cAMP-specific PDE4 phosphodiesterases and AIP in the pathogenesis of pituitary tumors

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Abstract

PDE4 cyclic nucleotide phosphodiesterases regulate cAMP abundance in cells and therefore regulate numerous processes, including cell growth and differentiation. The rat PDE4A5 isoform (human homolog PDE4A4) interacts with the AIP protein (also called XAP2 or ARA-9). Germline mutations in *AIP* occur in approximately 20% of patients with Familial Isolated Pituitary Adenoma (FIPA) and 20% of childhood-onset simplex somatotroph adenomas. We therefore examined the protein expression of PDE4A4 and the closely related isoform PDE4A8 in normal human pituitary tissue and in pituitary adenomas. PDE4A4 had low expression in normal pituitary but was significantly overexpressed in somatotroph, lactotroph, corticotroph and clinically nonfunctioning gonadotroph adenomas ($P < 0.0001$ for all subtypes). Likewise, PDE4A8 was expressed in normal pituitary and was also significantly overexpressed in the adenoma subtypes ($P < 0.0001$ for all). Among the different adenoma subtypes, corticotroph and lactotroph adenomas were the highest and lowest expressed for PDE4A4, respectively, whereas the opposite was observed for PDE4A8. Naturally occurring oncogenic variants in *AIP* were shown by a two-hybrid assay to disrupt the ability of *AIP* to interact with PDE4A5. A reverse two-hybrid screen identified numerous additional variants in the tetratricopeptide repeat (TPR) region of *AIP* that also disrupted its ability to interact with PDE4A5. The expression of PDE4A4 and PDE4A8 in normal pituitary, their increased expression in adenomatous pituitary cells where *AIP* is meant to participate, and the disruption of the PDE4A4–*AIP* interaction by *AIP* mutants may play a role in pituitary tumorigenesis.

Key Words

- ▶ pituitary adenomas
- ▶ phosphodiesterases
- ▶ FIPA
- ▶ AIP
- ▶ cAMP
- ▶ PKA
- ▶ PDE4A8
- ▶ PDE4A4
- ▶ PDE4A5

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Introduction

The PDE4 enzymes are members of a large superfamily of cyclic nucleotide phosphodiesterases (PDEs) that enzymatically break down the ‘second messengers’ cAMP

and/or cGMP and therefore regulate their abundance in cells (Bolger *et al.* 2007, Francis *et al.* 2011). cAMP signaling pathways, working through spatially distinct

subpopulations of the cAMP effectors, the cAMP-dependent protein kinase A (PKA) and the exchange protein activated by cAMP (Epac), differentially regulate a large range of distinct intracellular processes (Francis *et al.* 2011, Maurice *et al.* 2014). PDE4 enzymes are differentiated from other members of the PDE superfamily (PDE1 through PDE11, respectively), by their specificity for cAMP and by their ability to be inhibited by a class of highly selective inhibitors.

There are at least 20 different PDE4 isoforms in humans (Bolger 2007), which differ in their structure, pattern of expression in tissues and interactions with specific proteins. They are encoded by four genes (*PDE4A*, *PDE4B*, *PDE4C*, and *PDE4D*), with additional diversity produced by alternative mRNA splicing and the use of promoters unique to each isoform (Bolger 2007, Bolger *et al.* 2007). PDE4A5 (known as PDE4A4 in humans) is a well-studied PDE4 isoform that interacts with several proteins and has a number of characteristic biochemical properties (Bolger *et al.* 1993, 1994, Christian *et al.* 2010). An important and apparently unique property of rat PDE4A5, compared with other rodent PDE4 isoforms, is that it interacts selectively with the AIP protein, a member of the immunophilin family of proteins (Bolger *et al.* 2003b). The AIP protein interacts with PDE4A5 largely through its tetratricopeptide (TPR) domain (Bolger *et al.* 2003b, Igreja *et al.* 2010, Morgan *et al.* 2012), which is a well-characterized protein–protein interaction domain found on many proteins (Kinoshita *et al.* 1990, Sikorski *et al.* 1990) and which is often disrupted in patients with AIP mutations (Igreja *et al.* 2010). The interaction of AIP with PDE4A5 changes the enzymatic activity of PDE4A5 and also its ability to be inhibited by PDE4-selective inhibitors (Bolger *et al.* 2003b).

Mutations in genes encoding components of cAMP pathways have been implicated in the predisposition to several endocrine neoplasms and related disorders (Stratakis 2013, for a review). Germline mutations in the gene encoding the regulatory subunit type 1- α of PKA (*PRKAR1A*) produce the multiple neoplasia, myxoma and lentiginosis syndrome Carney complex (Horvath *et al.* 2010). Germline mutations in the PKA catalytic subunit can produce adrenal cortical adenomas (PRKACA), (Beuschlein *et al.* 2014) or Carney complex (PRKACB) (Forlino *et al.* 2014). Mosaic somatic mutations in the gene encoding the G-protein alpha subunit (*GNAS*), which regulates adenylyl cyclase, the enzyme that synthesizes cAMP, cause McCune–Albright syndrome, characterized by fibrous dysplasia of bone, café-au-lait nodules, and precocious puberty (Salpea & Stratakis 2014), whereas

somatic mutations can also be detected in 30–40% of somatotroph adenomas (Landis *et al.* 1989). Recently, microduplications in the G-protein-coupled orphan receptor GPR101, which activates adenylyl cyclase and therefore cAMP signaling, have been implicated in a disorder with very young-onset gigantism (Trivellin *et al.* 2014). These genetic syndromes implicate cAMP signaling in pituitary tumor pathogenesis and provide a rationale for study of additional cAMP signaling components in this process.

Germline mutations in the *AIP* gene are present in approximately 20% of patients with familial isolated pituitary adenoma (FIPA). The *AIP* mutation-positive patients have a characteristic clinical phenotype with usually young- or childhood-onset growth hormone (GH) and/or prolactin (PRL)-secreting adenomas and can be seen in cases with no apparent family history as well (Vierimaa *et al.* 2006, Daly *et al.* 2007, Leontiou *et al.* 2008, Heliovaara *et al.* 2009, Igreja *et al.* 2010, Gadelha *et al.* 2013, Beckers *et al.* 2013). AIP is expressed in the pituitary, and many pathogenic AIP variants block its interaction with PDE4A5 (Vierimaa *et al.* 2006, Leontiou *et al.* 2008, Igreja *et al.* 2010, Bolger *et al.* 2003b). Furthermore, impaired function of AIP in the pituitary attenuates cAMP signaling (Formosa *et al.* 2013, Formosa & Vassallo 2014, Tuominen *et al.* 2015).

Given the role of both cAMP signaling and AIP in the pathogenesis of pituitary tumors, we wished to explore further the potential role of the PDE4A5–AIP interaction in pituitary tumorigenesis. Therefore, an important aim of the current studies was to demonstrate that PDE4A4 is expressed in normal pituitary and in pituitary adenomas, where it is available for interaction with AIP. We had shown previously that mRNA encoding PDE4A4 and its rat/mouse ortholog, PDE4A5, is expressed in a number of tissues, including the brain (Bolger *et al.* 1994, 1996), but there was no data on its expression in the pituitary. We have been particularly interested in the tissue-specific expression of PDE4A8, an isoform encoded by the *PDE4A* gene that has undergone rapid evolutionary change (Mackenzie *et al.* 2008). Human PDE4A8 differs from its rodent counterpart almost exclusively at its alternatively spliced amino-terminus and related 5' genomic sequences, which are likely to control its tissue expression (Bolger *et al.* 1996, Mackenzie *et al.* 2008). Here we study PDE4A4 and PDE4A8 protein expression in normal pituitary and in pituitary adenomas using novel specific antibodies. We also identify additional variants in AIP that affect its ability to interact with PDE4A5.

Materials and methods

Tissue specimens

Samples from pituitary adenomas were removed at transsphenoidal surgery. The tumor type was determined on the basis of clinical and biochemical findings before surgery and immunohistochemical determination of the expressed pituitary hormones: for the adenoma samples, all slides utilized showed more than 90% of the specific tumor, as confirmed by hematoxylin and eosin. Autopsy pituitary samples were collected within 24 h of death from patients with no evidence of any endocrine abnormality. Consecutive cuts were prepared for histological analysis from normal pituitary tissue and different types of pituitary adenomas. Informed consent was obtained from all pituitary patients, and the protocol was approved by the Institutional Research Ethics Committee.

PDE4 antibodies

The mouse monoclonal antibody 4A4-1, specific to human PDE4A4, was generated specifically for this study against the peptide sequence SAERAERERQPHRPIERADA (Fig. 1), corresponding to the unique amino-terminal region of PDE4A4 amino acids 51 through 70 in both human PDE4A4 (GenBank Accession number L20965.1) and rat PDE4A5 (GenBank L27057.1, Fig. 1) (Bolger et al. 1993, 1994, Mackenzie et al. 2008). The rabbit polyclonal

antibody 7233 specific to human PDE4A8 was described previously (Mackenzie et al. 2008). The mouse monoclonal antibody 4E1.16, specific for human PDE4A8, was generated to the same peptide as the polyclonal antibody, of the sequence KGDESRSTPESDRAN, corresponding to the unique amino-terminal region of PDE4A8 (GenBank AY593872, (Mackenzie et al. 2008)). All monoclonal antibodies were purified by limiting dilution and then grown in culture. Antibodies were then purified from culture supernatants with a protein G-affinity matrix (Pierce, Thermo Fisher Scientific).

Immunoblotting

COS7 cells were transfected with the plasmid pcDNA46VSV to express human PDE4A4, or with pcDNA4A8VSV to express human PDE4A8, or with vector pcDNA3 (Life Technologies), using methods that we have described previously (McPhee et al. 1999, Mackenzie et al. 2008, Christian et al. 2010). The proteins were engineered to contain a vesicular stomatitis virus (VSV) epitope at their carboxy terminus. Extracts from the cells were prepared using methods that we have described previously (McPhee et al. 1999, Mackenzie et al. 2008, Christian et al. 2010) and were analyzed by LDS-PAGE (Novex, Life Technologies) and immunoblotting with either an antibody to VSV (Sigma) (Kreis 1986) or the monoclonal antibody of interest.

Human PDE4A4 and Rat PDE4A5, with AIP interaction sites

Figure 1

Human PDE4A4 and rat PDE4A5 with AIP interaction sites. Schematic representation of the human PDE4A4 protein structure. The human PDE4A4 consists of a unique amino-terminal region, upstream conserved regions 1 and 2 (UCR1 and UCR2) separated by linker regions 1 and 2 (LR1 and LR2), a catalytic domain, and the unique carboxyl-terminal region. The domain structure of rat PDE4A5 is identical. The amino acid sequences of the human PDE4A4 and rat PDE4A5 are shown on the specific regions implicated in the interaction of PDE4A5 with AIP: the unique N-terminal region and the EELD motif located at the beginning of UCR2. The middle row of amino acids shows the high identity of these conserved regions between the two species. The sequence SAERAERERQPHRPIERADA was used to generate a new PDE4A5/PDE4A4 antibody.

For this purpose, samples were run in parallel lanes on the same gel and then transferred to a single filter. For immunoblotting, the filter was then cut in half, and each half was incubated with the appropriate antibody; after washing, the two halves were rejoined for image generation with enhanced chemiluminescence (ECL), generating a single image with both antibodies. Primary antibody incubations (1:500 for PDE4A4 and PDE4A8 and 1:5000 for VSV) were performed for 1 h in Tris-buffered saline with 0.1% Tween 20, followed by two washes in the same buffer. Secondary antibody incubations (SC-5099, 1:10,000; Santa Cruz Biotechnology) were performed in the same buffer, followed by two washes in the same buffer. Signal development was with ECL (Pierce, Thermo Fisher Scientific).

Immunofluorescent staining and confocal microscopy

We performed confocal immunofluorescence analysis of somatotroph, corticotroph, lactotroph, and clinically nonfunctioning gonadotroph adenomas as well as normal pituitary tissue. Normal pituitary architecture was evaluated by reticulin staining. After deparaffinization and hydrating procedures, the slides were transferred into 400 mL of prewarmed (94–96°C) target retrieval solution (40 mL of stock into 360 mL water; Dako) in a glass container in a water bath. The sections were incubated in blocking solution (1% BSA and 0.1% Tween 20) at room temperature for 1 h and then incubated overnight at 4°C with one of the primary antibodies. The mouse monoclonal antibody 4A4-1 was used for all PDE4A4 comparisons, whereas the mouse monoclonal 4E1.16 was used for all PDE4A8 comparisons. In order to colocalize the selected proteins in different cells, polyclonal goat anti-GH (L20, 1:50; Santa Cruz Biotechnology), polyclonal goat anti-ACTH (P-12, 1:50; Santa Cruz Biotechnology), polyclonal rabbit anti-follicle-stimulating hormone (FSH) (87461:25; Abcam) or polyclonal mouse anti-FSH (C10, 1:25; Dako), and polyclonal goat anti-PRL (C-17, 1:50; Santa Cruz Biotechnology) or polyclonal rabbit anti-PRL (1:50; Dako), with one of the PDE4A4- or PDE4A8-specific antibodies described previously, were also added to the incubation buffer. After an overnight incubation, slides were rinsed five times in PBS and then incubated for 1 h in the dark at room temperature with anti-goat Alexa 546 (1:100; Life Technologies), anti-rabbit Alexa 546 (1:50, 1:100, or 1:200; Life Technologies), anti-mouse DyLight 488 (1:50, 1:200, or 1:400; Pierce, Thermo Fisher Scientific), anti-goat Alexa 488 (1:100; Life Technologies) or anti-mouse Alexa 546 (1:200; Life Technologies),

according to the primary antibodies previously used. Due to a change of PRL antibody used in our university hospital from goat to rabbit anti-PRL, secondary antibodies used in PRL experiments for the isoform PD4A8 had to be altered in order to reach the expected double labeling. Following washes with PBS, sections were mounted in 90% glycerol/10% Tris 1M, and images were captured through confocal microscope (Leica TCS SP5; Leica), 63× objective, and 630× original magnification. All confocal settings were determined at the beginning of the imaging session and remained unchanged. For quantitative analysis, images were captured at 8 bits and analyzed in gray scale, using the protocol we have published previously (Ribeiro-Oliveira Jr et al. 2008). Briefly, three different normal pituitary samples and four to seven distinct samples of each adenoma subtype were analyzed. Three to four images were captured randomly from each sample and three measurements were obtained for each image, with about 30–50 cells also chosen randomly in each analyzed sample. ImageJ (the National Institutes of Health, Bethesda, MD, USA) software was used to quantify fluorescence intensity and area intensity, as well as the area of each individual cell. The background fluorescence and the energy intensity threshold were then subtracted from the region of interest in the same level for all examined pictures. The relative fluorescence corresponded to the unit 'gray level', varying from 0 (black) to 255 (white), as an average of the area for each cell (the sum of gray value of all pixels divided by the number of pixels/area).

Two-hybrid analysis of protein–protein interactions

The effect of variants in AIP on its interaction with PDE4A5 was studied by the yeast two-hybrid system, as described previously (Bolger et al. 2003b, Leontiou et al. 2008, Igreja et al. 2010). For these studies, PDE4A5 was cloned into the *NotI*-site of pLEXAN, to generate a LexA DNA-binding domain fusion (i.e., the plasmid pLEXAR6). Wild-type (WT) AIP and various missense AIP sequence variants were cloned into the *NotI*-site of pGADN, to generate GAL4 activation domain fusions (i.e., the plasmid pGADNRB3 and mutants thereof). Site-directed mutagenesis was performed using the circular mutagenesis method (Weiner et al. 1994), and all mutants were verified by sequencing before use. Quantitative β -galactosidase assays were performed in the *Saccharomyces cerevisiae* strain L40 by the method of Guarente (Guarente 1983) using O-nitrophenyl- β -D-galactopyranoside as a substrate. Each variant was tested in at least two different yeast clones.

'Reverse' two-hybrid analysis

This was performed essentially as we have described previously for a different interaction (Steele *et al.* 2001). The plasmid pGADNRB3Sma was generated by site-directed mutagenesis of pGADNRB3. It encodes the full open reading frame of AIP, cloned into the *NotI* site of pGADN (Bolger *et al.* 2003b), with a *SmaI* site engineered at codon 168. The generation of this site created a variant (W168G) in AIP that had no effect on its ability to interact with PDE4A5 in two-hybrid assays (data not shown). To mutagenize the AIP TPR homology region (Bolger *et al.* 2003b), PCR primers were synthesized that amplified codons 168–330 of AIP, with some vector sequences at the 3' end of the insert. The PCR product overlapped approximately 30 nucleotides at each end with *SmaI*-cut pGADNRB3Sma DNA. Therefore, cotransformation of *S. cerevisiae* L40 with the PCR product and *SmaI*-cut pGADNRB3Sma1 DNA efficiently generated full-length GADNRB3Sma with PCR-generated AIP TPRs, by the use of recombination *in vivo*. The PCRs were designed to introduce a single-nucleotide substitution, on average, in the PCR product and used 25 U/mL of Taq DNA Polymerase (Thermo Fisher Scientific) in 20 mM Tris–HCl pH 8.4, 50 mM KCl, 5 mM MgCl₂, 0.2 mM dATP, 0.4 mM of each of dCTP, dTTP, and dGTP, 1 µg/mL of pGADNRB3Sma and each PCR primer at 1 nM/mL. Ten separate PCRs, each 100 µL, were performed for 25 cycles with an annealing temperature of 55°C. The PCR products were pooled and 20 µL were mixed with 50 ng of *SmaI*-cut pGADNRB3Sma DNA and transformed into *S. cerevisiae* strain L40 containing pLEXAR6. Colonies containing both plasmids were selected by their ability to grow in the absence of tryptophan and leucine, replica-plated onto nylon filters, and subjected to a filter β-galactosidase assay (Bolger 1998). The pGADNRB3Sma mutant plasmids were isolated from white colonies (i.e., those lacking β-galactosidase activity) by their ability to complement the *leuB*-genotype of *Escherichia coli* strain LB101. The PCR-generated region of the resulting plasmids was then sequenced with an ABI 3700 sequencer (Applied Biosystems).

Characterization of AIP variants

Three public databases were interrogated to check for the allele frequencies of the AIP variants tested in this study: the 1000 Genomes Project (Abecasis *et al.* 2012); the Exome Variant Server (EVS), NHLBI GO Exome Sequencing Project (ESP), Seattle, WA, USA (<http://evs.gs.washington.edu/EVS/>) (accessed on March 2016); and the Exome Aggregation Consortium (ExAC), Cambridge, MA, USA (<http://exac.broadinstitute.org>) (accessed on 1 March 2016)).

Statistical analysis

Variables were first checked for normal distribution through the Shapiro–Wilk test. The immunofluorescence data comparing each adenoma subtype with normal pituitary tissue were tested for statistical significance with the nonparametric Mann–Whitney *U* test, using GraphPad Prism 6.0 (GraphPad). Different adenoma subtypes were compared through Kruskal–Wallis followed by Dunn's multiple comparisons test. Quantitative β-galactosidase assays were analyzed by unpaired *t*-test (SigmaPlot; Systat, San Diego, CA, USA). Significance was taken as *P* < 0.05. Data are shown as mean ± S.E.M. or S.D.

Results

PDE4A4 is specifically expressed in pituitary adenomas

As the rodent homolog of PDE4A4 interacts with AIP (Bolger *et al.* 2003b), we wished to determine PDE4A4 distribution in normal human pituitary cells and adenomas (Fig. 2). For this study, we developed a monoclonal antibody, 4A4-1, specific for PDE4A4 (and also PDE4A5). Immunoblotting of protein extracted from cells transfected with PDE4A4 shows a band with a relative molecular mobility of 125 ± 3 kDa, as shown previously (Mackenzie *et al.* 2008). Immunofluorescence of normal pituitary showed that PDE4A4 is present in GH and FSH cells, with very low expression in PRL or ACTH cells. However, we detected a significantly higher expression of PDE4A4 in GH-, PRL- or ACTH-secreting pituitary adenomas, as well as FSH-positive non-functioning pituitary adenomas (NFPAs), compared with the corresponding cell type in normal pituitary (Fig. 2). By contrast, we did not see any expression in fat tissue, which was used here as a negative control.

PDE4A8 is expressed in normal pituitary cells and adenomas

We had previously developed a polyclonal antibody to PDE4A8 and used it to demonstrate its expression in several regions of the CNS (Mackenzie *et al.* 2008). For this study, we have developed a monoclonal antibody, 4E1.16, which has the same specificity as the polyclonal antibody (see 'Materials and methods' section for details) and used immunofluorescence to determine its expression in pituitary tissue (Fig. 3). The 4E1.16 antibody detected a protein of the correct size on immunoblotting of cells transfected to express PDE4A8.

Figure 2

Localization of PDE4A4 in the pituitary. (A) Immunofluorescence with the 4A4-1 antibody in fat tissue, as a negative control. (B) Immunoblotting with the 4A4-1 antibody. COS7 cells were transfected to express human PDE4A4 (4A4) or vector (Vect). All PDE constructs also encoded a VSV epitope at the carboxyl-terminus of the protein. LDS-PAGE and immunoblotting with an antibody against VSV, or with 4A4-1, were performed as described in the 'Materials and methods' section. The PDE4A4 protein migrated at 97 kDa. (C, D, E and F) Double immunofluorescent staining using monoclonal 4A4-1 (green staining) and polyclonal GH, ACTH, FSH, or PRL (red staining) antibodies in normal pituitary ($N=3$), and in GH-, ACTH-, FSH- (NFPA), and PRL-secreting adenomas ($N=4-7$ for each adenoma subtype; C, D, E, and F, respectively). The bar graphs to the right of each panel provide semiquantitation of the relative fluorescence of PDE4A4 in each adenoma type, compared with its respective normal cell. Scale bar = 25 μ m. All data are shown as mean $\pm$ S.E.M.; AU, arbitrary units; * $P < 0.0001$.

Immunofluorescence of normal pituitary showed that PDE4A8 was present in all cell types, with somewhat lower expression in ACTH cells. In pituitary adenomas, PDE4A8 was expressed at significantly higher levels, in all subtypes (Fig. 3). We did not see any expression of PDE4A8 with this antibody in control tissues, such as fat tissue, consistent with results obtained previously with the polyclonal antibody (Mackenzie et al. 2008).

Differences among different adenoma subtypes for PDE4A4 and PDE4A8

When different adenoma subtypes were compared for PDE4A4 (Fig. 4A), this isoform was significantly reduced in prolactinomas compared with the other adenoma subtypes (Fig. 4A). However, PDE4A4 abundance in corticotrophinomas was significantly

Figure 3

Localization of PDE4A8 in the pituitary. (A) Immunofluorescence with the 4E1.16 antibody in fat tissue, as a negative control. (B) Immunoblotting with the 4E1.16 antibody. Extracts from COS7 cells transfected to express PDE4A8-VSV or with vector (Vect) were immunoblotted with an antibody against VSV or with 4E1.16. On separate gels, PDE4A8-VSV extracts were immunoblotted with 4E1.16 and the peptide used as immunogen (P1), or an unrelated peptide (P2). All methods were otherwise as for Fig. 2B. The PDE4A8 protein migrated at 102 kDa. (C, D and E) Double immunofluorescent staining using monoclonal 4E1.16 (red staining) and polyclonal GH, ACTH, and FSH (green staining) antibodies in normal pituitary ($N=3$) and GH- and ACTH-secreting adenomas and NFPAs ($N=4-7$ for each adenoma subtype; C, D, and E, respectively). (F) Double immunofluorescent staining using monoclonal 4E1.16 (green staining) and polyclonal PRL (red staining) in normal pituitary ($N=3$) and PRL-secreting adenomas ($N=5$). The bar graphs to the right of each panel provide quantitation of the relative fluorescence of PDE4A8 in each adenoma type, compared with its respective normal cell. Scale bar = 75 μm . All data are shown as mean $\pm$ S.E.M.; AU, arbitrary units; * $P < 0.0001$.

increased compared with the other adenoma subtypes (Fig. 4A).

For the PDE4A8 isoform, in turn, prolactinomas showed significantly higher PDE4A8 expression than the other adenoma subtypes (Fig. 4B), whereas corticotrophinomas showed the lowest abundance of PDE4A8 (Fig. 4B).

Disease-associated variants in AIP attenuate its interaction with PDE4A5

AIP interacts specifically with PDE4A5 (Bolger *et al.* 2003b) and mutations in AIP that are associated with FIPA attenuate this interaction (Leontiou *et al.* 2008, Igreja *et al.* 2010). A yeast two-hybrid quantitative β -galactosidase assay can rapidly and conveniently assess the effects of

Figure 4

Comparison of PDE4A4 and PDE4A8 immunostaining in pituitary adenomas. (A) PDE4A4 expression in pituitary adenoma subtypes. All data are shown as mean $\pm$ S.E.M.; AU, arbitrary units; * $P < 0.0001$ compared with corticotroph adenomas and * $P < 0.0001$ compared with lactotroph adenomas. (B) PDE4A8 expression in pituitary adenoma subtypes. All data are shown as mean $\pm$ S.E.M.; AU, arbitrary units; * $P < 0.0001$ compared with corticotroph adenomas and * $P < 0.0001$ compared with lactotroph adenomas.

AIP variants on its interaction with PDE4A5, which in turn can inform about the possible pathogenicity of AIP variants. In this study, we analyzed/reanalyzed a large number of disease-associated AIP variants, including six variants that we have not reported with this assay previously (Leontiou et al. 2008, Igreja et al. 2010),

and found that attenuation of interaction generally correlated with the disease phenotype (Fig. 5). The new and previously studied single amino acid substitutions or deletions (C238Y, R271W, Y248del, and V291M) and the truncation mutations (R81*, Q164*, Q217*, and R304*), all of which are strongly disease associated, completely ablate the interaction (Fig. 5). We also saw ablation, but with borderline statistical significance, by the K241E and R304Q missense variants, which are also felt to be disease associated. The I257V and A299V variants, which are predicted to be unlikely to be pathogenic and have uncertain clinical significance, showed no clear difference. Conversely, the R16H and V49M missense variants, which are generally considered to be rare SNPs (Igreja et al. 2010), and are located outside the TPRs, have no effect, or have only a weak effect on the interaction (Fig. 5). Two apparently disease-causing variants, K103R and Δ G23/E24, have no clear inhibitory effect or may augment the interaction, as measured by this assay (Fig. 5). K103R is, however, a conservative substitution, and both K103R and Δ G23/E24 are located outside the TPRs, which are considered to mediate its interaction with PDE4A5 (Table 1).

Isolation of additional variants in the AIP TPRs

The AIP TPRs form a close association with two different regions of PDE4A5 (Bolger et al. 2003b). Given this close association, it seems unlikely that all the amino acids in the TPRs that are essential for this interaction have been identified in the analyses performed to date. These amino acids would also be potential sites of disease-associated variants. To identify such amino acids, we performed random mutagenesis of the AIP TPRs and then used two-hybrid approaches to select and identify variants that attenuated the AIP–PDE4A5 interaction. This approach, typically called ‘reverse two-hybrid’, has successfully been used to characterize many protein–protein interactions. With this screen, we identified seven single amino acid variants (K175E, C208R, N236H, C254R, H275L, L306P and L309P) and one double variant (W223R/V250A) in the AIP TPRs that strongly attenuated their ability to interact with PDE4A5 (Fig. 5). Intriguingly, one of these variants, C254R, has been recently identified as a disease-associated mutation in a patient with gigantism: this female patient was diagnosed at the age of 14 years due to tall stature (184 cm, midparental height 161.5 cm) and primary amenorrhea and had a large (46 $\times$ 44 $\times$ 31 mm) GH- and PRL-secreting macroadenomas causing hydrocephalus. Insulin-like growth factor 1 (IGF-1) was 5 $\times$ and PRL was

Figure 5

(A) Yeast two-hybrid quantitative β -galactosidase assay of human AIP mutants. AIP mutants were expressed in yeast cells, and their effect on AIP interaction with PDE4A5 was measured with a yeast two-hybrid quantitative β -galactosidase assay. Variants not studied previously are indicated in bold type in this figure legend; selected previously studied variants are included for comparison. A significant reduction in β -galactosidase activity (0–30% of WT; $P < 0.03$ by t -test) was observed for missense variants C238Y, R271W, **V291M**, and **Y248del**, as well as for all the truncation mutations tested (**R81***, **Q217***, Q164*, and **R304***). The K241E and R304Q mutants were of borderline significance ($P = 0.062$). The R16H, V49M, K103R, I257V, and A299V variants show activity 33–100% of WT (less than three-fold difference; $P =$ not significant (NS)) from WT AIP. Substantial increased activity is seen with the **G23/E24** deletion ($P = 0.011$). Each variant was tested in at least two different yeast clones, with identical results ($N = 3$ for each clone). Mean $\pm$ s.d. are shown for each mutant. Vector+4A5: PDE4A5 with the AD vector with no insert; Vectors X 2: both vectors with no insert. (B) Yeast two-hybrid quantitative β -galactosidase assay of AIP mutants identified in a reverse two-hybrid screen. AIP mutants identified in a reverse two-hybrid screen were expressed in yeast cells, and their effect on AIPs interaction with PDE4A5 was measured with a yeast two-hybrid quantitative β -galactosidase assay, as in panel A. All mutants showed a significant reduction in β -galactosidase activity (0–30% of WT; $P < 0.03$ by t -test). Each variant was tested in at least two different yeast clones, with identical results ($N = 3$ for each clone). Mean $\pm$ s.d. are shown for each mutant. Vector: PDE4A5 with the AD vector with no insert.

150 $\times$ upper limit of normal (Hernandez-Ramirez et al. 2015b, Iacovazzo et al. 2015).

Because of technical issues with the two-hybrid system, in that PDE4A8 tends to have intrinsic transactivation activity, detectable in several different two-hybrid vector/host systems, we have been unable to assess the effects of AIP variants on its interaction, if any, with PDE4A8.

Discussion

PDE4s play an essential role in cAMP signaling in many organs and tissues (Bolger et al. 2007, Conti & Beavo 2007, Houslay 2010, Francis et al. 2011, Maurice et al. 2014), in that they catalyze the breakdown of cAMP to AMP and therefore contribute to the regulation of cAMP levels in cells. cAMP is generated by adenylyl cyclases, which, in majority of cell types, including pituitary, are regulated by several stimuli, including G-protein-coupled receptors (GPCRs), calcium fluxes, and phosphorylation by a number of kinases (Willoughby & Cooper 2007). cAMP activates two downstream effectors, PKA and Epac, and PKA phosphorylates numerous substrates, which in turn reflect its protean physiologic functions in cells. GPCRs, cyclases, PKA, and PDEs are often found to be physically colocalized in cells and therefore regulate cAMP levels in specific subcellular compartments (Conti & Beavo 2007, Bolger et al. 2007, Willoughby & Cooper 2007, Houslay 2010, Francis et al. 2011, Maurice et al. 2014). The presence and/or activity of PDE4 isoforms in these compartments may be regulated, at least in part, by specific protein–protein interactions; for example, the PDE4D5 isoform interacts selectively with RACK1 and β -arrestin2 (Yarwood et al. 1999, Perry et al. 2002, Bolger et al. 2003a) and the PDE4A5 isoform (called PDE4A4 in humans) selectively interacts with AIP (Bolger et al. 2003b). The expression of cAMP signaling pathway components, including the PDE4s, varies substantially between tissues, thereby producing an additional level of specificity of action.

Overexpression of WT AIP in rat GH3 and human HeLa cells was shown to reduce forskolin-induced (but not basal) cAMP signaling (Formosa et al. 2013). This effect was observed even in the presence of the general PDE inhibitor IBMX or the selective PDE4 inhibitor rolipram, suggesting that the AIP–PDE interaction is either not required or only partially responsible for the reduction in forskolin-activated cAMP signaling by WT AIP. This may be explained by the fact that AIP, in addition to PDE, interacts with several other proteins. Conversely, knockdown of endogenous AIP resulted in increased cAMP

Table 1 Reported *AIP* variants tested in this study.

DNA change	Protein change	SNP id	Control MAF – EVS	Control MAF – ExAC	Control MAF – 1000 Genomes Project
c.47G>A	p.R16H	rs145047094	0.254	0.1956	0.1
c.66_71del	p.G23_E24del	rs267606567	NO	NO	NO
c.145G>A	p.V49M	rs1063385	0.0154	0.02395	NO
c.241C>T	p.R81*	rs267606541	NO	NO	NO
c.308A>G	p.K103R	rs267606548	NO	NO	NO
c.490C>T	p.Q164*	rs104895073	NO	NO	NO
c.649C>T	p.Q217*	rs267606566	NO	NO	NO
c.713G>A	p.C238Y	rs267606569	NO	8.42e–04	NO
c.721A>G	p.K241E	rs267606573	NO	4.212e–03	NO
c.742_744del	p.Y248del	rs267606574	NO	NO	NO
c.760T>C	p.C254R	NA	NO	NO	NO
c.769A>G	p.I257V	rs267606575	NO	NO	NO
c.811C>T	p.R271W	rs267606579	NO	NO	NO
c.871G>A	p.V291M	NA	NO	NO	NO
c.896C>T	p.A299V	rs148986773	0.1002	0.04275	NA
c.910C>T	p.R304*	rs104894195	NO	1.715e-03	NO
c.911G>A	p.R304Q	rs104894190	0.0693	0.1458	NO

The allelic frequencies identified in controls in three public online databases (EVS, ExAC, and 1000 Genomes Project) are shown. MAF frequencies are expressed in percentage. MAF, minor allele frequency; NO, not observed; NA, not applicable.

signaling even in basal conditions (Formosa *et al.* 2013). Another mechanism suggested for AIP–cAMP interactions via the inhibitory G protein, G α -i2, as this inhibitory protein had lower level in AIP-deficient cells and human samples, suggesting that failure to inhibit cAMP synthesis through dysfunctional G α -i signaling underlies the development of GH-secreting pituitary adenomas in AIP mutation carriers.

In this study, we show that PDE4A4 and PDE4A8 proteins are expressed in human pituitary cells and that their expression is increased in all studied adenoma subtypes. Although prior studies had demonstrated that mRNA transcripts from the *PDE4A*, *PDE4B*, and *PDE4D* genes were present in pituitary cells and adenomas (Persani *et al.* 2001), these studies did not use immunohistochemistry or immunofluorescence to determine the precise cellular distribution of the isoforms. Interestingly, although PDE4A4 and PDE4A8 are expressed in normal pituitary, they are expressed at higher levels in four different adenoma subtypes. Although the mechanistic effect(s) of this overexpression in adenomas will require further study, this level of PDE4 expression is highly likely to downregulate cAMP signaling pathways and therefore be functionally important.

Whereas differences in normal pituitary tissues obtained through consecutive cuts are expected due to the heterogeneous admixture of several different cell types, adenomas tend to be more uniform as they represent an expansion of a single neoplastic cell representing usually at least 90% of the adenoma

cells. Therefore, we have compared the PDE isoform expression of the different pituitary adenoma subtypes. Whereas PDE4A4 was expressed lower in tumor PRL cells and higher in tumor ACTH cells, the opposite was observed for PDE4A8 isoform. Although these observed results are quite of unknown origin, the differences between isoforms might be related to the evolutionary change from one isoform to the other (Mackenzie *et al.* 2008). We note that as we had to use a different secondary antibody for the prolactinomas for the PDE4A8 staining, this might have influenced the comparisons.

AIP mutations that predispose to adenomas also impair AIP's ability to interact with PDE4A5 (Leontiou *et al.* 2008, Igreja *et al.* 2010). However, AIP interacts with a number of proteins, including survivin and RET (Vargiolu *et al.* 2009, Kang *et al.* 2011), both important growth regulatory proteins, with the aryl hydrocarbon receptor (Carver & Bradfield 1997) and with cAMP signaling pathway members G α -2 and G α -3 (Tuominen *et al.* 2015) or PRKAR1A (Schemthaler-Reiter *et al.* 2014), as well as various chaperones, such as Hsp90 and Hsp70. AIP has also been described as interacting with a different PDE, specifically PDE2A; however, the biochemical and physiologic implications of this observation remain uncertain (de Oliveira *et al.* 2007, de Oliveira & Smolenski 2009). Therefore, we need to explore further the potential role of the PDE4A4/5–AIP interaction in the pathogenesis of pituitary tumors. It is possible that PDE4A4/5 in particular, in view of its

selective interaction with AIP, plays an important role in AIP's tumor suppressor effect; however, it is also possible that increased expression of PDE4 isoforms could be a cellular response to the pro-proliferative effects of AIP loss in pituitary adenoma cells. Finally, it is possible that PDE4A4/5 and other PDE4 isoforms play a role in pituitary processes other than adenoma formation.

Given that oncogenic mutations in *AIP* attenuate its interaction with PDE4A5, it is possible that PDE4A protein isoforms have important functional roles in pituitary and pituitary adenomas. We have shown previously that AIP inhibits the enzymatic activity of PDE4A5 and therefore acts to increase cAMP levels and activate PKA. Therefore, loss of AIP function should increase the activity of PDE4A4/5, lower cAMP levels, and downregulate PKA. Given that activation, rather than inactivation, of PKA predisposes to somatotroph adenomas ('Introduction' for details), it is most likely that the effect of the AIP mutants on PDE4A4/5 is to generate a compensatory response to tumorigenesis, rather than to promote tumorigenesis. AIP may support the function of inhibitory G-protein $G_{\alpha i-2}$ (Tuominen *et al.* 2015), and the lack of AIP leads to reduced $G_{\alpha i-2}$ function, increased adenylyl cyclase activity, and therefore higher cAMP levels. Overactivation of adenylyl cyclase due to *gsp* mutation, however, is known to produce overexpression of PDE4 as a possible compensatory process (Peverelli *et al.* 2014). Interaction of AIP and PDE4A4/5 may play a role in this process. However, further functional experiments will be needed to assess this possibility. Interestingly, AIP mutations do not associate with *gsp* mutations in human adenomas (Hernandez-Ramirez *et al.* 2015a).

In this study, we assay a number of newly identified AIP variants and show that almost all of them attenuate their interaction with PDE4A5. This is consistent with these variants having an important effect on the biochemical activity of PDE4A5. However, potential anomalies remain, in that two variants deemed pathogenic based on clinical data, K103R and Δ G23/E24, have no clear inhibitory effect, or may augment the interaction, as measured by our assay. K103R is a conservative substitution, and both K103R and Δ G23/E24 are located outside the TPRs, which are considered to mediate AIP protein's interaction with PDE4A5. It is possible that these mutations might destabilize the AIP protein generally in mammalian cells, as we have shown for several disease-causing missense variants (Hernandez-Ramirez *et al.* 2015b) or have other effects on AIP and/or PDE4A4/5, which might not be detected by our assay. Further

functional studies might provide additional insights into the apparent aberrant effects of these mutants.

Our study has several limitations. Although there is high homology between rat PDE4A5 and human PDE4A4, the human PDE4A4 protein has not been previously shown to interact with AIP. The number of human samples assessed for these protein expressions needs to be increased in the future.

In summary, our data suggest, to our knowledge for the first time, that PDE4A4 and PDE4A8 are expressed in normal human pituitary cells and are overexpressed in all studied pituitary adenoma subtypes. Differences among different adenoma subtypes for PDE4A4 and PDE4A8 isoforms might be related to the rapid evolutionary change from one isoform to the other. We have also tested new AIP mutants for their ability to disrupt the PDE4A5–AIP interaction. Our results suggest a number of further studies. We have yet to evaluate the AIP content, and its correlation with PDE4 isoform expression, in normal and adenomatous pituitary. Determining PDE4 expression in AIP-mutated tumors will possibly also shed light on this field. The physiological function(s) and possible role of PDE4s in counteracting pituitary tumorigenesis require further investigation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

G B B and M K developed the idea of the study. G B B provided overall supervision and planned and performed the two-hybrid experiments. A R O Jr also provided overall supervision and planned the immunofluorescence studies. M F B and S V B P performed all immunofluorescence studies. G T performed the human variant analysis and, with M K, provided samples. M A A and L S generated and characterized the monoclonal antibodies and performed immunoblotting. G B B, M K, and A R O Jr prepared the manuscript.

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