Intracellular localization of the PDE4A cAMP-specific phosphodiesterase splice variant RD1 (RNPDE4A1A) in stably transfected human thyroid carcinoma FTC cell lines

Linda POOLEY, Yasmin SHAKUR*, Graham RENA and Miles D. HOUSLAY†

Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, Wolfson Building, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

Cells of two human follicular thyroid carcinoma cell lines (FTC133, FTC236) were stably transfected with a cDNA encoding the PDE4A cAMP-specific phosphodiesterase (PDE) splice variant RD1 (RNPDE4A1A) so as to generate the cloned cell lines, FTC133A and FTC236A. This allowed the expression of a novel rolipram-inhibited cAMP-specific PDE activity in these cells. Unlike the parent cell lines in which Ca^{2+} /calmodulin caused a profound activation (approx. 3–4-fold) of homogenate PDE activity, no such stimulation was evident in the RD1-expressing cell lines, indicating loss of PDE1 activity. Reverse transcriptase-PCR analysis indicated that this was due to the down-regulation of the PDE1C isoform. The novel PDE4 activity in transfected cells was located exclusively in the membrane fraction, as was immunoreactive RD1. Low concentrations of

the detergent Triton X-100, but not high NaCl concentrations, allowed RD1 to be solubilized. Laser scanning confocal immunofluorescence analyses identified RD1 immunoreactivity in a discrete perinuclear region of these RD1-expressing transfected cell lines. A similar pattern of labelling was observed using the antiserum Tex1, which specifically identified the Golgi apparatus. Treatment of FTC133A cells with the Golgi-perturbing agents monensin and brefeldin A led to a similar redistribution of immunoreactive species detected using both the Tex1 and anti-RD1 antisera. It is suggested that the PDE4A splice variant RD1 contains a membrane-association signal which allows the targeted expression of RD1 within the Golgi complex of these human follicular thyroid carcinoma cell lines.

INTRODUCTION

cAMP has a key role as a second messenger and is able to mediate the action of a number of hormones and neurotransmitters. The sole means of degrading cAMP is via the action of cAMP-specific phosphodiesterases (PDEs) [1–5]. This enzyme activity is provided by a large multigene family which includes PDE1 [Ca^{2+} /calmodulin (CaM)-stimulated enzymes], PDE2 (cGMP-stimulated enzymes), PDE3 (cGMP-inhibited enzymes) and PDE4 and PDE7 (cAMP-specific enzymes). The cAMP-specific PDE4 family can be selectively and potentially inhibited by the antidepressant rolipram [2,3,6,7]. Four genes, located [8–10] on three chromosomes, encode PDE4 enzymes with further complexity added by the occurrence of alternative mRNA splicing [2,3,7,11,12]. In the case of the rat *PDE4A* gene family, at least three distinct splice variants have been characterized and shown to be differentially expressed [2,3,7,12–14]. These all differ by virtue of 5'-domain swapping, which leads to the expression of distinct N-terminal regions characterizing each member. The 'core' PDE4A enzyme can be considered to be the species that is encoded by the sequence in the *PDE4A* gene found up to the first splice junction, i.e. the junction at which the PDE4A cAMP-specific PDE splice variant RD1 diverges [2]. Met²⁶RD1, an engineered enzyme that approximates this species, is expressed as a fully active soluble protein in transiently transfected COS cells [15]. In contrast, native RD1 is found as a membrane-bound

protein in brain [14], and the two other PDE4A splice variants, RPDE6 [13] and RPDE39 [12], are found distributed between cytosol and particulate (cytoskeletal) fractions. Thus the N-terminal splice regions of PDE4A species appear to confer a propensity to associate with membrane/cytoskeletal fractions [13–16]. This may have relevance to the generation and control of compartmentalized signalling responses through cAMP [17].

The splice variant RD1 (RNPDE4A1) is found in brain where it is differentially expressed in various regions [2,7,8,13]. It has a unique N-terminal splice region, comprising some 23 amino acids [2], which has been demonstrated [16] to confer membrane association on the normally soluble bacterial protein, chloramphenicol acetyltransferase in studies using chimaeric species. We have recently determined the structure of this region by proton NMR and shown that membrane association depends on a distinct compact tryptophan-rich domain within this splice region [18]. From this analysis [18] it was difficult to see how such a structure could insert into a lipid bilayer and so it was suggested that membrane association probably ensued through the interaction of the N-terminal splice region of RD1 with a membrane 'anchor' protein. This would be expected to confer a unique intracellular distribution on RD1 reflecting that of the anchor protein and is consistent with observations that RD1 was not randomly associated with all intracellular membranes when expressed either natively in brain or in transfected COS cells [14]. The proposal that alternative splicing yields isoforms with distinct

Abbreviations used: PDE, cAMP phosphodiesterase; the PDE4 enzymes are rolipram-inhibited cAMP-specific phosphodiesterases for which there are four known genes, giving rise to PDE4A, PDE4B, PDE4C and PDE4D; RD1, rat 'dunc-like' PDE also referred to as either RNPDE4A1A or rPDE-IV_{A1} representing rat PDE4 isoform subfamily A splice variant 1 (GenBank accession number M26715); rolipram, 4-[3-(cyclopentoxyl)-4-methoxyphenyl]-2-pyrrolidone; CaM, calmodulin; SH3 domain, src homology 3 domain; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine.

* Present address: Laboratory of Cellular Metabolism, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A.

† To whom reprint requests and all correspondence should be addressed.

N-terminal regions that govern protein-protein interaction is also in accord with our recent demonstration [19] that the N-terminal splice region of the PDE4A splice variant RPDE6 confers the ability to be able to interact with certain SH3-domain-containing proteins.

Transfection of COS cells with the plasmid pSVL-RD1 leads to the high-level transient expression of RD1 [14,15]. However, in these transfected cells, initiation also appears to occur at Met²⁶ because of the presence of a suboptimal Kozak sequence, and this leads to the production of soluble N-terminally truncated Met²⁶-RD1 [13]. The presence of this soluble species has complicated attempts to determine the precise intracellular distribution of RD1 *in situ* by immunofluorescence analysis. No cell lines that express RD1 have been identified to date. In order to try to assess whether RD1 associates randomly with intracellular membranes or can show a degree of specificity, we have here used two human follicular carcinoma cell lines, FTC133 and FTC236 [20,21], to generate stable transfectants that express RD1. In these transfectants, only membrane-associated RD1 appeared to be expressed, and immunofluorescence analysis showed it to have a striking perinuclear localization indicative of association with the Golgi complex.

MATERIALS AND METHODS

Materials

The human follicular thyroid carcinoma cell lines FTC133 and FTC236 [20,21] were kindly supplied by Dr. D. Wynford-Thomas, Cancer Research Campaign, University of Wales College of Medicine, Cardiff, U.K. The rat *dunc* cDNA for RD1 [7] was kindly provided by Dr. R. L. Davis (Baylor College of Medicine, Houston, TX, U.S.A.). Restriction enzymes, RPMI, fetal calf serum, glutamine, penicillin/streptomycin and Geneticin G418 sulphate were from Life Technologies (Paisley, Scotland, U.K.). Tris, Hepes, BSA, cytochalasin B, benzamidine hydrochloride, PMSF, aprotinin, pepstatin A, antipain, EGTA, cAMP, cGMP, Dowex I (X8-400; 200–400 mesh; Cl[−] form), isobutylmethylxanthine snake venom (*Ophiophagus hannah*), bovine brain calmodulin (CaM), paraformaldehyde, monensin, brefeldin A, forskolin and fish skin gelatin were from Sigma Chemical Co. (Poole, Dorset, U.K.). [³H]cAMP was from Amersham International (Amersham, Bucks., U.K.). Leupeptin was from the Peptide Research Foundation (distributed by Scientific Marketing Associates, London, U.K.). Dithiothreitol, Triton X-100 and DOTAP transfection reagent were from Boehringer (U.K.) Ltd., (Lewes, Sussex, U.K.). Triethanolamine, acrylamide, Bromophenol Blue, glycine, β -mercaptoethanol, NaCl and NH₄Cl were from BDH (Glasgow, Scotland, U.K.). Bradford reagent and TEMED were from Bio-Rad. DMSO was from Koch-Light Ltd., (Haverhill, Suffolk, U.K.). MgCl₂, CaCl₂, bisacrylamide, glycerol, SDS and KCl were all from Fisons (Loughborough, Leics., U.K.). Ammonium persulphate was from May and Baker (Dagenham, Essex, U.K.). Goat serum was from SAPU (Carlisle, Scotland, U.K.). Mowiol was from Calbiochem. Milrinone was from Stirling Winthrop (Guildford, Surrey, U.K.). Cilostimide was from Pfizer (Sandwich, Kent, U.K.). Erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) was a gift from the Max Planck Institute. Rolipram was a gift from Schering Aktiengesellschaft (Berlin, Germany). Tri-Reagent™ was obtained from Sigma. 'First-strand cDNA synthesis kit' was from Pharmacia (Uppsala, Sweden). *Taq* DNA polymerase was from Promega (Madison, WI, U.S.A.). 'Taq Start' antibody was from Clontech Laboratories (Palo Alto, CA, U.S.A.). PCR 'Perfect Match' PCR additive was from Stratagene (La Jolla, CA, U.S.A.).

DNA constructs

The rat *dunc* gene (RD1) cDNA [7] was isolated from pIBI as a 2.2 kb *Eco*RI fragment and inserted into the unique *Eco*RI site of pBluescript SK[−], to give the recombinant vector pSK-RD1 [15]. The cDNA was then isolated from pSK-RD1 as a *Bam*HI-*Sal*I fragment and directionally cloned into the expression vector pDOL [22], generating the recombinant vector pDOL-RD1.

Cell culture

The human follicular thyroid carcinoma cell lines FTC133 and FTC236 [20,21] were cultured at 37 °C in an atmosphere of 5 % CO₂/95 % air in a complete growth medium containing RPMI (1 % v/v), penicillin (10⁴ units/ml), streptomycin (10⁴ µg/ml) and 1 % (v/v) glutamine (200 mM) and supplemented with 10 % (v/v) fetal calf serum.

Generation of anti-RD1 sera

This was carried out as described previously [14]. Briefly, a rabbit polyclonal antiserum was generated against a peptide corresponding to the last 12 amino acids of the RD1 protein [(C)-T-P-G-R-W-G-S-G-G-D-P-A]. The peptide was synthesized with a cysteine at the N-terminus to facilitate conjugation of the peptide to keyhole limpet haemocyanin.

Stable expression of RD1 in FTC133 and FTC236 cells

The cells were plated 24 h before transfection at 60 % confluency on 10 cm diameter plates. Just before transfection, the old culture medium was aspirated and replaced with fresh culture medium. For each 10 cm plate, the transfection mixture was prepared in a polystyrene reaction vial by diluting 70 µl of the transfection reagent DOTAP to 250 µl with HBS (20 mM Hepes, 150 mM NaCl, pH 7.4) and mixing this with 5 µg of DNA diluted in 250 µl of HBS. The solution was incubated at room temperature for 10 min and then added to the culture dishes, with gentle swirling to ensure even mixing. The cells were incubated under standard growth conditions for a further 18 h, after which the transfection mixture was aspirated, replaced with fresh growth medium and the cells grown for a further 48 h. Transfected cells were then selected by inclusion of 1 mg/ml Geneticin G418 sulphate in the growth medium. All untransfected cells had died back a week after application of the selection medium and colonies of transfected cells were visible after about 17 days. These colonies were individually picked into multiwell cell culture dishes, expanded and maintained.

SDS/PAGE and Western blotting

Polyacrylamide gels (10 %) were used and the samples were boiled for 5 min after being resuspended in Laemmli buffer [23]. Gels were run at 7 mA/gel overnight or 60 mA/gel for 4–5 h with cooling. For detection of RD1 by Western blotting, the protein samples were separated by SDS/PAGE and then transferred to nitrocellulose before being immunoblotted using RD1-specific antisera [14]. Labelled bands were identified by using anti-rabbit peroxidase-linked IgG and the enhanced chemiluminescence Western blotting visualization protocol (Amersham).

Preparation of cell extracts

Cells were grown to 90 % confluency under standard growth conditions in 75 ml flasks. The growth medium was removed and the cells incubated for 45 min at 4 °C in 2 ml of ice-cold

'complete' KHEM buffer [KHEM buffer: 50 mM KCl, 50 mM Hepes (pH 7.2) 10 mM EGTA and 1.92 mM MgCl_2 ; 'complete' KHEM buffer: KHEM buffer containing 1 mM DTT, 20 μM cytochalasin B and a 'cocktail' of protease inhibitors (2 μl of 1000-fold stock made by dissolving 2 mg of aprotinin, 2 mg of antipain, 2 mg of pepstatin, 2 mg of leupeptin, 313 mg of benzamidin and 80 mg of PMSF in 2 ml of DMSO)]. The 'complete' KHEM buffer was then removed and replaced with 5 ml of ice-cold TEA/KCl buffer (10 mM triethanolamine/150 mM KCl, pH 7.2) and left for 10 min at 4 °C. The TEA/KCl buffer was removed and the cells were washed briefly with 5 ml of ice-cold KHEM buffer and then 1 ml of ice-cold 'complete' KHEM buffer for 2 min. The buffer was drained off and the cells were scraped into 100 μl of 'complete' ice-cold KHEM buffer. The cell suspension was homogenized in a glass Dounce homogenizer, on ice, using 50 strokes and finally passed ten times through a 25G 5/8 syringe needle.

Reverse transcriptase (RT)-PCR

RNA was isolated from cells as described previously [24]. Preparation of first-strand cDNA was performed using the 'First strand cDNA synthesis kit' with a total reaction volume of 33 μl . Briefly, 3 μg of RNA, as determined by its A_{260} , was denatured at 65 °C for 10 min and then incubated with murine reverse transcriptase, 6 mM DTT, dNTPs and 0.2 μg of the supplied poly(dT)primer [*NotI*-d(T)₁₈] for 60 min at 37 °C.

A set of primers (GR18 sense 5'-RYCTYATCARCCGYTT-YAAGATTCC-3' and GR19 antisense 5'-RAAYTCYTCCAT-KAGGGGCCWTGG) was used as described previously [24] to detect a 601 bp fragment found in all *PDE1A* and *PDE1B* transcripts, including the recently published human *PDE1A* (U40370) [25] (In these primers, R = A/G, Y = C/T, K = G/T and W = A/T.) We also designed primers specific for phylogenetically stable regions of *PDE1C* on the basis of human and rat sequences deposited in GenBank (Human *PDE1C1* (U40371), *PDE1C3* (U40372) [25] and rat *PDE1C2* (L41045) [26]). We used the approx. 520 nucleotides (approx. 1550 to 2067 in human *PDE1C1*) of conserved (> 80% identity human/rat) unique *PDE1C* sequence found between residues that encode the putative catalytic region and those at the first of the 3' splice junctions [25,27]. Partially degenerate primers (GR45 sense 5'-CMAAGC-GATCAGGTGTCAAG-3' and GR46 antisense 5'-GTTRGAGTGATCCTTCTTGCTG-3', where M = C/A; GR45 starts at 1609 and GR46 ends at 2039 in *PDE1C1*) were designed to the ends of this sequence in order to detect an approx. 430 bp fragment in all the *PDE1C* transcripts then deposited in GenBank (see above). These sequences are also found in the mouse *PDE1C* forms recently deposited in GenBank [one mismatch with each of mouse *PDE1C1* (L76944), mouse *PDE1C4* (L76947) and mouse *PDE1C5* (L76946) forms]. However, they are not found in any deposited *PDE1A* or *PDE1B* sequences [bovine *PDE1A* (M90358), human *PDE1A* (U40370), bovine *PDE1B* (M94867), Chinese hamster *PDE1B* (U40585), human *PDE1B* (U40584), mouse *PDE1B* (M94538)]. Amplification was performed in 1 $\times$ PCR buffer (50 mM KCl, 20 mM Tris/HCl) containing 200 μM each dNTP, 0.5 μM each primer (except for 1.5 μM of GR18 and GR19), 1.5 mM MgCl_2 , 5 μl of first-strand cDNA mixture and 5 units of *Taq* DNA polymerase that had first been mixed with 'Taq Start' antibody for 'Hot-Start' PCR according to the manufacturer's instructions. Then 0.5 unit of the PCR additive 'Perfect Match' was added to a total volume of 50 μl . The amplification protocol consisted of 40 cycles of denaturation for 60 s at 94 °C, annealing for 70 s at 51 °C (except for PCR with GR18 and GR19 where the annealing segment was 80 s at 50 °C)

and extension for 70 s at 72 °C. A 10 μl aliquot from each reaction mixture was resolved by electrophoresis on a 2% agarose gel and visualized with ethidium bromide under UV light.

Salt and detergent treatment of membranes

This was performed as previously described [14,15]. Briefly 0.8 ml of freshly prepared membrane (160 μg) was divided into 8 $\times$ 100 μl aliquots. Now 100 μl aliquots of stock NaCl solutions were added to give the final indicated range of [NaCl]. Each tube was incubated on ice for 30 min before centrifuging at 200 000 g_{av} for 30 min. Each resulting pellet was then boiled in SDS/PAGE loading buffer, electrophoresed, blotted and developed. Washing of membranes with Triton X-100 was performed essentially as the NaCl treatment but using the indicated range of Triton X-100 concentrations.

Confocal analyses

FTC133 cells expressing RD1 were plated out on to coverslips (18 $\times$ 18 mm) at about 60% confluency. After 24 h the cells were fixed to the coverslips using paraformaldehyde, labelled with anti-RD1 serum and stained with goat anti-IgG Rhodamine B conjugate (TCS Biologicals) as previously described [14].

Cells were not only treated with anti-RD1 antibody but also, in separate experiments, with preimmune antisera and goat anti-IgG rhodamine B conjugate, and further controls containing both detergent-permeabilized and non-permeabilized cells were used. In these control situations, only a weak background immunofluorescence was seen in contrast with studies using the anti-RD1 sera where a strong immunofluorescence was seen. The parental cell line FTC133 was similarly labelled as a control but again no specific immunofluorescence could be seen.

The Golgi complex was visualized using Tex1, a gift from Dr. Graham Warren (Imperial Cancer Research Fund, London, U.K). This antibody was raised against a batch of rat Golgi proteins and predominantly recognizes mannosidase II [28].

Analyses were performed using a Zeiss laser-scanning confocal Axiocvert 100 microscope with a $\times$ 63/1.4NA plan apochromat lens. A 543 nm laser line was selected with appropriate filters (LP570; KT 488/543) and a pinhole size of 8-14. Three-dimensional reconstruction was carried out using either Zeiss LSM software on an IBM Pentium system or Imaris (2.2.4) software (Bitplane AG, Zurich; Fairfield Imaging Ltd.) on a Silicon Graphics Indigo system.

PDE assay

PDE activity with 1 μM cAMP as substrate was assayed by a modification of the two-step procedure of Thomson and Appleman [29] and Rutten et al. [30] as described previously by Marchmont and Houslay [31]. All assays were conducted at 30 °C and in all experiments a freshly prepared slurry of Dowex/water/ethanol (1:1:1, by vol.) was used; initial rates were taken from linear time courses of activity.

The buffer was modified from that previously reported (20 mM Tris/HCl, 10 mM Mg^{2+}) to include 2 mM EGTA, to ensure that sufficient endogenous Ca^{2+} /CaM was present to activate any PDE1 enzymes fully. For the determination of PDE1 activity, Ca^{2+} was added to a final concentration of 5 mM together with 10 units of CaM. The effects of Ca^{2+} /CaM, cGMP and the PDE inhibitors milrinone, cilostamide, EHNA and rolipram on PDE activity were all determined as described previously [24,32,33].

Protein assays

Protein concentration in the membrane and cytosolic fractions was determined by the Bradford [34] method using BSA as standard. Membranes were solubilized with 0.2% Triton X-100 for 30 min on ice before assay. When Triton X-100 was used in the assay it was also included in the standards.

RESULTS

Expression of RD1 in stably transfected FTC133 cells

Transfected FTC133 cells were selected by their resistance to Geneticin G418 sulphate as detailed in the Materials and methods section. In line FTC133A we uniquely detected an immunoreactive species that co-migrated with RD1 expressed in COS cells (Figure 1). This band was not seen using homogenates of the

parental cell line (Figure 1). RD1 immunoreactivity was maintained at an identical level through at least 25 passages of the FTC133A cell line and hence it was taken as being a stable RD1-transfected cell line of FTC133. Interestingly, a number of cell lines were obtained that were resistant to Geneticin G418 but in which we were unable to detect the presence of RD1 either by immunoblotting or an increase in rolipram-inhibited PDE4 activity. One of these, FTC133G (Figure 1), was used as a 'control' to identify any effects of transfection that might have occurred without detectable expression of RD1.

Immunoblotting of membrane and soluble cytosol fractions showed that RD1 immunoreactivity was essentially found associated only with the membrane fraction (> 96%) of FTC133A cells (Figure 1C). Consistent with this, we observed no increase in cytosolic PDE4 activity in these RD1-transfected cells (Table 1).

Expression of RD1 in FTC133 cells increases their cAMP-hydrolysing capability

Particulate and soluble fractions were prepared from the homogenates of these cell lines. A much higher (approx. 8-fold) cAMP-specific PDE activity was observed in the membrane fraction of the FTC133A cell line than in membranes from either native cells or the FTC133G cell line (Table 1). This enhanced PDE activity could be completely negated (> 96%) by the addition of the PDE4-selective inhibitor rolipram (10 μ M) (Table 1). Under these conditions the residual PDE activities in all the cell lines were very similar (Table 1). Indeed, rolipram failed to exert any significant inhibitory effect (< 5%) on the membrane PDE activity of either the native FTC133 cells or the FTC133G cell line, indicating the absence of any membrane-associated rolipram-sensitive PDE4 activity in these cell lines (Table 1).

Cytosolic PDE activity was remarkably similar in all three cell lines (Table 1), showing that the novel rolipram-sensitive PDE activity observed in FTC133A cells was associated solely with the particulate fraction. This is in accord with the immunoblotting studies described above, which identified the novel RD1 immunoreactivity as occurring solely in the particulate fraction of the FTC133A cell line (Figure 1).

Whereas the levels of rolipram-inhibited cytosolic PDE4 activity in native FTC133 cells and the 'control'-transfected FTC133G cell line were similar, that of the RD1-expressing FTC133A cell line was lower (Table 1). It is possible that the increase in total (basal) cellular PDE activity caused by stable transfection with RD1 (Table 2) may have led to the down-regulation of endogenous PDE4 isoforms. This could have occurred if, for example, intracellular cAMP levels were decreased as a result of the expression of RD1 in FTC133A cells, as the expression of certain PDE4 isoforms can apparently be influenced by changes in intracellular cAMP levels [35–38].

Effect of PDE-isoform-selective activators and inhibitors on the PDE activity of FTC133 cells

Compounds selective for effects on specific PDE isoforms were employed to gauge the presence and magnitude of the activity of various PDE isoforms in homogenates of these cell lines. All PDE assays were carried out as a routine in the presence of EGTA to negate Ca^{2+} /CaM-stimulated PDE1 activity. This strategy was crucial, as otherwise (results not shown) carryover of endogenous Ca^{2+} and CaM led to the activation of any endogenous PDE1 activity.

Homogenates of the RD1-expressing FTC133A cells exhibited markedly increased levels of total cAMP-specific PDE activity compared with the native FTC133 cells (Table 2). In contrast, the FTC133G cells showed no change in their total cAMP-specific

Figure 1 Immunodetection of RD1 expressed constitutively in stably transfected human thyroid carcinoma cell lines

Immunoblots using an antibody to the rat PDE4A isoform RD1 [13,14] are shown. Panel A gives results for homogenates of COS-7 cells transfected transiently so as to express RD1 [13,14] (lane 1), the FTC133A cell line (lane 2), the FTC133G cell line (lane 3) and the native FTC133 cell line (lane 4). Panel B gives results for homogenates of untransfected COS cells (lane 1), the FTC236 cell line (lane 2) and the FTC236A cell line (lane 3). Panel C, results for homogenates from both the native FTC133 cell line (lane 1) and the RD1-expressing FTC133A cell line (lane 2) as well as the membrane (lane 3) and cytosol (lane 4) fractions from the RD1-expressing FTC133A cell line. These results are typical of experiments performed at least three times.

Table 1 Distribution of PDE activity between membrane and cytosol fractions in native and transfected FTC133 cells: action of rolipram

Cells were cultured, harvested, homogenized and membrane and high-speed supernatant (cytosol) fractions prepared as described in the Materials and methods section. Activity is expressed as pmol of cAMP hydrolysed/min per mg of protein with 1 μ M cAMP as substrate; where indicated, 10 μ M rolipram was added. Activity is shown in the absence (—) and presence (+) of rolipram. The 'net' rolipram-inhibited PDE4 activity is also shown. Data represents means $\pm$ S.D. for six separate experiments carried out on different cell preparations with PDE assays performed in triplicate.

Rolipram...	PDE activity (pmol/min per mg of protein)								
	FTC133			FTC133A			FTC133G		
	—	+	Net	—	+	Net	—	+	Net
Cytosol	27 $\pm$ 3	16 $\pm$ 4	11	18 $\pm$ 4	12 $\pm$ 3	6	28 $\pm$ 6	16 $\pm$ 2	12
Membrane	19 $\pm$ 5	17 $\pm$ 5	2	150 $\pm$ 13	18 $\pm$ 5	132	28 $\pm$ 6	26 $\pm$ 5	2

Table 2 Effect of selective inhibitors and activators on PDE activity in normal and transfected FTC133 cells

Cells were cultured, harvested and homogenized as described in the Materials and methods section. Activity is expressed as pmol of cAMP hydrolysed/min per 10^6 cells using 1 μ M cAMP as substrate, with the basal activity shown for each cell line (no additions). Various agents were then added to determine the presence and magnitude of the various other PDE types as indicated in the Materials and methods section. Ca^{2+} /CaM (50 μ M; 20 ng/ml) was added in order to detect PDE1 activity (indicated by the increase in PDE activity); the presence and magnitude of PDE3 activity was determined by assessing the degree of inhibition achieved by the PDE3-selective inhibitor cilostimide (10 μ M); the presence and magnitude of PDE2 activity was determined by assessing the degree of inhibition achieved by EHNA (20 μ M) in the presence of a maximally stimulatory concentration of cGMP (10 μ M); the presence and magnitude of PDE4 activity was determined by assessing the degree of inhibition achieved by rolipram (10 μ M) (indicated by the decrease in PDE activity). Data represent means $\pm$ S.D. for six separate experiments performed on different cell preparations with PDE assays carried out in triplicate.

	PDE activity (pmol/min per 10^6 cells)		
	FTC133	FTC133G	FTC133A
No additions	8 $\pm$ 3	11 $\pm$ 2	36 $\pm$ 7
Ca^{2+} /CaM-stimulated component	26 $\pm$ 5	22 $\pm$ 6	0.6 $\pm$ 0.3
EHNA-inhibited component	1.2 $\pm$ 0.3	1.2 $\pm$ 0.6	3.7 $\pm$ 1.5
Cilostimide-inhibited component	1.3 $\pm$ 0.7	2.5 $\pm$ 0.5	5.8 $\pm$ 1.8
Rolipram-inhibited component	3.6 $\pm$ 0.9	3.0 $\pm$ 0.9	31 $\pm$ 6
Total PDE activity	34 $\pm$ 5	33 $\pm$ 8	37 $\pm$ 7
observed in presence of added Ca^{2+} /CaM			

PDE activity (Table 2). PDE4 species are cAMP specific and can be inhibited selectively by rolipram [1–3,5,6,12,13,39–44]. They typically exhibit IC_{50} values of 0.1–1 μ M rolipram when assayed at 1 μ M cAMP, whereas other PDE species are either insensitive to rolipram or show IC_{50} values some 50–100 times higher. Thus, in the presence of 10 μ M rolipram with 1 μ M cAMP as substrate, complete or near-complete inhibition of PDE4 activity can be expected. The profound increase in PDE activity seen in the FTC133A cell line was clearly due to a species of enzyme that could be potently inhibited by the selective PDE4 inhibitor, rolipram (Table 2), consistent with it being due to RD1 expression. In contrast, rolipram exerted a similar small inhibitory effect on the PDE activity of both the parental FTC133 cell line and the 'control' FTC133G cells (Table 2). In the presence of rolipram to inhibit cellular PDE4 activity (10 μ M; [2,6,12,13,39,45]) and EGTA to prevent activation of PDE1 by endogenous Ca^{2+} /CaM, the residual PDE activity seen in all three cell lines was very similar (Table 2). This observation on homogenates is

consistent with the results for isolated membrane and cytosol fractions (Table 1).

PDE1 activity can be selectively stimulated by the addition of Ca^{2+} /CaM (50 μ M; 20 ng/ml) [1,5]. Indeed, we noted here that there was a profound increase (approx. 4-fold) in the total homogenate PDE activity of the native FTC133 cells on addition of Ca^{2+} /CaM (Table 2). A similar stimulation (approx. 3-fold) was also apparent in the 'control' FTC133G cell line (Table 2). In marked contrast, an inhibitory effect of Ca^{2+} /CaM on the homogenate PDE activity of the RD1-expressing FTC133A cell line was noted (Table 2). This loss of PDE1 activity was not unique to the FTC133A cell clone. We also studied a clone (FTC236A) of the related FTC236 cell line that had been stably transfected to express RD1 (Figure 1B). It exhibited a homogenate PDE activity of 67 $\pm$ 12 pmol of cAMP hydrolysed/min per 10^6 cells compared with 7.6 $\pm$ 0.6 pmol of cAMP hydrolysed/min per 10^6 cells for the native non-transfected cells (means $\pm$ S.D.; $n = 4$). Such a profound increase in PDE activity was clearly attributable to the stable expression of RD1, as the addition of rolipram (10 μ M) yielded residual PDE activities that were similar ($< 5\%$ difference; $n = 4$) in the native FTC236 and the RD1-expressing FTC236A cell line. Whereas the addition of Ca^{2+} /CaM to PDE assays of homogenates of the native FTC236 cells led to a profound increase in PDE activity (12 $\pm$ 2 pmol of cAMP hydrolysed/min per 10^6 cells; mean $\pm$ S.D.; $n = 3$ separate experiments), the addition of Ca^{2+} /CaM to PDE assays using homogenates of the RD1-expressing FTC236A cell line yielded no observable increase in PDE activity ($< 4\%$ change; $n = 4$ separate experiments).

The absence of PDE1 activity from the FTC133A cells could not be simply reversed by culturing them for up to 24 h in the presence of the PDE4-selective inhibitor rolipram (10 μ M). After such treatment the addition of Ca^{2+} /CaM to PDE assays elicited no observable effect ($< 5\%$ change; $n = 3$ experiments). Neither did challenge of either FTC133 or FTC133A cells with the phorbol ester phorbol 12-myristate 13-acetate (10 nM for periods of up to 6 h) elicit any increase in PDE1 activity, as has been reported using CHO cells [24].

There are a number of distinct PDE1 forms, which are the products of separate genes [25,27,46]. We have previously reported the design of sets of RT-PCR primers aimed at detecting PDE1 transcripts in a species-independent fashion [24]. These primers were designed to a phylogenetically stable sequence in order to amplify a 601 bp fragment in all published transcripts of *PDE1A* and *PDE1B* [47]. Using such a degenerate set of primers, however, we were singularly unsuccessful in detecting transcripts for either *PDE1A* or *PDE1B* in RNA preparations from native FTC133 cells, FTC133G cells and the RD1-expressing FTC133A

Figure 2 Detection of PDE1 transcripts in human thyroid carcinoma cell lines

The details are described in the Materials and methods section and elsewhere [24]. Top, primers specific for PDE1C: rat brain (lane 1); FTC133A cells (lane 2); markers (lane 3); FTC133G cells (lane 4); FTC133 wild-type cells (lane 5); no-DNA control lane (lane 6). Bottom, primer pairs able to detect both PDE1A and PDE1B but not PDE1C: a no-DNA control lane (lane 1); rat brain (lane 2); markers (lane 3); rat brain (lane 4); FTC133A cells (lane 5); FTC133 wild-type cells (lane 6); FTC133G cells (lane 7). These results are typical of experiments performed at least three times. Arrows indicate the position of the 431 bp fragment.

cells (Figure 2, bottom). Recent sequence data [25,27], however, indicate that such a set of degenerate primers [24] would be unable to detect transcripts for the newly described PDE1C form [25,27]. We thus designed primers specific for the PDE1C sequence, conserved between human and rat, in the C-terminal region found downstream of the catalytic domain and which is not conserved in either PDE1A or PDE1B (see the Materials and methods section). Using these primers we were able to amplify (Figure 2) a fragment (431 bp) of the expected size, indicating the presence of PDE1C in the native FTC133 cell line. Although no attempt was made to perform quantitative studies, the magnitude of this signal was similar using identical amounts of RNA from both the native FTC133 cells and the 'control' transfected FTC133G cells but was dramatically reduced in the RD1-expressing FTC133A cell line (Figure 2, top). Such data are consistent with the loss of PDE1 activity in the RD1-expressing FTC133A cell line.

The compound EHNA has been demonstrated to serve as a selective PDE2 inhibitor [33,48,49]. However, it appears to inhibit PDE2 activity most effectively under conditions in which this isoform is stimulated by low concentrations of cGMP [33], which acts by binding to the regulatory site on the enzyme [5]. Under such conditions of cGMP stimulation (1 μ M cGMP) we observed a small inhibitory effect exerted by EHNA, indicative of the presence of PDE2 activity. This was noted in all the cell lines but appeared to be slightly greater in the RD1-expressing FTC133A cell line (Table 2).

Figure 3 Release of RD1 from the particulate fraction of FTC133A cells by Triton X-100 but not by high NaCl concentrations

Membranes from FTC133A cells were treated with increasing concentrations of either NaCl (■) or the non-ionic detergent Triton X-100 (●) as detailed in Materials and methods section. RD1 was detected by immunoblotting. The immunoreactive RD1 associated with the pelleted membrane fraction is given as a function of increasing dose of both NaCl and Triton X-100 (means $\pm$ S.D.; $n = 3$).

The PDE3 family can be selectively inhibited by low concentrations of cilostimide, with IC_{50} values of 0.1–1 μ M when assayed at 1 μ M cAMP [5]. In contrast, other PDE isoforms are either insensitive to cilostimide or have IC_{50} values that are typically some 500-fold higher [5,39]. In order to gauge the magnitude of PDE3 activity in these cells, assays were carried out with 1 μ M cAMP as substrate and 10 μ M cilostimide. This indicated the presence of PDE3 activity in all the cell lines, but it appeared to be somewhat higher in the RD1-expressing FTC133A cell line (Table 2).

Native FTC133 cell lines appear to express PDE1, PDE2, PDE3 and PDE4 isoforms. Using 1 μ M cAMP as substrate, approx. 45% of the total PDE activity in such cells is contributed by the PDE4 family and about 16% by PDE3 activity with the remaining approx. 39% contributed by the PDE1 and PDE2 enzymes. However, when Ca^{2+} levels are raised, the profound activation of PDE1 that ensues means that the activity of this isoform predominates, such that it provides more than 76% of the total PDE activity in the native FTC133 cell line with 1 μ M cAMP as substrate. A similar scenario was apparent for the FTC133G cell line, where elevation of Ca^{2+} levels again allowed PDE1 activity to predominate, providing in this instance more than 66% of the total PDE activity. Such a scenario is very different in the RD1-expressing FTC133A cell line in which PDE1 activity was ablated. Interestingly, however, when total homogenate PDE activity was assayed in the presence of Ca^{2+} /CaM, the values obtained for all the cell lines were remarkably similar (Table 2).

RD1 solubilization by the detergent Triton X-100

Repeated washing by centrifugation and resuspension of the particulate fraction did not release immunoreactive RD1 (< 5% change; $n = 3$ separate experiments), neither did treatment with high NaCl concentrations (Figure 3). However, treatment with

Figure 4 Intracellular distribution of RD1 and Tex1 immunoreactivity in stably transfected human thyroid carcinoma cells

As described in the Materials and methods section, cells were grown on coverslips, fixed, permeabilized and then treated with either anti-RD1 serum specific for the C-terminus of RD1 or Tex1 antiserum specific for antigens within the Golgi complex. These were then each treated with Rhodamine-conjugated secondary antibody. Control experiments were carried out, as described in the text to show that the immunofluorescence recorded was specific for permeabilized cells and dependent, where appropriate, on the appropriate antiserum. A generalized, faint background fluorescence only was seen in both the FTC133 and the FTC133G cell lines treated with anti-RD1 serum. Sections are either shown as 'xy,' horizontally through the cell and parallel to the anchoring surface the cell is growing on, or vertically down through the cell in the 'z-x' direction, as indicated. The xy sections are examples of single confocal sections through the centre of the cells under study. Examples of RD1 and Tex1 immunoreactivity are indicated by arrows and nuclei are indicated by 'N' or 'n'. The horizontal white bar is a scale marker and is 25 μ M in size. RD1 immunoreactivity is shown in panels A, B and C and Tex1 immunoreactivity in panels D and E. Panels A and C are xy confocal sections of FTC133A and FTC236A cells respectively, identifying RD1; panel B is a zx confocal section through a single FTC133A cell identifying RD1; panels D and E are xy confocal sections through FTC133A and FTC236A cells respectively, identifying Tex1 antigens.

the non-ionic detergent Triton X-100 did solubilize immunoreactive RD1 from the FTC133A cell particulate fraction (Figure 3). This showed a similar dose-dependence to that noted for endogenously expressed RD1 in brain and RD1 expressed in COS cells [14,15].

Immunofluorescence studies of FTC133A cells

Cells were grown on coverslips, fixed, permeabilized and then probed with specific antisera followed by Rhodamine-labelled anti-rabbit serum (see the Materials and methods section). With primary antiserum to RD1, a strong fluorescent signal was apparent with FTC133A cells (Figure 4). In contrast, as for immunoblotting (Figure 1), no fluorescent signal was evident in either the native FTC133 cells or the FTC133G 'control' cell line (results not shown). In addition, either no signal or a very weak diffuse background fluorescence was observed in cells that had (a) not been permeabilized with detergent, showing that the

Figure 5 Intracellular distribution of RD1 and Tex1 immunoreactivity in FTC133A cells treated with either monensin or brefeldin A

FTC133A cells were treated with either monensin or brefeldin A, as described in Materials and methods section, before fixation and analysis by laser scanning confocal microscopy. Panels A and B show examples of xy sections of cells treated with monensin and then probed for RD1 and Tex1 immunoreactivity respectively. Panels C and D show examples of xy sections of cells treated with brefeldin A and then probed for RD1 and Tex1 immunoreactivity respectively.

antigenic species was intracellular, (b) not been treated with a rabbit antiserum able to recognize RD1, (c) not been treated with anti-rabbit antiserum, (d) been exposed to primary antiserum that had been pretreated with the peptide immunogen before use and (e) been exposed to preimmune serum only (results not shown). From this we can conclude that the strong immunofluorescent signal observed in the FTC133A cells (Figure 4) was due to the specific expression of RD1 within FTC133A cells.

Laser scanning confocal microscopy was used to generate 'optical slices' through FTC133A cells. These identified a discrete localization of RD1 immunofluorescence which took the form of a strong fluorescent signal asymmetrically distributed about the nucleus (Figures 4A and 4C). This was apparent in optical sections taken both horizontally across the cells (xy sections; Figure 4A) and vertically down through the cells (zx sections; Figure 4B). Such a well-defined localization of RD1 was not unique to the FTC133A cell line as it was also clearly observed for the RD1-expressing FTC236A cell line (Figure 4C). Such a focused perinuclear localization is highly reminiscent of Golgi-associated proteins [14,50]. Indeed, a similar pattern of immunofluorescence was obtained with the Tex1 antiserum (Figures 4D and 4E), which was raised against a mixture of Golgi-specific proteins, mannosidase II being one of the major antigenic species [28]. Such confocal studies clearly support biochemical data that indicate an exclusively membrane association for RD1 in FTC cells. Furthermore they show that RD1 is localized to a highly distinct intracellular structure probably the Golgi complex.

Monensin and brefeldin A cause specific disruption of the Golgi complex [50–53]. Monensin causes expansion of the Golgi complex and, consistent with the localization of RD1 in this structure, treatment of FTC133A cells with monensin led to a profound change in the pattern of RD1 immunofluorescence (Figure 5A). Thus, instead of a sharp focus at a pole of the nucleus, a markedly expanded perinuclear immunolocalization

was identified using antisera against RD1. Indeed, immunofluorescence spread around the entire nucleus and even extended to punctate structures found dispersed through the cell. Monensin also caused the redistribution of the Tex1 antigen (Figure 5B), consistent with perturbation of the Golgi structure. Brefeldin A treatment elicited a marked change in the pattern of immunofluorescence obtained for both RD1 and Tex1 antigens (Figures 5C and 5D). Again, instead of the extremely tight and focused pattern of fluorescence seen in the untreated cells (Figure 4), immunofluorescence was dispersed over the entire nucleus and even into punctate structures found throughout the cytosol (Figure 5), consistent with disruption of the Golgi complex [50–52,54]. However, the precise patterns of distribution of RD1 and Tex1 antigens are slightly different. This may be because Tex1 primarily detects antigens in the *trans*-Golgi stack [28], perhaps implying that RD1 is localized to a rather different region of the Golgi. Nevertheless, our data are consistent with localization of RD1 to the Golgi of FTC cells.

DISCUSSION

We show here that two thyroid carcinoma cell lines can be stably transfected with the rat PDE4A species RD1. Intriguingly, the RD1-expressing FTC133A and FTC236A cell lines did not exhibit the potent Ca^{2+} /CaM-stimulated PDE1 activity seen in the parental cell lines. The reduction in the RT-PCR signal for PDE1C in these RD1-expressing cells might indicate that loss of PDE1 activity was due to lowered levels of transcripts for this PDE isoform. Whether such a change is specific for this particular cell type or is due to overexpression of specific PDE4 isoforms remains to be determined, as does the underlying mechanism of down-regulation.

In the FTC133A and FTC236A cell lines all of the novel PDE4 activity and all of the immunoreactive RD1 was found exclusively in the membrane fraction. Indeed, the single immunoreactive species found in these transfected thyroid cells co-migrated with immunoreactive RD1 expressed in the membranes of pSVL-RD1-transfected COS cells. No novel cytosolic PDE4A species was observed in these transfected thyroid cells. This supports our contention [13,16] that the novel soluble PDE4 activity seen in pSVL-RD1-transfected COS cells was in fact a truncated form of RD1 (Met²⁶RD1) which lacked the N-terminal membrane-targeting splice region. That this was produced in COS cells [13,16] and not transfected thyroid cells may reflect the action of the strong SV40 promoter in COS cells on the suboptimal Kozak sequence preceding Met²⁶. As seen for RD1 expressed in brain [14] and transfected COS cells [15], membrane-bound RD1 in these stably transfected thyroid cells was not released by repeatedly washing the membranes or by using high salt concentrations. However, it was solubilized by concentrations of Triton X-100 similar to those causing solubilization of RD1 in brain [14] and transfected COS cells [15]. RD1 thus appears to achieve a membrane-associated state in these human thyroid carcinoma cells which shows similar characteristics to those demonstrated by the native brain-located species and the membrane-bound form found in transfected COS cells.

The absence of cytosolic immunoreactive PDE4A species in transfected cells has allowed us to define the intracellular location of RD1 using immunofluorescence analysis. In the RD1-expressing FTC133A and FTC236A cell lines, immunoreactive RD1 had a highly distinctive perinuclear localization (Figure 4). This distribution pattern was strikingly asymmetrical and showed a profound similarity to that identified using the Tex1 antiserum (Figure 5) which primarily detects proteins in the *trans*-Golgi stack [28]. This suggests that expression of RD1 in these thyroid

cell lines is exclusively in the Golgi compartment. The slightly different labelling patterns seen for RD1 and the antigenic species identified by the Tex1 antiserum (Figure 4) may reflect differences in localization of these species within the compartments of the Golgi stack. Treatment of RD1-expressing transfected thyroid cells with either brefeldin A or monensin, agents known to specifically perturb the Golgi complex [50–53], caused similar profound changes (Figure 5) in the distribution of both RD1 and Tex1 antigens. This is consistent with our contention that RD1 was exclusively localized to the Golgi apparatus in these cells. Whether or not such an intracellular distribution reflects that of native RD1 found in brain remains to be determined. However, these studies demonstrate that RD1 does not associate randomly with intracellular membranes. Thus the N-terminal splice region of RD1 is unlikely to achieve membrane association by inserting into lipid bilayers as this might be expected to lead to a more general association with various intracellular membranes. We have [18] suggested that a more likely alternative is that membrane association is achieved through association of RD1 with an anchoring species. This might be expected to confer on RD1 the intracellular distribution of such a putative anchoring species. That RD1 showed such a proscribed localization in transfected thyroid cells is consistent with such a notion. Analysis of transfected COS cells also pointed to an association of RD1 with the Golgi, although a major fraction of RD1 appeared to localize to punctate vesicles underlying the cell surface plasma membrane [14]. As vesicles traffic between the Golgi and the plasma membrane, it is possible that the distribution of RD1 in COS cells reflects this phenomenon, perhaps indicating that it binds to a protein associated with such vesicles.

It is thus intriguing that RD1, when expressed in non-neuronal cell types, is exclusively membrane-associated, as it is in brain. If RD1 does interact with an anchoring species, then either this anchoring species or a related entity would appear to be expressed in a number of cell types and at a level sufficient to accommodate all of the RD1 produced in these transfected cells. Thus alternative splicing of the *PDE4A* gene appears to generate isoforms able to locate to distinct intracellular compartments as a result of their unique N-terminal regions. This is apparent for the splice variants RPDE6 [13] and RPDE39 [12], which can be found both in cytosol and associated with cytoskeletal fractions. The mode of anchoring these two species has yet to be defined, although for RPDE6 it has been suggested [19] that this may be via interaction with an SH3-domain-containing protein. An appreciation of the mode and functional significance of the localization of PDE4 isoforms may aid our understanding of the control of intracellular signalling mechanisms.

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