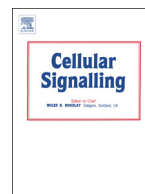




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Q1 Dimerization of cAMP phosphodiesterase-4 (PDE4) in living cells 2 requires interfaces located in both the UCR1 and catalytic unit domains

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ABSTRACT

PDE4 family cAMP phosphodiesterases play a pivotal role in determining compartmentalised cAMP signalling through targeted cAMP breakdown. Expressing the widely found PDE4D5 isoform, as both bait and prey in a yeast 2-hybrid system, we demonstrated interaction consistent with the notion that long PDE4 isoforms form dimers. Four potential dimerization sites were uncovered using a scanning peptide array approach, where recombinant purified PDE4D5 fusion protein was used to probe a 25-mer library of overlapping peptides covering the entire PDE4D5 sequence. Key residues involved in PDE4D5 dimerization were defined using a site-directed mutagenesis programme directed by an alanine scanning peptide array approach. Critical residues stabilising PDE4D5 dimerization were defined within the regulatory UCR1 region found in long, but not short, PDE4 isoforms, namely the Arg¹⁷³, Asn¹⁷⁴ and Asn¹⁷⁵ (DD1) cluster. Disruption of the DD1 cluster was not sufficient, in itself, to destabilise PDE4D5 homodimers. Instead, disruption of an additional interface, located on the PDE4 catalytic unit, was additionally required to convert PDE4D5 into a monomeric form. This second dimerization site on the conserved PDE4 catalytic unit is dependent upon a critical ion pair interaction. This involves Asp⁴⁶³ and Arg⁴⁹⁹ in PDE4D5, which interact in a *trans* fashion involving the two PDE4D5 molecules participating in the homodimer. PDE4 long isoforms adopt a dimeric state in living cells that is underpinned by two key contributory interactions, one involving the UCR modules and one involving an interface on the core catalytic domain. We propose that short forms do not adopt a dimeric configuration because, in the absence of the UCR1 module, because residual engagement of the remaining core catalytic domain interface provides insufficient free energy to drive dimerization. The functioning of PDE4 long and short forms is thus poised to be inherently distinct due to this difference in quaternary structure.

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46 1. Introduction

Cyclic AMP is a ubiquitous second messenger that plays a pivotal role in regulating many key cellular processes [1–5]. cAMP signalling in mammalian cells is compartmentalised so that spatially distinct sub-populations of the cAMP effectors, PKA and Epac can differentially regulate a range of distinct intracellular processes [1,5–7]. The differential activation of such effectors is achieved through gradients of cAMP formed by spatially distinct sub-populations of both adenylyl cyclase and cAMP degrading phosphodiesterases [1,8]. 9 different PDE sub-families are capable of degrading cAMP and, in addition exhibiting cell-type specific patterns of expression, they are differentially located in cells, conferring distinct roles upon enzymes from this super-family

[2,3,9–13]. Differences in intracellular targeting, coupled with regulated changes in both their activity and targeting elicited by post-translational modification, place PDEs firmly as critical enzymes regulating cellular function [1]. Indeed, the ability to generate inhibitors selective for each PDE sub-family has been judiciously exploited in order to both generate therapeutic agents and garner understanding of the functional significance of these enzymes [3,9,14].

Members of the PDE4 enzyme family play a pivotal role in cell functioning. These enzymes are encoded by four genes (*PDE4A/PDE4B/PDE4C/PDE4D*), which generate over 20 distinct isoforms through alternate mRNA splicing and the use of distinct promoters [1,2,9,10,15–18]. PDE4 isoforms critically define the compartmentalization of cAMP signalling through their ability to be recruited to specific signalling complexes, where they shape cAMP gradients in a temporal and spatial manner [1]. As such individual isoforms have specific, non-redundant roles acting in defined intracellular compartments as elucidated

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through dominant negative, siRNA-mediated knockdown and peptide displacement approaches [19–22]. Their functioning in these distinct locales is dynamically regulated through phosphorylation by kinases such as PKA [23–26], Erk [27–29], MK2 [30,31] and AMPK [30] as well as modification by ubiquitination [32] and sumoylation [33].

Many proteins can undergo dimerization, which can lead to functional differences [34]. In this regard, enzymes from the various PDE families have a highly conserved catalytic unit and many sub-families are also characterized by distinct, paired domains located N-terminal to this. These include the Ca^{2+} /calmodulin binding domains of PDE1, the Gaf domains of PDE2/PDE5/PDE6/PDE10/PDE11 and the UCR1/2 domains of PDE4 [2,9,15,17]. Such domains have been implicated in dimer formation [35–46].

Alternative mRNA splicing of all four PDE4 genes yields a plethora of isoforms. These can be sub-categorised as ‘long’ forms that possess both UCR1 and UCR2 regulatory domains, ‘short’ forms that lack UCR1 and ‘super-short’ forms that lack UCR1 and have a truncated UCR2 [1,3,9,10,14–16,18,47]. Also identified are ‘dead-short’ forms that lack both UCR1 and UCR2 and have a truncated catalytic unit, making them catalytically inactive [48].

There is now good evidence that PDE4 isoforms can form dimers [41–43]. These elegant studies have shown that dimerization in cells is restricted to the long, but not the short, isoforms as UCR1 is fundamental to this process. Consistent with this, 2-hybrid studies using isolated domains have demonstrated that UCR1 can interact with UCR2 and this might, through *trans* interaction, facilitate assembly of a long isoform dimer [49]. Notwithstanding the apparent requirement for UCR1, X-ray crystallographic analyses of active, but highly truncated PDE4 core catalytic units, reveal that the isolated catalytic domain can dimerize, at least under crystallisation conditions, despite the absence of UCR1 and UCR2 [50]. The dimerization interface within the catalytic unit comprises a focal contact surface at a C2 symmetry axis that is bounded at each end by an Asp/Lys charge pairing that is conserved in all four PDE4 sub-families [50].

Here we use two novel approaches to gain further insight into the nature of PDE4 dimers formed in living cells, focusing on the widely expressed PDE4D5 long isoform as an exemplar [51]. Amongst other things, this isoform has particular functional importance in regulating the β_2 -adrenoceptor through its ability to bind to the β -arrestin signalling scaffold [52–56], and also in the migration and polarity of cells through its ability to bind to the RACK1 signalling scaffold [21,32,53,56,57]. In one approach we employed a yeast 2-hybrid methodology to evaluate dimerization in living cells and, in a second approach we used scanning peptide array analyses to determine the details of the PDE4 dimerization site located in the long form-specific UCR1 domain. These studies have allowed us to engineer a catalytically active mutant form of PDE4D5 that, unlike the native dimeric enzyme, exists as a monomer in living cells.

2. Materials and methods

2.1. Materials

Primary antibodies used are rabbit-polyclonal anti-VSV (Abcam Ltd., Cambridge, CB4 0FL, UK), mouse polyclonal anti-HA (Covance, Alnwick, NE66 2JH, UK), mouse anti-FLAG-horseradish peroxidase conjugate and VSV (vesicular stomatitis virus)-affinity agarose beads were from Sigma (Gillingham, Dorset, SP8 4XT, UK). Anti-GST antibody (Santa Cruz/Insight Biotechnology Ltd, Wembley, Middlesex HA9 7YN, UK). Secondary antibodies used are anti-mouse horseradish peroxidase linked Ig (GE Healthcare, Amersham Place, Little Chalfont Bucks, HP7 9NA, UK) and anti-rabbit horseradish peroxidase linked Ig (Sigma, Gillingham, Dorset, SP8 4XT, UK). Stock solutions of rolipram were prepared in DMSO. Bradford reagent was from Bio-Rad (Hemel Hempstead, Herts, HP2 4PD, UK). Polyfect transfection reagent was from Qiagen (Lloyd Street North, Manchester M15 6SH). Protease

inhibitor tablets were from Roche. Plasmid DNA was prepared using the QIAprep® Spin Miniprep kit from Qiagen (Lloyd Street North, Manchester M15 6SH). $[8\text{--}^3\text{H}]\text{cAMP}$ was from GE Healthcare (Amersham Place, Little Chalfont Bucks, HP7 9NA, UK) and unlabelled cAMP together with all other biochemicals were from Sigma (Gillingham, Dorset, SP8 4XT, UK). NuPAGE was from Invitrogen (Paisley PA4 9RF, UK). ECL reagents were from Pierce/ThermoFisher (Northumberland, NE23 1WA, UK).

2.2. Yeast 2-hybrid analyses

Yeast 2-hybrid techniques are identical to those used previously by us to identify and analyse protein–protein interactions [57,58]. In all experiments, one of the two interacting proteins was expressed as “bait”, as a LexA DNA-binding domain fusion. The second protein was expressed as “prey”, as a GAL4 activation-domain protein. In some experiments, a third protein (*i.e.*, either RACK1 or β -arrestin2) was also expressed, but as a native species and not as a fusion protein. All bait were expressed either in pLEXAN or in pBridge as LexA DNA-binding domain fusions. All prey were expressed in pGADN as GAL4 activation-domain fusions. All non-fusion co-expressed proteins were expressed in pBridge (*i.e.*, pBridge expresses two proteins, one as a LexA DNA-binding domain fusion and the other with only a nuclear localization signal). All proteins were targeted to the nucleus. 158

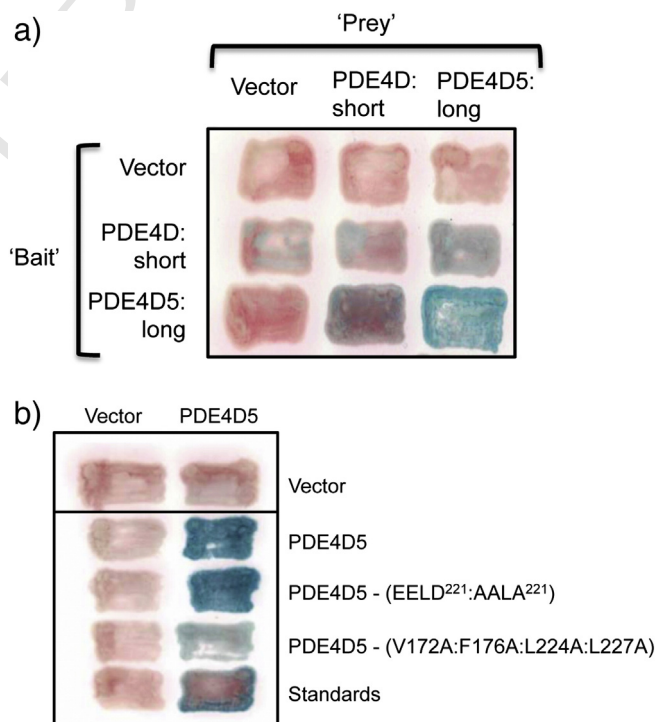


Fig. 1. PDE4 dimerizes in living cells. Yeast 2-hybrid experiments using PDE4D5 as both ‘bait’ and ‘prey’. PDE4D5 was expressed unmutated (“wild-type”) or with the indicated point mutations. PDE4D5 was expressed unmutated (“wild-type”) or with the indicated deletion or point mutations. All patches in each column use identical ‘prey’ and all patches in each row use identical ‘bait’, as described in Materials and methods. Controls are vectors alone and standards are Ras^{V12}–Raf, as done before by us [57]. Positive interactions, assessed with a filter β -galactosidase assay, produce blue patches, while negative interactions produce pink patches. (a) Full-length PDE4D5 (amino acids 1 to 749; “long”), or PDE4D5 lacking its unique N-terminal domain and UCR1, but containing UCR2 and the catalytic region (amino acids 206 to 747 of PDE4D5; “short”), was expressed as either a fusion to LexA (rows) or to GAL4 (columns) and the various mutants tested for interaction. (b) The EELD:AALA mutant reflects that ablating the UCR1:UCR2 interaction [49] while the VFL:AAAA mutation reflects that used to ablate PDE4D3 dimerization [42]. This shows data typical of experiments performed at least 3 times.

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