Identification and characterization of the human homologue of the short PDE4A cAMP-specific phosphodiesterase RD1 (PDE4A1) by analysis of the human HSPDE4A gene locus located at chromosome 19p13.2

Michael SULLIVAN^{*1}, Graham RENA^{*}, Fiona BEGG^{*}, Laurie GORDON[†], Anne S. OLSEN[†] and Miles D. HOUSLAY²

^{*}Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, IBS, Davidson and Wolfson Buildings, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K., and [†]Human Genome Center, Biology and Biotechnology Research Program, L-452, Lawrence Livermore National Laboratory, Livermore, CA 94550, U.S.A.

The HSPDE4A gene spans 50 kb, consists of at least 17 exons and is orientated 5′–3′, telomere to centromere. It is located at chromosome 19p13.2, being 350 kb proximal to the gene encoding TYK2 and 850 kb distal to the gene encoding the low-density lipoprotein receptor. Its structure is consistent with the production of active ‘long’ and ‘short’ isoenzymes as the result of alternative mRNA splicing at two splice junctions. Identified is the single alternatively spliced 5′ exon encoding the unique N-terminal region of the long isoenzyme HSPDE4A4B (pde46). The upstream conserved regions, UCR1 and UCR2, which form characteristic domains of PDE4 long forms are each encoded by three exons. The PDE4A-subfamily-specific linker region LR1, which joins UCR1 and UCR2, is encoded by two exons, whereas LR2, which joins UCR2 to the catalytic unit, is encoded by a single exon. Identification of exons encoding an enzymically inactive product of this gene, HSPDE4A8A (2el), indicates that this is an authentic gene product. The 5′ exon encoding the unique N-terminal region of the human homologue of the rodent isoform RNPDE4A1A (RD1) was located, and the splice junction used

to produce this short PDE4A isoform shown to occur at a different position from that seen in both the rat PDE4B and PDE4D genes. Reverse transcriptase PCR analysis indicates that RD1 homologues are conserved across species, having a conserved membrane-targeting region and a hypervariable LR2 region. Human RD1 was expressed transiently in COS-7 cells and detected as an 83 kDa species primarily associated with the high-speed membrane fraction. Human RD1 exhibited a K_m for cAMP of about 3 μ M, an IC_{50} value for inhibition by the PDE4-selective inhibitor rolipram of about 0.3 μ M and was considerably more thermostable than rat RD1. Human RD1 was generated as a mature 80 kDa species in an *in vitro* transcription–translation system and shown to be capable of binding to membranes. Knowledge of the gene structure and the associated sequence information should facilitate analysis of the involvement of PDE4A in hereditary disorders that may result from alterations in enzyme expression, activity, regulation and intracellular targeting and serve as a resource for determining authenticity of cloned PDE4A species.

INTRODUCTION

Cyclic nucleotide phosphodiesterases (PDEs) catalyse the hydrolysis of cAMP and cGMP to the corresponding nucleoside 5′-monophosphates [1–6]. In higher eukaryotes, PDEs form a diverse group of enzymes that are currently divided into seven classes according to substrate specificity, inhibition and substrate requirements. Each of the seven classes are encoded by separate genes with a number of these represented by multigene families.

The PDE4 enzymes have been the focus of much interest as potential therapeutic targets for inflammatory diseases, such as asthma, multiple sclerosis and rheumatoid arthritis [2–4,7,8]. They have specificity for cAMP, are selectively inhibited by the compound rolipram and are encoded by four separate genes (A–D) in man, rat and mouse. Such diversity is probably further enhanced by the occurrence of splice variants of the human and rat PDE4 genes [2–4,9–12]. This predominantly takes the form of unique 5′ ends, which results in the production of enzymes possessing distinct N-terminal domains. The presence of different

N-terminal regions in PDE4A isoenzymes has been linked with distinct functional roles [6]. These include conferring susceptibility for membrane association and intracellular targeting regulation of the V_{max} of enzyme activity, influencing thermostability, conferring susceptibility to regulation by phosphorylation and the ability to bind SH3 domains [3]. The implication of such studies is that the regulated expression of distinct PDE4 splice variants may play an important functional role in controlling cAMP signalling in different cell types. Consistent with this, analyses of putative splice variants have identified distinct tissue-specific expression and developmentally controlled expression patterns [3].

In addition to the highly conserved domain containing the catalytic region, PDE4 isoforms are uniquely characterized [9] by exhibiting upstream conserved regions 1 and 2 (UCR1 and UCR2). ‘Long’ PDE4 isoforms possess both UCR1 and UCR2 whereas the ‘short’ isoforms have only UCR2. Whereas all known short-form variants of PDE4B and PDE4D contain a complete UCR2, the sole identified PDE4A short form, namely

Abbreviations used: BAC, bacterial artificial chromosome clone; FISH, fluorescence *in situ* hybridization; PDE, cyclic nucleotide phosphodiesterase; HSPDE4, *Homo sapiens* type 4 (IV) cAMP phosphodiesterase; pde46, human PDE4A isoform (HSPDE4A4B; GenBank L20965); 2el, human PDE4A isoform (HSPDE4A8A; GenBank U18088); TM3, human PDE4A cDNA (GenBank L20967); h6.1, a truncated human PDE4A (HSPDE4A4C; GenBank U18087); h-pde1, a truncated human PDE4A (HSPDE4A4; GenBank M37744); RD1, a rat PDE4A (RNPDE4A1A; GenBank M26715, L27062); RD2, a rat PDE4A cDNA (RNPDE4A2; GenBank M26717); RD3, a rat PDE4A cDNA (RNPDE4A3; GenBank M26716); RPDE-6, a rat PDE4A (RNPDE4A5; GenBank L27057); hRD1, HSPDE4A1A (GenBank U97583), a short form PDE4A homologue of rat RD1; rolipram, 4-[3-(cyclopentoxyl)-4-methoxyphenyl]-2-pyrrolidone; UCR, upstream conserved region; RT-PCR, reverse transcriptase PCR; LDLR, low-density lipoprotein receptor; ORF, open reading frame; LR, linker region.

¹ Present address: Astra Charnwood, Bakewell Road, Loughborough, Leicestershire LE11 0RH, U.K.

² To whom correspondence should be addressed.

rat RD1, appears to contain a partial UCR2 region [2–4]. It is not known whether this reflects a cloning artifact or a true difference in splicing of the PDE4A gene compared with the other PDE4 genes. Another apparent difference between PDE4A isoforms was revealed by the identification [13] of a human PDE4A cDNA (2el), which provides evidence for alternative mRNA splicing of a PDE4 transcript. This generates a species (HSPDE4A8A) with novel N- and C-termini, which, unusually, is catalytically inactive. The physiological role of this species and whether it is encoded in the PDE4A gene have yet to be ascertained.

The four PDE4 genes have been located to three different human chromosomes [13–16]. PDE4B maps to chr1p31, PDE4D to chr5q12, PDE4C to chr19 and PDE4A to the p13.2-q12 region of chromosome 19. A partial characterization [10] of rat PDE4B and 4D genomic clones has delineated the structure of exons encoding the extreme C-terminal and catalytic regions as well as identifying exons encoding the unique N-terminal regions of the short-form products of these genes.

Here we present the first characterization of a PDE4A gene and the first identification of the exons encoding a long PDE4 isoform. We identify those exons encoding the unique N-terminal region as well as the UCR1 and associated linking regions LR1 and LR2. Fine mapping of the HSPDE4A gene on to a chromosome 19 cosmid contig placed it within the region p13.2, between the genes encoding TYK2 and the low-density lipoprotein receptor (LDLR). This analysis shows that the catalytically inactive 2el product is indeed encoded within the HSPDE4A gene together with appropriate consensus splice sites. Also identified is the organization of the exons encoding a short PDE4A isoform, which demonstrates that the point of alternative mRNA splicing is distinct from that used in the generation of PDE4B and PDE4D short forms. This indicates that the rat PDE4A short-form RD1 is an authentic isoform. We have identified, cloned and characterized the short human homologue of rat RD1, demonstrating that it is an active species with a propensity for association with membranes.

MATERIALS AND METHODS

cDNA library screening

A human T-lymphoblastoid cell line (CCRF-CEM) cDNA library made in λ ZAP II was purchased from Clontech. A total of 1×10^6 plaques were plated, transferred to Hybond-N filters (Amersham International) and hybridized with a human PDE4A-specific probe using conditions described previously [17]. Positively hybridizing phage were plaque-purified, and inserts were subcloned by *in vivo* excision into pBluescript II SK. The plasmid generated from the plaque with the largest PDE4A-positive insert, called 10A, was sequenced completely (GenBank accession number U68532).

Isolation of genomic clones

The chromosome-specific cosmid library has been previously described [18]. A probe specific for human PDE4A was hybridized to colony filters of this library using previously described procedures [18]. A 480 bp fragment derived from the extreme 5' end of the HSPDE4A cDNA, clone 10A, was radiolabelled and used to probe a high-density gridded filter of chromosome 19-specific clones as reported previously [18].

RD1 reverse transcriptase (RT)-PCR and cDNA cloning

Human, guinea-pig, mouse, rabbit, cat and pig RD1 cDNAs spanning the unique 5' exon of RD1 to exon 9 were amplified by

PCR using brain-derived cDNA as template and the primer pair GR73 and GR36, both derived from the original RNPDE4A1 (RD1) sequence [19]. The forward primer GR73, 5'-TTCTTCTGCGAGACCTGCTCCAAG-3', was generated so as to bind within the unique 5' end coding region of RD1, at nucleotides 71–95 with respect to the rat RD1 sequence (GenBank accession no. L27062). The reverse primer GR36, 5'-CAGGCCCCATTTGCTCAAGTTCTCC-3', was generated so as to be complementary to RD1 at nucleotides 418–442. In addition, human RD1 (hRD1) cDNA was amplified from human brain cDNA using the primer pair GR99 and GR36. GR99, 5'-CCAGGCGGGCTAAGTCTCCAAG-3', was generated so as to bind upstream of the human RD1 open reading frame (ORF), in the 5' untranslated region, and produced a fragment of 430 bp when used with GR36. Human brain mRNA was purchased from Clontech. For other species, total RNA was prepared from snap-frozen brain tissue (150 mg) after thawing and homogenization in 1 ml of Tri-Reagent (Sigma). First-strand cDNA was generated using the PDE4A-specific antisense primer GR29, 5'-CAGGGTGRGCCACATCGTGG-3' (nucleotide position 727–746 in RD1; R being A or G) and a cDNA synthesis kit used according to the manufacturer's instructions (Pharmacia). RD1-specific sequences were PCR amplified from 10 μ l of first-strand cDNA reaction mixture using the primers GR36 and GR73 or GR99 (500 μ M each) and *Taq* DNA polymerase (1.5 units). Thirty cycles of 94 °C (1 min), 50 °C (1 min) and 72 °C (1 min) were carried out, and reaction products were separated on a 2% agarose gel. For each species, single bands of the expected size were isolated, and DNA was extracted using the Qiaex II Gel Extraction Kit (Qiagen). The isolated DNA was then ligated with the pCR2.1 TA cloning vector (Invitrogen) with blue/white screening. A total of six individual positive colonies for each species were sequenced in both directions using the *Taq* DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and analysed as described below. Sequences were deposited in GenBank (accession nos. U97584–9).

Chromosome 19 mapping and HSPDE4A localization

The metric physical map of chromosome 19 has been described previously [20]. Localization of PDE4A on this map was accomplished by fluorescence *in situ* hybridization (FISH) of a PDE4A cosmid to chromatin targets of increasing resolution [21]. A contig spanning, and extending beyond, the PDE4A gene was constructed by hybridization walking [22] in the chromosome 19 cosmid library and a human genomic bacterial artificial chromosome clone (BAC) library (Research Genetics Inc., Huntsville, AL, U.S.A.). Cosmid and BAC clones identified by hybridization were analysed by *Eco*RI digestion. Fluorochrome-labelled *Eco*RI fragments were analysed on the Applied Biosystems Genescanner. Fragments less than 0.5 kb were generally not detectable under the conditions used. An approximate *Eco*RI restriction map of the PDE4A contig was assembled on the basis of the pattern of restriction fragments common among overlapping clones.

DNA manipulation and sequence analysis

A number of overlapping restriction fragments from cosmid clones 33405, 28065 and 27826 were subcloned as a series of overlapping restriction fragments into derivatives of pUC19. Large subclones were treated with the Double Stranded Nested Deletion Kit (Pharmacia) to produce a series of deletions that allowed rapid sequence analysis of plasmid inserts, as described previously [17]. Sequence analysis of HSPDE4A-positive cosmids, 33405, 28065 and 27826, was accomplished by direct

sequencing of cosmid DNA with the Prism Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit (Perkin–Elmer). Sequence products were analysed using an Applied Biosystems model 373A automated sequencer. *Escherichia coli* strains NM522 and K803 were used for plasmid transformation and propagation. Plasmid DNA was purified using the WizardTM Miniprep Kit (Promega). Cosmid DNA was purified by Qiagen column chromatography, and sequence cycling was performed on a Perkin–Elmer PCR System 9600.

Generation of a hRD1 expression plasmid

The ORF encoding hRD1 (HSPDE4A1A; GenBank U97583) as predicted from the HSPDE4A genomic sequence was engineered for expression. The complementary oligonucleotides hRD1-5' up (5'-ATCCACCATGCCTTTGGTGGATTCTTC-TGCGAGACCTGCTCTAAGCCTTGGCTGGTGGGCT-GGTGGGACCAGTTTAAACTTAAAGG-3') and hRD1-5' lo (5'-TCGACCTTAAGTTTAAACTGGTCCCACCAGCCCA-CCAGC CAA GGCTTAGAGCAGGTCTCGCAGAAGAAA-TCCACCAAAGGCATGGTG-3') were annealed and ligated into *Bam*HI–*Sal*I-digested pK19 to create pK19RD5'. Clones were sequenced to ensure that the cloned oligonucleotides were as designed and contained no sequence errors. The annealed oligonucleotide cassette contains *Bam*HI and *Sal*I compatible ends, a Kozak consensus sequence upstream of the initiation codon (underlined and in italics in hRD1-5' up) followed by the sequence that encodes the N-terminal 22 amino acids from the 5' exon of hRD1 (see Figure 2) and a *Dra*I restriction site created by a silent C to T change of the last base of the first triplet codon of exon 7. The region encoding the C-terminus of human PDE4A shared by both PDE46 and hRD1 was amplified by PCR using pSV-SPORT-pde46 as template with primers FB15 and FB3'*Sal*-I. Oligonucleotide FB15 (5'-AAGTTTAAAGGAT-GTTGAACC-3') binds at the extreme 5' end of exon 7 and contains a *Dra*I site created via the same silent C to T change (underlined) incorporated into hRD1-5' up. The 3' primer FB3'*Sal*-I (5'-GTCGGACGAGGTCTGGGGATCCAGGTA-3') is complementary to the upper strand and binds downstream of the PDE4A ORF stop codon and incorporates a unique *Sal*I site. The product of the PCR was cloned into pZero (Invitrogen) and clones were sequenced to select one that contained no base changes that would lead to altered amino acids encoded by the PDE ORF. The insert was released by digestion with *Dra*I and *Sal*I and ligated with *Dra*I–*Sal*I-digested pK19-hRD1-5' to create pK19-hRD1-FL. The full-length hRD1 ORF was then removed by digestion with *Bam*HI and *Sal*I and ligated with *Bam*HI–*Xho*I-digested pcDNA3 (Invitrogen) to create the expression plasmid pCMV-hRD1.

PDE assays

PDE activity was assayed by a modification of the two-step procedure of Thompson and Appleman [23] as described previously by Marchmont and Houslay [24]. 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 for determination of activities. Initial rates were taken from linear time courses of activity. For the determination of kinetic parameters, the PDE assays were conducted using 10–15 different cAMP concentrations over the range 0.1–100 μ M. As indicated below, the COS-7 cell transfection procedure utilized in this study led to very high levels of novel PDE4 activity being produced such that this comprised more than 98 % of the total COS cell PDE activity. Mock transfections, with vector only, as indicated in previous studies [25–28], did not alter the endogenous

COS cell PDE activity. As a routine, however, we subtracted the residual endogenous COS cell PDE activities performed in parallel experiments from those activities found in the PDE4B-transfected cells. Protein was routinely measured by the method of Bradford [29] using BSA as a standard.

Cell culture, transfection and fractionation

COS-7 cells were seeded at approx. one-third confluency on to 10 cm-diameter plates, 18 h before transfection, as described previously by us in some detail [11,25,30]. Disruption of COS cells was performed, as described previously by us [25], in KHEM buffer (50 mM KCl, 50 mM Hepes/KOH, pH 7.2 10 mM EGTA, 1.92 mM MgCl₂) containing 1 mM dithiothreitol and a mixture of protease inhibitors at a final concentration of 40 μ g/ml PMSF, 156 μ g/ml benzamidine and 1 μ g/ml each aprotinin, leupeptin, pepstatin A and antipain. Pellet fractions were resuspended in this mixture also. The high-speed (P2) pellet fraction was generated essentially as described previously by us [25,27,28]. This procedure routinely yielded a P1 pellet (1000 g_{av} for 10 min) and a P2 pellet (60 min at 100000 g_{av}) as well as a high-speed supernatant. The homogenization procedure was complete in that there was no detectable latent lactate dehydrogenase activity present in the P1 pellet, indicating an absence of cytosolic proteins. Pellet fractions were treated with either the non-ionic detergent Triton X-100 (5 %), high [NaCl] (2 M) or both Triton X-100 and high [NaCl] added together as described previously [26].

SDS/PAGE and Western blotting

Polyacrylamide gels of concentration 8 % were used and the samples boiled for 3 min after being resuspended in Laemmli [31] buffer. Gels were run at 8 mA/gel overnight or 50 mA/gel for 4–5 h with cooling. For detection of transfected PDE by Western blotting, 2–50 μ g of protein samples were separated by SDS/PAGE and then transferred to nitrocellulose before being immunoblotted using human PDE4A-specific antiserum [30]. Labelled bands were identified by using anti-rabbit peroxidase-linked IgG and either the Amersham ECL Western blotting system used as a visualization protocol or ¹²⁵I-labelled anti-rabbit serum used for detection and quantification.

Analysis of thermostability

Thermostability was analysed as described previously by us [27], with samples incubated at either 55 or 50 °C, removed to ice before assay of PDE activity at a cAMP concentration of 1 μ M.

Generation of hRD1 using an *in vitro* transcription–translation system

Both the generation of a mature protein using an *in vitro* transcription–translation system and the subsequent analysis of the potential of this species to interact with P2 membranes from COS cells was carried out for intact hRD1 essentially as described previously [32]. As in that study, labelled protein was generated using [³⁵S]methionine (0.8 mCi/ml) with detection on a PhosphorImager subsequent to SDS/PAGE. Here, however, a Promega T7 TNT Coupled Reticulocyte Lysate System was used.

RESULTS AND DISCUSSION

Localization of HSPDE4A on the chromosome 19 map

We and others [13–16] have located the human PDE4A gene to chromosome 19. We have previously refined this to the p13.2-q12

region using a panel of human somatic cell hybrids and used FISH analysis to show association with the short arm of chromosome 19 [13,14]. In this study, we have screened a high-density array of chromosome 19-specific cosmid clones in an attempt to

isolate cosmids containing the entire human PDE4A gene locus. As a probe we used the extreme 5'-terminal 480 bp of the PDE4A cDNA clone 10A isolated here from a human T-cell library. Clone 10A is very similar to the human long PDE4A splice

variant HSPDE4A4B (pde46) [9] but lacks 158 bp seen at the extreme 5' end of pde46. The PDE4A-5' probe identified nine cosmids (21018, 25495, 27270, 27826, 28065, 29158, 29774, 31069 and 31310), which were members of a 75 kb cosmid contig (contig 1003) previously assembled by fingerprinting but not yet incorporated into the metric map of chromosome 19. Cosmid 31310 has been mapped to chromosome 19p13.2 by metaphase FISH, but not ordered further. To incorporate PDE4A into the ordered map, the location of cosmid 31310 relative to other chromosome 19p13.2 markers was refined by a series of two-cosmid two-colour metaphase FISH experiments, followed by three-cosmid three-colour pronuclear FISH. These experiments extend the previously published [33] pronuclear FISH map of chromosome 19p with several additional markers and distance estimates for the approx. 1.4 Mb region in chromosome 19p13.2 which occurs between the genes encoding DNA methyltransferase and the erythropoietin receptor (Figure 1). Distance measurements between pairs of cosmids across the region indicated that PDE4A was about 350 kb proximal to the DNA methyltransferase gene and 850 kb distal to the LDLR gene (Figure 1).

To extend the contig 3' of PDE4A, cosmid and BAC libraries were screened with a 13 kb *EcoRI* fragment from cosmid 27826. The probe identified a single BAC (BC101351) and no additional cosmids. Hybridization of BC101351 to chromosome 19 cosmids identified clones in the PDE4A contig, as well as several additional cosmids which had been assembled into a 70 kb contig (contig 1821). Analysis of *EcoRI* digests of the cosmids and BAC indicated that BC101351 joined the two cosmid contigs, forming a single contig of about 210 kb (Figure 1). The orientation of this contig on the chromosome was determined by incorporating cosmid 28201 into the pronuclear FISH map. These results demonstrate that the PDE4A gene is in a 5–3' orientation, running from telomere to centromere (Figure 1).

Identification of exons encoding the PDE4A long form, pde46 (HSPDE4A4B)

Nine cosmids with overlapping inserts that formed a contig were picked for further analysis (Figure 1). The sequence of all exons and flanking intronic sequences were determined (see Figures 2 and 3) on either appropriate subclones of 33405, 28065 and 27826 or through the use of exon-specific oligonucleotide primers which were used to sequence directly cosmid DNA from clones 33405, 28065 and 27826.

HSPDE4A4B (pde46) is the largest identified human PDE4A species [9]. It is the only known long form of human PDE4A and has been shown to be expressed in a variety of different cell types [9]. The rodent homologue of this, RNPDE4A5 (rpde6), has also been characterized [25,34] and shown to occur in a range of cell types as well as being differentially expressed in various brain areas [25]. These long PDE4A enzymes are found in both the cell cytosol and particulate fractions, with confocal and biochemical analyses indicating an interaction of this isoform with the cell cytoskeleton [25,30]. The 5' exon of pde46 encodes a unique N-terminal region (Figure 3) which contains three PxxPxxR motifs, suggesting an ability to interact with SH3 domains [35]. Consistent with this, it has been shown [36] that rpde6, the rat homologue of pde46, can bind preferentially to the SH3 domains of src family tyrosyl kinases. Recently, the presumed murine homologue of pde46 has been shown [37] to be activated by growth hormone through a process involving phosphatidylinositol-3-kinase and p70S6 kinase and which is dependent on the unique N-terminal region encoded by the equivalent of the 1⁴⁶ exon identified here. This study demonstrates the authenticity of the long PDE4A cDNA pde46 as within the contig are

sequences that encode the unique 5' region of pde46. Thus exon 1⁴⁶ contains the pde46 initiation codon and encodes the first N-terminal 107 residues, which confer SH3 interaction and the ability to be regulated by a phosphatidylinositol 3-kinase-triggered signalling cascade on this long PDE4A isoform.

A characteristic feature of all known long PDE4 isoforms is the presence of both UCR1 and UCR2 [9]. In analyses to characterize the short isoforms of rodent PDE4B and PDE4C genes, it was shown [10] that three exons (exons 6, 7, 8) encode UCR2. Here we show (Figure 3) that three novel exons (exons 2, 3, 4) encode UCR1 which characterizes PDE4 long-form enzymes. UCR1 has been shown to be of functional significance from studies [38] on PDE4D3 demonstrating that protein kinase A can cause the phosphorylation of a serine residue located in the motif RRES, which characterizes UCR1 of all long PDE4 isoforms [3]. This, in PDE4D3, leads to a profound increase in enzyme activity and altered sensitivity of the catalytic unit to inhibition by the PDE4-selective inhibitor rolipram. Thus conformational changes in UCR1 can apparently be transmitted to the catalytic unit. This may involve the so-called [3] linker region 1 (LR1), which joins the two UCRs, and LR2, which joins UCR2 to the catalytic unit. The sequence of both LR1 and LR2 are highly heterogeneous between the various PDE4 species [3], and, from the organization of the PDE4A gene (Figure 3), it is apparent that they are encoded by distinct exons. Thus LR1 is encoded by both exons 4 and 5 whereas LR2 is encoded entirely by exon 8. The heterogeneous form of LR1 and LR2 from the different PDE4 families may be of importance in conferring the distinct functional properties of the isoforms from each of the four PDE4 gene families. Thus exons 4, 5 and 8 may define PDE4 subfamily characteristics. In this regard we note (Figure 2) that the PDE4A LR2 region is rich in proline and arginine residues, which may indicate an additional site for SH3 domain interaction. Given that this is generated by a hypervariable exon and occurs in a region that links the catalytic unit to the PDE4 'signature' UCRs, it is possible that this may have functional significance.

The HSPDE4A catalytic region is encoded essentially by five exons (10–14) with some additional contribution from the most 3' exon (15). The splice sites for these exons demonstrate high conservation with those found in the rat RNPDE4B and RNPDE4D genes (Table 1 of ref. [10]), with the sole exception being the junction between exons 12 and 13, where the splice site appears to have moved by a single base.

In the genomic sequence, we have been unable to find a consensus polyadenylation signal in the immediate vicinity of the sequence representing the 3' end of the reported human PDE4A cDNAs [9]. We have found two polyadenylation sequences in the downstream genomic sequence, 2363 bp and 2721 bp after the start of exon 15 (Figure 2). The first sequence is a canonical sequence, AAUAAA, while the second sequence, AUUAAA, is a functional common variant of the canonical sequence [39]. Searching the EST database, dbEST, with sequences flanking these two polyadenylation sequences identified four distinct unassigned ESTs (accession numbers AA975441, AA402329, AA402738, AA613574) whose 3'-ends terminate 16 bases (3 ESTs) and 17 bases (1 EST) downstream from the second non-canonical polyadenylation signal. Viewing the chromatographic traces for two of the ESTs (AA402329 and AA402738) revealed that these cDNAs contain poly A tracts that have been trimmed and do not appear in the GenBank database. In the absence of any evidence that the first polyadenylation signal is used, we conclude that the second polyadenylation site is used in the maturation of PDE4A transcripts and that poly A addition occurs 16/17 bases downstream of this site.

ATCTTGTCCTCCA**ATTAA**ACCGAGGCTTTCCACCGATtttctgget

At the top, the exons encoding the PDE4 conserved motifs UCR1 and UCR2 and the PDE common catalytic region-containing domain are labelled with double-headed arrows. The arrow depicting UCR2 is split to exclude the 5' exon of the human homologue of RD1 (hRD15'). The exons found in the published HSPDE4A cDNAs are labelled with letters from 1 to 15 (5' to 3'). The exons that make up the HSPDE4A cDNAs, pde46 (HSPDE4AAB) [9], 2EL (HSPDE4A8A) [13,14], and the human homologue of RD1 [19] are shown. The extreme 5' exon of the purported non-functional HSPDE4A, TM3 [9], is indicated as 1^{TM3}. Exons found in the longest functional HSPDE4A cDNA, pde46, are shown as clear boxes. Alternative exons found in the non-functional HSPDE cDNA 2EL and in TM3 and are represented by black boxes. The 5' end of RD1 is represented by a thick hatched box. The predicted ORFs in the different variants are marked at the initiation codon by an arrow and at the termination codon by a filled diamond. The calculated mass of the predicted protein produced by each variant is displayed on the right (kD, kDa).

The first human PDE4A cDNA clone to be isolated [40] was called h-pde1. However, subsequent to the cloning of pde46 [9], it was realized that h-pde1 and the species h6.1 engineered to reflect it [17] were N-terminally truncated. Recently, it has been suggested by the original investigators [41] that the 5' end of the h-pde1 clone may have originated as a cloning artifact rather than h-pde1 reflecting a distinct PDE4A splice variant. Our delineation of the structure of the HSPDE4A gene is consistent with such an assertion. We have been unable to identify any cosmids that encode the first nine amino acids of h-pde1, and neither are there consensus splice sites, which would allow for the disruption of exons encoding UCR2 as seen in h-pde1.

We [13] have previously isolated a cDNA that encodes a catalytically inactive human PDE4A isoform called 2el

It is likely that truncations at the extremities of the conserved catalytic region lead to the loss of PDE catalytic activity in 2el, although it is also possible that the unique N- and C-terminal regions may serve to confer inhibition, as it has been suggested

Exons present in the largest human PDE4A cDNA that encodes functional enzyme, pde46 [9], are shown in uppercase letters and are labelled above the encoded amino acids; introns are in lowercase letters. Alternative exon sequences (nucleotides) present in the cDNA TM3 [9] are underlined, and those found in the cDNA 2EL [13] are in italics and are underlined. The exon labelled hRD1 5' is homologous to the unique 5' rat PDE4A variant RD1 and is found between exon 6 and exon 7 and is shown in bold capitals. The start and stop codons that define the functional ORF of pde46 are underlined and are in bold in exon 1⁴⁶ (1-46) and exon 15 respectively. The ORF of pde46 is translated and the corresponding amino acids are shown in italics above the nucleotide sequence. The alternative N-terminal amino acids encoded by different PDE4A cDNAs, pde46, TM3, human RD1 and 2EL, are shown in bold above the corresponding nucleotide sequences. The 5' end of TM3, exon 1^{TM3} (1-TM3), is translated and the corresponding amino acids shown above the DNA sequence; the initiation codon used in this Figure lies in the flanking sequence just upstream of the extreme 5' end of the published TM3 sequence (accession no. L20967). The nucleotide representing the extreme 3' end of the pde46 cDNA sequence that we find in the genomic sequence is underlined in exon 15. This base represents nucleotide 3643 of pde46. The two polyadenylation signals, 5'-AATAAA-3' and 5'-ATTAAA-3', in exon 15 are in bold and are underlined. The two sites of poly A addition, 16 and 17 bases downstream of the second polyadenylation signal are underlined. These sequences have been deposited in the GenBank database with accession numbers AF069487-91 inclusive.

CAGCCTCCTCCTGGGACCCCTTGCCTGCCCCCTCC**CAT**GGGCAC-GGAC
 CA-C-TGC-CCAGGG-CAC**T**CACCTGCCTGTC-CC**CAT**GGGCACAGGTC
 CCCCCACCGCCTCCACCCACTGCCGCGGGGGGGCCGTTGGGGCCAGGG
 CCCC-**ACT**GCCCTCCTGCCTCTGCTGCAGAGGTGCTGTCAGGACC-AGGG
 CT-GGCGGG**CATGTAA**CCAGGGCTGCTGCTGGGAGCGCGGAGGGGAAGG
 CTTGGCAGGCG**GATGTAA**CCAGGACTGCTGCTGGGAGCGCGGAGGGGAAGG
 GAGCCCCAGCCCTGCTGGGCGGGCCAGGCCCTCCGCGGCTCC---
 GGGCCCCAGTCTGCTGGGCTGGCCAGGCCCTCCGCGGCTCCTTTTG
 --CCTT--CCACTAC-CCAC--CTGC-CCGG-C-AC--CCCCT---CCCC
 GACCTTGTCCTCCTCCAGCTGCTCCCTCCACTGCCCTGGTCCCTC
 AGTGG-T---TGTAAACCCCGGACTCC-CCAAGCCAGCCTCTG--TGT
 AG-GAATCACTG--A-CCAGGACTCGGCCAAACCTA-CCT-TACCTGT
 GCAGC-AGCCCCAGCGGG**TAAG**TCT-CCAAGATGCCCTTGGTGGATT
 GC-GCCAGCCC-AGA-G--**CTAAG**-CTTCATCATGCCTCTGGTTGACTT
METProLeuValAspPh
 CTTCTGCGAGACCTGCTCTAAGCCTTGGCTGGTGGGCTGGTGGGACCAG
 CTTCTGCGAGACCTGCTCCAAGCCCTGGCTGGTGGGCTGGTGGGACCAG
ePheCysGluThrCysSerLysProTrpLeuValGlyTrpTrpAspGln

Figure 4 Alignment of nucleotides from the intron separating human PDE4A exon 6 and exon 7 with the 5' end of the rat PDE4A1 cDNA variants RD1 and RPDE8

Human sequence is shown above the longest rat RD1 sequence (L27062). Identical nucleotides are identified with a vertical dash. The conserved upstream initiation codons and stop codons are depicted in bold. The unique N-terminal 22 amino acids are shown in triple-letter format in italics below the rat RD1 sequence.

[30] that the unique N-terminal region of pde46, which is encoded by exon 1⁴⁶, serves to attenuate catalytic activity. The generation of so-called 'inactive' splice variants from genes is highly unusual and it may be that such species play an important regulatory role. This warrants investigation on the basis that 2el appears to be an authentic PDE4A splice variant.

Identification of PDE4A exons associated with the plasmid TM3

It has been suggested [9] that the human PDE4A cDNA, TM3, might generate a C-terminally truncated catalytically inactive PDE such as was seen for 2el [13]. Here we have identified TM3 sequence in our contig and have been able to identify a unique 5' exon, called 1^{TM3} for this presumptive PDE4A long form. This exon is found 10 kb downstream from exon 1⁴⁶ and is 15 kb upstream from exon 2 (Figures 2 and 3). However, the sequence of the extreme 3' 360 bp of the TM3 cDNA clone bears no resemblance to that of any known PDE. Rather this sequence reflects that of the inverted 3' end of a previously reported [43] cDNA, called pM5, which has similarities to that of members of the collagenase gene family. We suggest that the TM3 clone may be composed of two unrelated cDNAs which became inappropriately ligated during the cloning process.

It thus remains to be seen whether the unique 5' exon of TM3 reflects a novel long PDE4A isoform. If this should be the case, inspection of the cDNAs for both pde46 and TM3 suggests that, although they may share certain exons, TM3 has distinct exons 3 and 6 which appear to be generated through the use of alternative 3' splice acceptor sites.

	RD5'	Exon 7

Human	PWLVGWWDQFKRMLNRELTHLSEMSRSGNQVSEYIStTFL	
G. Pig	PWLVGWWDQFKRMLNRELTHLSEMSRSGNQVSEYIStTFL	
Cat	PWLVGWWDQFKRMLNRELTHLSEMSRSGNQVSEYIStTFL	
Pig	PWLVGWWDQFKRMLNRELTHLSEMSRSGNQVSEYIStTFL	
Rabbit	PWLVGWWDQFKRMLNRELTHLSEMSRSGNQVSEYIStTFL	
Mouse	PWLVGWWDQFKRMLNRELTHLSEMSRSGNQVSEYISnTFL	
Rat	PWLVGWWDQFKRMLNRELTHLSEMSRSGNQVSEYISnTFL	
		Exon 8
Human	DKQnEVeIPSPtMkErEkQqAPRprpsqppPppv-phlQP	
G. Pig	DKQnEVeIPSPtMkdrEpQeaPRqrpcqqlPppv-phlQP	
Cat	DKQnEVeIPSPtMkdrEkQqAPRqrpsqppPppg-pqfQP	
Pig	DKQnEVdIPSPtMkdhEkQqAPRqrpsqppPppg-pqfQP	
Rabbit	DKQnEVeIPSPtSKerE--qtPRqkpsqppPapv-ppfQP	
Mouse	DKQhEVeIPSPt-----PRqrpfqppPpaavqqaQP	
Rat	DKQnEVeIPSPt-----PRqrafqppPpsvlrqsQP	
Human	MsQITGlKklmHsnsLn--nsniPRFGVKtDQeELLAQE	
G. Pig	MsQITGvKrlsHnsgLn--nasiPRFGVKtDQeELLAQE	
Cat	MsQITGlKklmHssLS--dssiPRFGVKtDQeELLAQE	
Pig	MsQITGlKklmHssLSnedssiPRFGVKtDQeELLAQE	
Rabbit	MaQITGvKrpThsL---gdsqiPRFGVKtDQeELLAQE	
Mouse	MsQITGlKklvHtgsLn--tnvPRFGVKtDQeELLAQE	
Rat	MsQITGlKklvHtgsLn--tnvPRFGVKtDQeELLAQE	

Figure 5 Conservation of RD1 across species: comparison of amino acids encoded by RD1 species expressed in the brains of man, guinea pig (G. Pig), cat, pig, rabbit, mouse and rat

RT-PCR of human brain cDNA was carried out using an RD1-specific forward primer, GR99, and a PDE4A-specific reverse primer, GR36 (see the Materials and methods section and Figure 2). For other species the RD1-specific forward primer GR73 was used. The amino acids essential for membrane targeting of RD1 are marked by * above the residues. Completely conserved residues are shown in uppercase letters, and non-identical residues are shown in lowercase letters. Exons are marked with bold arrowed lines and labelled above. These sequences have been deposited in the GenBank with accession numbers U97584-9 inclusive.

Identification of the 5' exon encoding the unique N-terminal region of the human homologue of RD1

To date, no short human PDE4A isoform has been identified. However, the single identified rat short isoform, RD1, provided the first mammalian PDE4 enzyme to be cloned [19]. This isoform is able to interact with and bind to specific intracellular membranes, being found in the Golgi apparatus and also with punctate structures underlying the cell surface plasma membrane [25,27,32,44]. Such specific intracellular targeting is due exclusively to the unique N-terminal region of this protein. NMR analysis of this region [44] has identified two helical structures separated by a mobile hinge region, with membrane association being primarily associated with the second tryptophan-rich helical region. Distribution analysis, by immunoblotting [28], suggests that RD1 is uniquely expressed in brain, where it is differentially expressed between various regions.

However, concerns have been expressed [2] over whether RD1 provides an authentic splice variant because the splice junction that can be inferred from the sequence of RD1 differs from that seen for the splice junction of short-form products of the PDE4B and PDE4D genes [10]. Analyses of the HSPDE4A gene show that within the intron that separates two of the three exons that encode UCR2 (exons 6 and 7) is a 55 bp sequence that encodes

precisely the unique N-terminal region of RD1 (Figures 3, 4 and 5). The location of this 5' exon for RD1 explains why the PDE4A short-form RD1 has a disrupted UCR2. In contrast, UCR2 is intact in the PDE4B and PDE4D short forms because their unique 5' exons are located between exons 5 and 6 (Figures 2 and 3).

Comparison of the rat PDE4B and PDE4D genomic clones [10] with the HSPDE4A gene structure reveals an explanation for the intriguing difference found in their respective short-form cDNA variants. Rat and human PDE4B and PDE4D short forms contain unique 5' ends that are spliced on to the equivalent exon 6. The unique 5' ends of these cDNAs exhibit no similarity to exon 5 of PDE4A or the predicted exon 5 found in the long-form cDNAs of PDE4B and PDE4D. The expression of PDE4D short forms is controlled by alternative promoters [45] located in the intron between exon 5, found in the long-form variants, and exon 6, expressed in both the long and short forms of the human and rat PDE4B and PDE4D genes. Whereas exon 5 and exon 6 of rat PDE4B and rat PDE4D are separated by introns of several kilobase pairs [10], the sequence of the HSPDE4A locus reveals that exon 5 is separated from exon 6 by an intron of only 138 bp (Figures 2 and 3). This effectively precludes the presence of an alternative promoter that would control the expression of 'PDE4A homologues' of the PDE4B and PDE4D short-form variants.

The presence of a conserved 5' splice site that allows in-frame fusion with the downstream exon 7 provides compelling evidence that there is a human homologue of RD1. In addition, comparison (Figure 4) of the sequence directly upstream from this small ORF with the 321 bp of 5' untranslated sequences of *rpde8*, the longest RD1 clone identified to date [34], reveals a remarkable homology between rat and man for a 5' non-coding region. In both these sequences are two upstream initiation codons and two upstream stop codons, which is consistent with RD1 translation being subject to regulation. Also present are two (C-A)_n·(G-T)_n dinucleotide repeats. Further upstream are (C-A)₂₂ and (C-A)₁₀ found 1203 bp and 630 bp respectively upstream from the RD1 5' exon initiation codon. Such repeats form the second most common simple repeat sequence found in the human genome, although they are under-represented on chromosome 19 [46]. In the absence of further 5' exons of RD1, we predict that the C-A repeats are close to the putative RD1 promoter region. The size of the repeat sequence more distal to the RD1 exon makes it highly likely to be polymorphic in the human population.

When RD1 was originally described [19], two further cDNAs, called RD2 and RD3, were reported. RD3 appears to encode an N-terminally truncated species, whereas RD2 has an apparently novel N-terminal region. It has been a matter of conjecture [2] as to whether RD2 represents an authentic splice variant or is a cloning artifact. Certainly no evidence consistent with their expression has been reported. In this study we were unable to find any sequence with similarity to the unique 5' region of RD2. Indeed the unique 133 bp found at the 5' end of RD2 exhibits a high degree of homology (93% identity) within the ORF of the murine homologue of the *Drosophila* retinal degeneration B gene [47]. Of a total of nine single-base changes, seven represent silent third-base changes. We conclude that the sequence found at the extreme 5' end of RD2 represents the rat homologue of the *Dres9/rdgB/mpt1* gene and that the RD2 clone represents the result of ligation of two separate cDNAs during the cloning process. The absence from the human PDE4A gene locus of sequence with homology to the unique sequence with the RD3 cDNA indicates that this species also probably represents a cloning artifact.

Figure 6 Expression of hRD1 in COS-7 cells

In (a) COS-7 cells were transfected so as to transiently express hRD1. Cells were then disrupted and separated into P1 (lane 2), P2 (lane 3) and supernatant (cytosol) fractions (lane 4) from the pCMV-hRD1-transfected cells. A 5 µg portion of protein was applied for the P2 fraction, 20 µg for the P1 and cytosol fractions and 20 µg of the homogenate from the 'mock' transfected cells. An immunoblot analysis of the homogenate from mock (vector only)-transfected COS-7 cells is also shown (lane 1). Fractions were subjected to SDS/PAGE with immunoblotting using a human PDE4A-specific antiserum [30]. In all fractions a single major band of about 83 kDa was observed. In (b) a plasmid encoding hRD1 was used in an *in vitro* transcription-translation system using [³⁵S]methionine to produce radiolabelled protein. SDS/PAGE analysis of this and subsequent detection using a PhosphorImager identified a single species of about 83 kDa (lane 1). hRD1 produced in this way was incubated with a cell pellet fraction and then subjected to centrifugation in order to isolate a treated supernatant (lane 2) and a treated pellet fraction (lane 3). These data show typical experiments performed three times.

Identification of RD1 homologues in other species

To provide further support for the authenticity of RD1, we carried out RT-PCR and sequence analyses on brain-derived cDNA from human, mouse, rabbit, guinea pig, cat and pig (Figure 5). We focused on the seven-residue region that has been shown [44] to be essential for membrane association of this isoform. Thus, with the use of an RD1-specific forward primer and a PDE4A-specific antisense primer that is complementary to sequence in exon 9, RT-PCR allowed the amplification of distinct cDNA fragments which were subsequently cloned and subjected to sequence analysis. Analysis of the cDNA clones derived from human brain confirmed that the RD1-homologous sequences found in the human PDE4A genomic clones are indeed expressed in human brain (Figure 5). Moreover, translation of the composite cDNA sequences for each species, derived from analysis of six individual clones per species, revealed (Figure 5) that they all appeared to express RD1 homologues and that the region shown [44] to be essential for membrane targeting of the protein encoded by RD1, namely Pro¹⁴-Trp²⁰, is conserved in all species analysed.

Expression studies of hRD1

The human homologue of RD1, called here hRD1, was engineered for expression in mammalian cells. Transfection of COS-7 cells with pCMV-hRD1 led to a profound increase in PDE activity. This was evident (Figure 6) on analysis of subcellular fractions, with activity in the P1 'low-speed' pellet, P2 'high-speed' pellet and high-speed supernatant fractions being 0.9 ± 0.2 , 12.1 ± 0.8 and 0.8 ± 0.1 nmol of cAMP hydrolysed/min per mg of protein respectively in pCMV-hRD1-transfected cells. These can be compared with values of 0.027 ± 0.005 , 0.019 ± 0.004 and 0.050 ± 0.009 nmol of cAMP hydrolysed/min per mg of protein in mock (vector only)

Figure 7 Properties of hRD1 expressed in COS-7 cells

Top, P2 fraction from hRD1-transfected COS-7 cells was incubated for the indicated times at 55 °C before being placed on ice and subsequently assayed for cAMP PDE activity. The logarithm of the percentage residual activity is plotted as a function of time. A linear result, indicative of a single exponential decay, was observed. The broken line indicates the decay plot observed previously [27] for rat RD1 when treated similarly. Bottom, hRD1 in the P2 fraction was assayed at 1 μ M cAMP in the presence of increasing concentrations of the PDE4-selective inhibitor rolipram. Activity is plotted as a percentage of that determined in the absence of added inhibitor. These data show typical experiments performed three times.

transfected cells (means $\pm$ S.D.; $n = 5$). The observed increases in PDE activity were entirely ($> 95\%$) inhibited by the PDE4-selective inhibitor rolipram (10 μ M). The highest specific activity noted was in the P2 fraction, being increased some 637-fold over that of the mock-transfected cells. In contrast, increases were only 33- and 16-fold for the P1 and supernatant fractions respectively. Distribution analysis of hRD1, on either an activity basis or by immunodetection using an HSPDE4A-specific antiserum [30], showed that $22 \pm 6\%$ was located in the P1 fraction, $63 \pm 8\%$ in the P2 fraction and $15 \pm 5\%$ in the supernatant fraction (means $\pm$ S.D.; $n = 5$). Thus it appears that hRD1 is preferentially localized in the P2 fraction, indicating that it does

not interact non-specifically with all intracellular membranes but is targeted. Presumably this feature has functional importance in establishing the demonstrated compartmentalization of cAMP signalling that occurs in many cell types [6]. The membrane association of hRD1 is consistent with studies [28,48] on rat RD1 in which the major fraction of this isoform was also associated with the particulate fraction in transfected COS cells.

Using a human PDE4A-specific antiserum [30], immunoblotting analysis (Figure 6) of COS-7 cells transfected with pCMV-hRD1 identified a novel immunoreactive species of 83 ± 1 kDa (mean $\pm$ S.D.; $n = 5$). The size of this species is considerably greater than the 72 kDa predicted from the amino acid sequence. Indeed, a protein of a similar size, 81 ± 3 kDa (mean $\pm$ S.D.), was also identified when hRD1 was synthesized *in vitro* using a coupled transcription-translation system (Figure 6), indicating that the lowered mobility on SDS/PAGE was probably a property of the protein *per se* rather than any post-translation modification. Similar aberrant migration on SDS/PAGE appears to be characteristic of PDE4 isoforms and may reflect the conserved poly(acidic regions) found towards the C-terminus of the catalytic region of these proteins [3].

As shown [28] with rat RD1, treatment of the particulate hRD1 with the detergent Triton X-100 (0.5–5%) led to the release of all the enzyme ($> 96\%$; $n = 3$), whereas treatment with 1 M NaCl did not release enzyme ($< 5\%$; $n = 3$), as assessed by both analysis of PDE activity and identification of immunoreactive hRD1 on SDS/PAGE (results not shown). This indicates that the interaction of hRD1 with its membrane anchor is not simply by charge but that hydrophobic interactions are needed. This is consistent with structural and mutational studies on RD1 that indicate that the membrane-interacting domain in the N-terminal region of this protein, and which is encoded by the exon RD1-5' (Figure 2), is highly hydrophobic [44]. Membrane interaction appears to be a post-translational event involving binding to an as yet unknown membrane-bound anchor [32,44]. Thus chimaeras of normally soluble proteins made with the unique N-terminal region of RD1 now become membrane associated in a similar fashion to RD1 [32]. Furthermore, such chimaeras, generated as mature species using an *in vitro* transcription-translation system, possess the ability to interact with membranes [32,44]. We show here (Figure 6) that hRD1, produced as a mature protein in such a system, was similarly able to bind to cell membranes. Thus mature hRD1 possesses an inherent ability to associate with membranes.

Thermal denaturation of proteins occurs as a first-order process which is defined as a single exponential. Thus for enzymes, semi-log plots of the residual activity against time will be linear and can be used to derive half-lives [27]. Such analyses offer a simple way of ascertaining whether the human and rat RD1 forms are conformationally distinct. Treated at 55 °C, membrane-bound hRD1 activity decayed with linear semi-log plots (Figure 7) from which a half-life of 31 ± 2 min was derived (mean $\pm$ S.D.; $n = 3$). In contrast, we have shown previously [27] that rat RD1 activity was much more thermolabile at 55 °C, exhibiting a half-life of about 4 min. The basis for this may be differences between the rat and human homologues which are predominantly restricted to LR2 encoded by exon 8 and the C-terminal region encoded by exon 15 (Figure 3).

The K_m values for cAMP hydrolysis by particulate (P2 bound) hRD1 was 1.5 ± 0.5 μ M (means $\pm$ S.E.M.; $n = 3$) which is comparable with that of 4.1 ± 2.3 μ M observed for rat RD1 [27]. The PDE4-selective inhibitor, rolipram, can act as both an anti-depressant and an anti-inflammatory agent [7]. Assayed at 1 μ M cAMP, it inhibited particulate (P2 pellet) hRD1 in a dose-dependent fashion (Figure 7, bottom panel) with an IC_{50} value of

$0.29 \pm 0.09 \mu\text{M}$ (mean $\pm$ S.D.; $n = 5$). This is slightly lower than the IC_{50} value of $0.7 \mu\text{M}$ observed [27] for rat RD1.

It would seem from these studies that the rat and human RD1 homologues are very similar with regard to their intracellular location, sensitivity to rolipram inhibition and K_m for cAMP. Nevertheless, they are markedly different with regard to their thermostability. This implies that there may be conformational differences between these two proteins outside of the active site. It will be of interest to see whether these formulate into differences in regulation and turnover of these homologues.

Conclusion

The complex gene structure of HSPDE4A allows the generation of isoforms with functionally distinct N-terminal regions. These exhibit distinct regulatory and targeting properties which may underpin the notion that cAMP signalling is compartmentalized in many cells and that this is tailored in a cell-type-specific fashion [6]. Here we have provided the first description of the gene structure of a long PDE4 isoform. A contig containing the human PDE4A gene has been identified and mapped to a precise location in chromosome 19p13.2. The definition of the intron/exon boundaries should provide a unique resource for studying the possible involvement of this gene locus in genetic disorders. Given that inhibitors of PDE4 enzymes serve as potent anti-inflammatory agents and also have anti-depressant properties [3,7,8], it seems probable that disruption of PDE4 enzyme exons would give distinct phenotypes.

Here we have shown that the rodent PDE4A short isoform RD1 not only has a human homologue, which we have characterized, but that the site of alternative splicing that produces short PDE4A forms is distinct from that which produces short isoforms from the PDE4B and PDE4D genes. Thus short PDE4A isoforms have a truncated UCR2, whereas this remains intact for PDE4B and PDE4D short isoforms. Encoded within the unique 5' exon of RD1 is a site that has been defined [44] as being essential for the intracellular targeting of this species. We show here that this region is absolutely conserved for RD1 homologues found in a number of different mammalian species, implying the importance of this in the function of this short PDE4 isoform.

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