, we tested the protein interaction *in vitro*. p76^{RBE} was expressed as a fusion protein with GSt in bacteria and purified as described under Experimental procedures. ^{35}S–labeled cytokeratin 18 was synthesized using TnT transcription/translation kit (Promega). As shown in Fig. 7B GSt-p76^{RBE}, but not GSt alone, was able to bind cytokeratin 18.

Human tissular distribution

To examine the human tissue distribution of p76^{RBE}, a Multiple Tissue Expression (MTE^{TM}) Array representing a

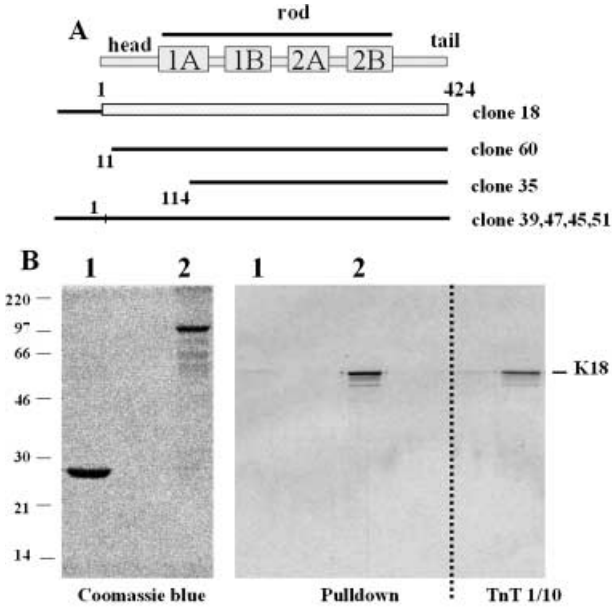

Fig. 7. (A) Schematic representation of the different cytokeratin clones isolated by the two-hybrid screening and interacting with p76^{RBE}. The dashed box represents the coding sequence. (B) Precipitation of cytokeratin 18 by GSt-p76^{RBE}. The left panel shows the Coomassie blue staining of the GSt (1) and GSt-p76^{RBE} (2) proteins engaged in the assay. The right panel shows the autoradiography of ^{35}S-labeled cytokeratin pulled-down in the same conditions (1 and 2) and a fraction of the cytokeratin TnT synthesized as a size control.

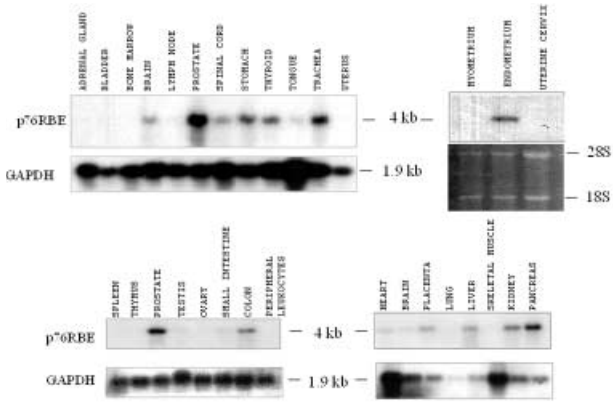

Fig. 8. Northern blot analysis of human tissue distribution of p76. Northern blots from BD Clontech were hybridized with full-length p76 and with GAPDH probes. For the upper right panel, the total mRNA loaded on the gel is controlled by acridine orange staining of the ribosomal RNAs 28S and 18S.

variety of adult and fetal tissues was used (data not shown). The results indicated strong mRNA expression in the uterus. Expression was also detected in other tissues including prostate, colon, lung, rectum, kidney, trachea, salivary and pituitary gland. To confirm these results by Northern blotting, we hybridized MTN^{TM} blots with a full-length human p76 probe. As shown in Fig. 8, p76 is abundantly expressed in prostate, trachea, stomach, colon, thyroid and pancreas. A lower expression is revealed in brain, spinal cord, kidney, placenta and liver. The expression of p76 in the uterus is restricted to the endometrium tissue and is not detectable in myometrium or uterine cervix.

DISCUSSION

Thyrotropin, via the adenylyl cyclase/cAMP pathway, is the most important regulator of gene expression in normal thyrocytes [43]. It represents one of the three thyroid mitogenic pathways along with the epidermal growth factor/ras/MAPK and phorbol esters/phospholipase C/protein kinase C pathways. The thyrotropin signaling cascade is different from the other two in its ability to induce both proliferation and expression of differentiation characteristics and to stimulate function, including the synthesis and secretion of thyroid hormones. The mitogenic pathways elicited by EGF and phorbol esters are associated with the loss of the differentiation specific genes [43]. Identifying the players of this thyrotropin signaling cascade, as well as the interactions with other effectors, is therefore highly important to understand cell function.

Dog p76^{RBE} cDNA was isolated by differential screening of a MM/PTU (methymazole/propylthiouracil) treated dog thyroid cDNA library [23]. This screening was aimed at isolating new proteins whose expression was regulated by the thyrotropin-dependent pathway. Analysis of the amino acid sequence of p76^{RBE} revealed that it is homologous to Rhophilin, which was identified on the basis of its interaction with RhoA [13], and to a conserved family of signal transduction proteins composed of the budding yeast Bro1 [37], *Xenopus* Xp95 [38], filamentus fungus *Aspergillus nidulans* Pal A [39], mouse AIP1/Alix [40] and nematode *Caenorhabditis elegans* YNK1 [41](Fig. 1). Functional and genetic evidences relate Bro1 to components of the yeast PKC1p-MAP kinase cascade [37] and Pal A to pH-dependent gene expression [39]. The homology with these proteins is located in the central part of p76^{RBE}. The presence of at least two potential phosphorylation sites in p76^{RBE} sequence among which one for CKII and one for PKC raises the possibility that the protein may be regulated by phosphorylation.

In vitro mRNA regulation of p76^{RBE} was assessed by Northern blotting in dog thyrocytes in primary culture treated by the different mitogenic agents. p76^{RBE} mRNA was mainly modulated by the thyrotropin-cAMP dependent pathway, with a transient elevation observed 4–6 h after stimulation. No new protein synthesis was required for the action of thyrotropin, as the up-regulation was not influenced by the addition of either cycloheximide or puromycin. On the contrary, an even more pronounced and more sustained increase was observed, suggesting that newly synthesized protein(s) may be involved in the destabilization of the mRNA. Thus p76^{RBE} behaves as an immediate early gene of the cyclic AMP cascade in the thyroid.

Experiments performed in the presence of actinomycin D showed that p76^{RBE} mRNA was quite stable, with a half-life estimated to be approximately 12 h. Taken together with the transient thyrotropin-promoted up-regulation, these data suggest that an active mechanism might be involved in the decline of the raised mRNA level.

The thyrotropin-induced up-regulation of p76^{RBE} was confirmed at the protein level. In contrast, dedifferentiation of the cells by treatment with EGF, PMA or HGF resulted in a down-regulation of the mRNA and protein levels (not shown). The weaker down-regulation observed with HGF is in accordance with its weaker dedifferentiation action on thyroid cells, as opposed to EGF or PMA [44]. Thus the expression of p76^{RBE} is correlated with differentiation but not with mitogenesis.

We have previously shown that activation of the thyrocyte thyrotropin/cAMP pathway induces characteristic morphological changes, associated with complete disruption of actin-containing stress fibers. Cells display a cubical epithelial morphology correlated with a profound redistribution of both actin microfilaments and cytokeratin intermediate filaments, and with the appearance of a marked cytokeratin and actin immunoreactivity at the cell junctions [28,42]. The latter cytoskeleton changes in culture may be related to the *in vivo* secretory process which, in the thyroid, involves the macropinocytosis of thyroglobulin and its digestion in secondary lysosomes [45,46]. This process is dependent on the integrity of the actin microfilament system [47].

Here we have demonstrated, by two-hybrid and GSt pulldown techniques, that intact p76^{RBE} can bind to cytokeratin 18, a major intermediate filament of simple epithelia. Whether p76^{RBE} could be a potential linker between thyrotropin/cAMP signal and the cytoskeleton changes observed in thyrocytes will be adressed in further experiments.

The existence of a Rho-binding domain (HR-1) in the amino terminal part of the protein suggests an implication in transduction pathways involving the Rho proteins. By use of the two-hybrid system and of *in vitro* coimmunoprecipitation experiments, we showed a strong and specific association of p76^{RBE} with constitutively activated RhoB.

As confirmed by the two-hybrid system, this interaction involves the HR-1 domain. This differentiates $p76^{RBE}$ from Rhophilin, which is associated with RhoA, and shows that, even if the homology between RhoA and RhoB is very high (92%), the specificity of association is well controlled. The association of $p76^{RBE}$ with RhoB depends clearly on the G protein stimulation resulting from the binding of GTP. As shown in Fig. 3, EGF, which is known to stimulate RhoB in different cell types, is able to induce the binding of $p76^{RBE}$ to the activated G protein.

The specificity of association of $p76^{RBE}$ with the activated form of RhoB suggested that it could act as a GAP protein or as a RhoB effector or inhibitor. Because $p76^{RBE}$ does not contain the Rho GAP consensus sequence Gha*R*h*SG* [48], we propose its role as a potential effector or inhibitor of activated RhoB.

Overexpression studies of $p76^{RBE}$ in kidney COS cells to determine the subcellular localization of the protein were carried out. When expressed alone, $p76^{RBE}$ showed a cytoplasmic distribution with a more intense labeling around the nucleus and with a subset of the protein attached to the plasma membrane. As observed before for PRK1, the overexpression of $p76^{RBE}$ in COS cells caused an increase in the number of multinucleate cells (observed in Fig. 4) which could reflect a disturbance in cytokinesis as already documented for yeast with double-mutants *bni1*,*bnr1* [49].

When coexpressed with activated RhoB, $p76^{RBE}$ immunolabelling shows a punctate pattern, compatible with an endosomal localization. This means that $p76^{RBE}$ could behave as a RhoB effector, changing its subcellular location following activation of RhoB by GTP binding.

Studies on RhoB distribution by immunofluorescence [50] or electron microscopy [6] show that RhoB is associated predominantly with structures resembling multivesicular bodies, a prelysosomal compartment. The pathways downstream of RhoB are still unknown, but RhoB seems to regulate cellular traffic through activation of the PRK kinases [8]. However the identity of the PRK substrates is still unknown. RhoB also retards the progress of the activated EGF receptor on its way to lysosomes for degradation [51] and is thought to be involved in the sorting of internalized receptors for degradation [7]. However, nothing is known about the molecular actors of this regulation. $p76^{RBE}$ could thus also be proposed as participating in endocytotic processes, for example polarized epithelial cells which are also dependent on cytoskeleton rearrangements. In the thyroid, endocytosis of thyroglobulin is the first step of the thyrotropin stimulated secretion of thyroid hormones.

In the elucidation of the cellular function of the small G proteins, RhoB has not been extensively investigated and little is actually known about the molecular targets of this endosomal protein. In this study, we have identified $p76^{RBE}$ as a direct and selective interacting protein of the small GTPase RhoB in its GTP-bound form. $p76^{RBE}$ is recruited to an endosomal compartment when coexpressed with the activated form of RhoB, and also binds *in vitro* to components of the cytoskeleton. Taken together, $p76^{RBE}$ could be proposed as an effector of RhoB perhaps linking it to proteins of the cytoskeleton and thereby playing a role in intracellular movement including endocytosis. Its immediate early expression in the thyroid in response to the thyrotropin cyclic AMP secretary cascade certainly suggests specific roles in these cells.

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