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Cyclic AMP (cAMP) specific phosphodiesterase-4 (PDE4) enzymes underpin compartmentalised cAMP signalling by localising to distinct signalling complexes. PDE4 long isoforms can be phosphorylated by mitogen-activated protein kinase-activated protein kinase 2 (MK2), which attenuates activation of such enzymes through their phosphorylation by protein kinase A (PKA). Here we show that MK2 interacts directly with PDE4 long isoforms and define the sites of interaction. One is a unique site that locates within the regulatory UCR1 domain and contains a core Phe141, Leu142 and Tyr143 (FLY) cluster (PDE4A5 numbering). Located with the second site is a critical core Phe693, Glu694, Phe695 (FQF) motif that is also employed in the sequestering of PDE4 long forms by an array of other signalling proteins, including the signalling scaffold beta-arrestin, the tyrosyl kinase Lyn, the SUMOylation E2 ligase UBC9, the dynein regulator Lis1 (PAFAH1B1) and the protein kinase Erk. We propose that the FQF motif lies at the heart of a multi-functional docking (MFD) site located within the PDE4 catalytic unit. It is clear from our data that, as well as aiding fidelity of interaction, the MFD site confers exclusivity of binding between PDE4 and a single specific partner protein from the cohort of signalling proteins whose interaction with PDE4 involves the FQF motif.
Identification of a multifunctional docking site on the catalytic unit of phosphodiesterase-4 (PDE4) that is utilised by multiple interaction partners.

By

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Abstract

Cyclic AMP (cAMP) specific phosphodiesterase-4 (PDE4) enzymes underpin compartmentalised cAMP signalling by localising to distinct signalling complexes. PDE4 long isoforms can be phosphorylated by mitogen-activated protein kinase-activated protein kinase 2 (MK2), which attenuates activation of such enzymes through their phosphorylation by protein kinase A (PKA). Here we show that MK2 interacts directly with PDE4 long isoforms and define the sites of interaction. One is a unique site that locates within the regulatory UCR1 domain and contains a core Phe141, Leu142 and Tyr143 (FLY) cluster (PDE4A5 numbering). Located with the second site is a critical core Phe693, Glu694, Phe695 (FQF) motif that is also employed in the sequestering of PDE4 long forms by an array of other signalling proteins, including the signalling scaffold beta-arrestin, the tyrosyl kinase Lyn, the SUMOylation E2 ligase UBC9, the dynein regulator Lis1 (PAFAH1B1) and the protein kinase Erk. We propose that the FQF motif lies at the heart of a multi-functional docking (MFD) site located within the PDE4 catalytic unit. It is clear from our data that, as well as aiding fidelity of interaction, the MFD site confers exclusivity of binding between PDE4 and a single specific partner protein from the cohort of signalling proteins whose interaction with PDE4 involves the FQF motif.

Keywords: PDE4; phosphodiesterase; cAMP; cyclic AMP; MK2; mitogen-activated protein kinase-activated protein kinase 2; rolipram; UBC9; Lyn; beta-arrestin; Lis1.
1. Introduction

The ubiquitous small molecule, cyclic AMP was the first intracellular second messenger to be described and is known to play a pivotal role in regulating many key cellular processes (Houslay, 2010; Lugnier, 2006; Maurice et al., 2014; Rogne and Tasken, 2014; Zaccolo, 2009). Such cAMP signalling processes are now well-understood to be compartmentalized in cells with intracellular gradients of cAMP formed by spatially constrained degradation that is mediated by sequestered populations of cAMP phosphodiesterases (PDEs). Thus the tethering of particular PDEs to specific signalling complexes, located at distinct intracellular sites, confers a spatial element to cAMP signal processing (Baillie, 2009; Houslay, 2010; Willoughby and Cooper, 2007). Such intracellular gradients of cAMP are then interpreted by spatially discrete sub-populations of the cAMP effectors, PKA and Epac, which differentially regulate different cellular processes (Houslay, 2010; Skroblin et al., 2010; Troger et al., 2012; Zaccolo, 2009).

Members of the PDE4 cAMP specific phosphodiesterase family play pivotal roles in forming and shaping cAMP gradients within cells. 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 (Bolger et al., 1993; Conti and Beavo, 2007; Conti et al., 2003; Houslay, 2001; Houslay, 2010; Houslay and Adams, 2003; Houslay et al., 2007; Lugnier, 2006). The fidelity of PDE4 targeting to particular signalling complexes invariably involves specific binding sites that are located within the N-terminal region unique to each PDE4 isoform. It is such precise intracellular targeting that confers specific, non-redundant roles on particular PDE4 isoforms. Such characteristics have been uncovered through the use of dominant negative, siRNA and peptide displacement approaches (Bolger et al., 2006; Lynch et al., 2005; McCahill et al., 2005; Serrels et al., 2010; Sin et al., 2011). This has allowed the appreciation of a large and growing collection of partner proteins that are able to bind PDE4 isoforms: this collection is known as the PDE4 interactome (Houslay, 2010).

PDE4 isoforms provide pivotal nodes for cross-talk between the cAMP and other signalling pathways via the ability of particular PDE4 isoforms to be phosphorylated by a range of important protein kinases. Thus long PDE4 isoforms can be phosphorylated and activated by cAMP dependent protein kinase A (PKA), which provides the key mechanism responsible for cellular desensitization to cAMP signalling (Hoffmann et al., 1998; MacKenzie et al., 2002; Oki et al., 2000; Sette and Conti, 1996; Sette et al., 1994). Furthermore, they can be phosphorylated by Erk, MK2, AMPK and an as yet unidentified kinase activated by reactive oxygen / stress pathways (Baillie et al., 2001; Baillie et al., 2000; Hoffmann et al., 1999; MacKenzie et al., 2011; Sheppard et al., 2014).
The p38 MAPK signalling cascade is a key signal transduction pathway involved in the control of cellular immune, inflammatory and stress responses (Cowan and Storey, 2003). Activated p38 MAPK exerts important effects on cell functioning by phosphorylating, and so activating, the downstream kinases, MK2 (Duraisamy et al., 2008). We have previously demonstrated (MacKenzie et al., 2011) that PDE4 long isoforms can be phosphorylated by MK2. Intriguingly, such phosphorylation appears to have little or no discernible effect on PDE4 activity per se (MacKenzie et al., 2011). Rather, its effect appears to be targeted to attenuate the stimulatory effect that PKA exerts when it phosphorylates long PDE4 isoforms (MacKenzie et al., 2011). In that way, MK2 phosphorylation of PDE4 long forms attenuates the cellular desensitization to cAMP that stimulatory PKA phosphorylation PDE4 long forms provides.

PDE4 enzymes from the PDE4B/C/D sub-families can also be phosphorylated by Erk. This leads to the activation of short forms and, contrastingly, the inhibition of long forms (MacKenzie et al., 2000). Interestingly, such modifications require physical association that involves distinct docking and specificity sites that straddle the Erk phosphorylation site located within the PDE4 catalytic unit (MacKenzie et al., 2000).

Here we show that MK2, as with Erk, interacts directly with PDE4 through distinct docking sites. One such docking site is located, along with the phosphorylation site, in the regulatory UCR1 region that is unique to PDE4 long isoforms. We also uncover an additional docking site, which is located within the PDE4 catalytic unit, which is employed by an array of proteins able to interact with PDE4. We propose that this forms a multi-functional docking site in that, as well as aiding fidelity of interaction, confers exclusivity of binding between PDE4 and specific partner proteins.


SPOT synthesis of peptides and peptide array probing

Peptide libraries were generated by automatic SPOT synthesis on Whatman 50 cellulose membranes using Fmoc (9-fluorenylmethyloxycarbonyl) chemistry with the Autospot-Robot ASS 222 (Intavis Bioanalytical Instruments, 50933 Koeln, Germany). The interaction of peptide spots with GST and GST-fused purified proteins by overlaying the cellulose membranes with 10 mg/ml of recombinant protein was determined as described previously by us in some detail (Baillie et al., 2007; Bolger et al., 2006; Murdoch et al., 2007). Bound recombinant proteins were detected with specific primary antisera and complementary HRP-coupled secondary antibody as for immunoblotting.

Cell culture and preparation of lysates
COS1 and HEK (human embryonic kidney)-293 cells were propagated as previously described (Huston et al., 1996; Lynch et al., 2005). Cell lysate for western blotting or immunoprecipitation were prepared using the protocol outlined in (MacKenzie et al., 2011).

**Cell Transfection**

PolyFect® (QIAGEN®) was used for transfection of COS1 and HEK293 cell lines as previously described (MacKenzie et al., 2011).

**Co-immunoprecipitation.**

Immunoprecipitations were carried out as previously described in (MacKenzie et al., 2011) (Baillie et al., 2007) using a specific antibody against MK2 (Cell Signaling Technology, Cat no 3042).

**SDS-PAGE**

Protein samples were separated on NuPAGE 4-12% Bis-Tris polyacrylamide gels (Invitrogen, UK) as previously described (MacKenzie et al., 2011).

**Western blotting**

The proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane for western blotting using the procedure outlined previously (MacKenzie et al., 2011). The commercially obtained primary antibodies used in this study were against MK2 (Cell Signaling Technology, Cat no 3042), P38 (Cell Signaling Technology, Cat no 9212), phospho-P38 (Cell Signaling Technology, Cat no 9215), p44/42 ERK MAPK (Cell Signaling Technology, Cat no 4696), β Arrestin1 (Cell Signaling Technology, Cat no 4674) and GST (Cell Signaling Technology, Cat no 2624). The antibodies to PDE4A5, PDE4B, PDE4C, PDE4D and phospho-PDE4A5 were raised by the Baillie lab and have been described previously (MacKenzie et al., 2011; MacKenzie et al., 2002; McCahill et al., 2005). Arrestin and ERK were detected concomitantly during competition overlay experiments on peptide arrays using Licor Odyssey Scanner as previously described (Bolger et al, 2006).

**3. Results and Discussion**

MK2 co-immunoprecipitates with PDE4 long isoforms.
We have previously shown (MacKenzie et al., 2011) that activated MK2 phosphorylates the long PDE4A5 isoform at a single site, namely Ser147, which is located within the regulatory UCR1 domain that characterises PDE4 long isoforms from all four sub-families (Bolger et al., 1993; Houslay, 2001; Houslay, 2010) where this residue and the surrounding MK2 consensus phosphorylation motif are conserved.

In order for the MAP kinase, Erk to phosphorylate authentic cellular substrates, such as PDE4, it binds directly to them via docking and specificity sequences that lie on each side of the phosphorylation site (MacKenzie et al., 2000). With this in mind we set out to investigate whether MK2 might bind to PDE4A5 in a similar fashion. Thus we first transfected Cos1 cells with the long PDE4A5 isoform in order to determine whether this ectopically expressed PDE isoform could bind to endogenous MK2. Lysates from such transfected cells were then subjected to immunoprecipitation, using a pan rodent PDE4A isoform, which allowed us to demonstrate that PDE4A5 does indeed co-immunoprecipitate together with MK2 (Fig. 1a).

We then subjected lysates of Hek293 cells to immunoprecipitation using MK2-specific antisera and probed these, together with the lysates, with antisera specific for each of the PDE4B, PDE4C and PDE4D subfamilies (Fig. 1b). This allowed us to demonstrate that endogenous long isoforms from each of these three PDE4 sub-families was able to co-immunoprecipitate with endogenous MK2 (Fig. 1b). Note that any putative endogenous PDE4A isoforms that might be expressed in these cells are below levels detectable by our antisera.

**Peptide array analyses to locate MK2 binding sites on PDE4A.**

We, and others, have successfully used a peptide array approach to delineate various protein-protein interaction surfaces (see e.g. (Baillie et al., 2007; Houslay, 2010; Hundsrucker et al., 2010; Murdoch et al., 2007)). This provides a novel and powerful technology for gaining insight into the basis of specific protein-protein interactions and driving intelligent mutagenesis approaches to confirm or refute binding site authenticity in the intact protein. We thus utilised this strategy to identify the sites within PDE4A5 that MK2 binds to.

A full-length peptide array of PDE4A5 was made using procedures utilised by us previously to evaluate the interaction of other PDE4 isoforms with partner proteins (Baillie et al., 2007; Bolger et al., 2006; Murdoch et al., 2007; Murdoch et al., 2011). This consisted of a library of peptide “spots” of 25 amino acids that sequentially span the PDE4A5 sequence and where each peptide overlapped the previous peptide by five amino acids. This array was then overlaid with purified recombinant GST tagged MK2. The location of putative binding sites was determined by...
probing with GST specific anti-sera and processed in a similar fashion to a western blot as described before in some detail by us (Baillie et al., 2007; Houslay, 2010; Murdoch et al., 2007). Such analyses showed that when spots previously shown to bind GST alone were discounted, then four potential binding sites for MK2 on PDE4A5 were identifiable (Fig. 2). These sites are associated with amino acids 121-155 located within UCR1, amino acids 577-606 and 637-681 both located within the catalytic region and amino acids 677-716 located within the C-terminal region (Fig. 2).

To gain further insight into particular amino acids that are important in each of these regions and to confirm the fidelity of interaction at such sites, alanine scanning peptide arrays were created for part of each region identified above. In undertaking this we screened a family of peptides derived from distinct 25-mer parent peptides that positively interacted with MK2. As utilised previously in studies of other protein-protein interactions (Baillie et al., 2007; Houslay, 2010; Murdoch et al., 2007), the peptide progeny from specific 25-mer parents were generated such that each had a single substitution, to alanine, of successive amino acids in the sequence to form a scanning peptide array. If alanine residues existed in the sequence to be scanned, these were substituted with an aspartate. The alanine scanning arrays were each probed with purified recombinant GST tagged MK2 (Fig. 3).

Examining the potential MK2 interaction site within the regulatory UCR1 of PDE4A5, we see that substitution, to alanine, of any of the amino acids within the cluster of Phe141, Leu142 and Tyr143 essentially ablated interaction (Fig. 3a). Binding was also compromised with several other alanine substitutions of amino acids within this region, which was particularly evident for Tyr149 (Fig. 3a). In the first catalytic region sequence, amino acids identified as being potentially required for MK2 binding by alanine scanning peptide analysis were Trp591, Thr592, Ile595, Glu598 and Phe599 where alanine substitution compromise MK2 interaction (Fig. 3b). In the second catalytic region sequence, amino acids identified as being potentially required for MK2 binding by alanine scanning peptide analysis were Ile654, Leu655, Asp656, Trp664, Tyr665, His666, Ser667 and Ile669 (Fig. 3c). Finally in the C-terminal region, amino acids identified as being potentially required for MK2 binding by alanine scanning peptide analysis were Phe693, Gln694, Phe695, Thr698, Leu699, Glu700, Glu701, Glu702, Glu703 and Glu704(Fig. 3d).

In order to evaluate the potential importance of such regions for PDE4A5-MK2 interaction we adopted a mutagenesis strategy (Fig. 4). In this we focused on, firstly, the Phe141, Leu142 and Tyr143, which we call the FLY motif, located in the regulatory UCR1 domain conserved in all PDE4 long isoforms and where the MK2 phosphorylation site is located (Fig. 4a). Interestingly, individual
mutations of any single one of these amino acids to alanine partially compromised binding, while the triple alanine mutation of all three residues fully ablated interaction, uncovering the pivotal importance of the FLY motif in allowing MK2 to bind to PDE4A5 (Fig. 4b).

Unlike for UCR1, the structure of the PDE4A catalytic unit is known (Wang et al., 2007). Thus we adopted our previous strategy for mapping binding sites (Baillie et al., 2007), namely to focus on residue clusters identified from alanine scanning peptide arrays that have surface availability (Fig. 4b). In the PDE4A catalytic unit these are the Ile654, Leu655, Asp656 cluster; the Trp664, Tyr665, His666, Ser667 cluster; the Phe693, Gln694, Phe695 cluster and the Thr698, Leu699, Glu700, Glu701 cluster. We generated mutants of PDE4A5 for each of these clusters separately, where all of the residues in each cluster were mutated in their entirety to alanine. Such PDE4A5 mutant constructs were individually expressed at similar levels in COS1 cells, which do not express detectable levels of endogenous PDE4A isoforms. Lysates were then generated and MK2 immunoprecipitates probed for the presence of PDE4A5 (Fig. 4a, b). These experiments showed that, in marked contrast to native PDE4A5, a PDE4A5 construct with a triple alanine mutation of the FLY domain, namely FLY:AAA-PDE4A5, failed to co-immunoprecipitate with MK2 (Fig. 4a) Additionally, alanine mutation of each of the catalytic site clusters examined here showed compromised interaction with PDE4A5, with the FQF:AAA-PDE4A5 and TLEE:AAAA-PDE4A5 species showing the most severe reduction in interaction (Fig. 4b). The attenuated binding of the mutated PDE4A5 species highlights the importance of these docking domains in maintaining the fidelity of complex formation with MK2. Nevertheless, the possibility has to be entertained that these reductions could be a result of a dramatic change in conformation of the PDE protein elicited by such discrete mutations.

**FLY domain is essential for phosphorylation of PDE4A5 by MK2**

We have demonstrated previously (MacKenzie et al., 2011) that MK2 can phosphorylate PDE4A5 on residue Ser147 that is located within UCR1. Such a modification serves to attenuate the activation of PDE4A5 that is engendered via PKA phosphorylation of residue Ser140 (MacKenzie et al., 2002), which is also located within UCR1.

In this study we have shown that MK2 docks with PDE4 and identified putative docking sites for MK2 on PDE4A5. We thus surmised that, as for Erk interaction with PDE4 and other substrate proteins, then PDE4A5 mutants showing reduced MK2 binding might also exhibit compromised phosphorylation by MK2 on Ser147. We set out to examine this using the well-established approach of using anisomycin to promote activation of the p38 MAPK pathway and
thus, of MK2 (Cowan and Storey, 2003). As shown previously by us (MacKenzie et al., 2011), anisomycin challenge of COS1 cells expressing PDE4A5 promoted its phosphorylation at Ser147 by MK2 and this was ablated in PDE4A5 mutants where the target for phosphorylation, namely Ser147 was substituted with alanine (Fig. 5; Ser147Ala-PDE4A5 mutant). Intriguingly, both the FLY:AAA-PDE4A5 and FQF: AAA-PDE4A5 mutants showed marked reductions in the anisomycin-driven Ser147 phosphorylation when compared with wild type (Fig. 5). These observations demonstrate that the interaction of PDE4A5 with MK2, through its two distinct docking sites, facilitates the efficient phosphorylation of PDE4A5 at Ser147.

_FQF lies at the core a multi-functional docking (MFD) site._

The FLY motif appears to be essential for MK2 binding to PDE4A5. However, as is the case with all known PDE4 partner proteins, fidelity of interaction appears to be driven through the involvement of more than one binding site (Houslay, 2010). Invariably this allows the partner protein to straddle the catalytic unit, with one site in either the regulatory domains (UCR1/2) or the isoform-specific N-terminus and another in the catalytic unit. Interestingly, the Phe693:Gln694:Phe695 (FQF) motif on the catalytic unit of PDE4A5 that we have identified here as contributing to MK2 binding also is intimately involved in the binding of Erk (MacKenzie et al., 2000), beta-arrestin (Baillie et al., 2007), Lis1 (PAFAH1B1) (Murdoch et al., 2011) and AKAP18 (McSorley et al., 2006). This suggested to us that FQF is located at the core of a multi-functional docking (MFD) site that confers fidelity on partnerships by preventing multiple partner proteins to interact with any one PDE4 isoform simultaneously. Indeed, have shown that competition exists between different binding partners of PDE4 for the FQF domain using simultaneous overlay of multiple purified proteins onto PDE4 peptide arrays (Bolger et al., 2006).

PDE4A5 was reported some time ago to bind to the SH3 domain of Lyn tyrosyl kinase (Beard et al., 2002; O'Connell et al., 1996) and, additionally, we have shown that PDE4 isoforms can functionally interact with the SUMOylation E2 ligase, UBC9 (Li et al., 2010). We thus set out to investigate whether the FQF motif might be highlighted in PDE4 peptide arrays probed with these two species as well as with other binding partner proteins that we have reported on, namely beta-arrestin, ERK (Baillie et al., 2000; MacKenzie et al., 2000) (Fig. 6).

Lyn and MK2 appear to interact specifically with PDE4A rather than isoforms of the other three PDE4 sub-families (McPhee et al., 1999). The C-terminal regions of all four sub-families are distinct, with no regions of homology. Indeed, such a difference has been exploited to make sub-family specific antisera (MacKenzie and Houslay, 2000). Thus we set out to probe peptide arrays...
representing the C-terminal region of PDE4A5 with purified Lyn, UBC9, Arrestin2 and ERK2 (Fig. 6a, b). These data show that all these very different proteins were able to bind to PDE4A5 peptides containing the FQFELTL sequence, however RACK1, a protein that is known to bind elsewhere in the catalytic unit, did not. (Fig 6a, b).

Alanine scanning peptide arrays were created for each region and probed with the PDE4 binding partners as before (Fig 6c). In all cases, the dual phenylalanines and leucines within the FQFELTL motif were shown to be crucial for partner binding to the PDE4 sequence. This suggests a common mode of interaction employed by all these very different proteins in the way that they bind to this multi-functional docking platform. As before (Bolger et al, 2006), we show using competition overlay on alanine scans of the MFD motif that both ERK1 and βArrestin1 compete for the wild type peptide (arrestin in green, ERK in red and dual binding in yellow: Fig. 6d) and that substitution of the dual phenylalanines and leucines are equally effective in ablating the binding of both PDE4 interacting proteins. We envisage that such binding may serve to aid in orientating such proteins to locate additional, partner-specific binding sites on the PDE4 host.

Interestingly, the structure of the proposed MFD site can be identified from observations of several PDE4 crystal structures as located within the so-called CR3 region (Burgin et al., 2010; Card et al., 2004; Felding et al., 2014; Fox et al., 2014; Goto et al., 2013; Hagen et al., 2014; Kranz et al., 2009; Xu et al., 2000). In these, PDE4-only structures, the MFD site can be seen as lying across the opening of the catalytic pocket (Figure 7a), typically through insertion of a single phenylalanine from the FQF motif. Although some variation is seen in the mode of interaction with the core catalytic domain in these structures, it has been proposed that this crystallographically observed capture of CR3 by the catalytic pocket might reflect an auto-inhibitory capping role that is relevant to PDE4 function, (Fox et al., 2014). In all structural determinations to date the CR3 region containing the FQF motif exhibits helical secondary structure, albeit that some conformational variability is seen across the available crystal structures (Figure 7b/c). Thus this region appears to be positionally mobile with respect to its point of attachment (helix-16) on the PDE4 core catalytic domain, which would be consistent with an ability to associate with appropriate partner proteins.

The binding of the CR3 region across the PDE4 catalytic pocket typically involves insertion of one of the two Phe residues of the FQF motif into the catalytic pocket. As such, one of the Phe residues would be inaccessible to a partner protein, whilst the other Phe would be solvent exposed and therefore accessible to a partner protein. In principle, then partner protein binding might potentially occur with this region in such a capped position. However, the results of the
peptide array analyses on PDE4A5 with arrestin2, UBC9, ERK, Lyn and MK2 suggest that for maximal interaction then both Phe residues are likely to be involved in the binding of these partner proteins. The implication of both Phe residues being involved in partner protein binding with PDE4 indicates that the MFD sequence would be removed from any association with the catalytic pocket when such partner proteins bound to PDE4. This would be fully consistent with the flexible, hinge-like nature of CR3.

4. Conclusion.

Specifically sequestered PDE4 isoforms play a major role in underpinning the compartmentalisation of cAMP signalling cells (Houslay, 2010). Associated with this, such species also provide nodes of cross-talk between a wide range of signalling pathways that is achieved through the multi-site phosphorylation of PDE4 isoforms (Houslay, 2010; Mika and Conti, 2016). The p38 MAPK phosphorylation cascade plays a fundamental role in regulating the immune and inflammatory response to infection and tissue injury (Cowan and Storey, 2003). MK2, the downstream effector kinase of p38 MAPK, has been shown (MacKenzie et al., 2011) to play a key role in attenuating cellular desensitization to cAMP by phosphorylating PDE4 long isoforms such that their ability to be activated by positive feedback PKA phosphorylation (Oki et al., 2000) is diminished. Here we show that in order for MK2 to phosphorylate PDE4 long isoforms effectively this kinase interacts with docking sites located on PDE4. Such a docking and consequential phosphorylation process is akin to that employed by Erk when it phosphorylates PDE4 (MacKenzie et al., 2000) and other physiological substrates, each of which is defined by the presence of a conserved docking site (Roskoski, 2012; Sharrocks et al., 2000).

PDE4 enzymes thus have binding domains that allow interaction with various kinases as well as domains that target them to scaffold proteins. These allow for the spatially discrete degradation of cyclic AMP so as to confer compartmentalised signalling, which can then be regulated through cross-talk with other signalling processes (Houslay, 2010).

The ability to form functionally discrete cAMP signalling complexes (signalosomes) involving a specific anchor protein and a specific PDE4 isoform will thus depend upon the fidelity of partnerships. However, various signalling scaffold proteins are able to interact with a wide range / all PDE4 isoforms. In such instances, if a particular PDE4 isoform was able to interact with more than one scaffold at a time then this would likely lead to multimerisation into aggregates with loss of integrity of spatial compartmentalisation of cAMP degradation. Similarly, the ability of PDE4 enzymes to be phosphorylated by various kinases will depend upon the availability of free
docking sites. Here, through our analyses of the interaction of the functionally important long PDE4A5 isoform, we have identified one means through which multimerisation through interaction with multiple scaffolds is prevented and availability for modification by a specific regulatory kinase is effected, namely through the identification of a cohort of interaction proteins that require docking to a common site, the Multifunctional Docking (MFD) site.

The sequestration of PDE4 isoforms by signalling scaffolds invariably involves binding at two or more sites on the PDE4 (Houslay, 2010). If partnerships are either isoform specific or show preference for one particular isoform then one binding site is located within the isoform-specific N-terminal region. However, various PDE4 isoforms are able to interact with a range of signalling proteins with scaffolding attributes. Here we have identified a MFD site on PDE4A5, which is conserved in all PDE4 isoforms from all four sub-families, that provides one of the essential sites used by a cohort of signalling proteins to interact effectively with PDE4 enzymes, namely the protein kinases, MK2 and ERK; the signalling scaffold proteins, beta-arrestin and Lis1 (PAFAH1B1); the SUMO E2 ligase, UBC9, and the tyrosyl protein kinase and scaffold protein, Lyn. The requirement of a scaffold protein / kinase for binding to such a MFD would indicate that PDE4 sub-populations sequestered by one scaffold using this MFD would be unable to interact with other scaffolds using this MFD, so ensuring spatial fidelity. Furthermore, when a PDE4 isoform interacts with a protein partner via the MFD, the PDE4 enzyme is unlikely to effectively phosphorylated by a kinase that also requires the MFD.

Thus interaction of PDE4 isoforms with MFD-binding partner proteins will define not only sub-populations that are spatially distinct but also that show attenuated susceptibility to phosphorylation by kinases such as MK2 and Erk, both of which modify the functional outcome of stimulatory PKA phosphorylation (Hoffmann et al., 1999; MacKenzie et al., 2011).

5. Acknowledgements.
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6. References.


with the N-terminal splice region of the PDE4A cAMP-specific phosphodiesterase RPDE-6 (RNPD4A5). Biochem J. 318 (Pt 1):255-261.


**Figure Legends.**

*Figure 1. Co-immunoprecipitation of PDE4A5 with MK2 from mammalian cells.*

(a) COS-1 cells were transiently transfected to express the long PDE4A5 isoform. Endogenous MK2 was then immunoprecipitated using a specific antisera (Cell Signaling Technology, Cat no 3042). The lysates and the immunoprecipitates were each probed with either a MK2 specific antisera (lower panel) or a rodent PDE4A specific antisera (upper panel). (b) Cos-1 cells were transiently transfected to express the long isoforms of PDE4 as indicated. Endogenous MK2 was immunoprecipitated using the MK2 specific antisera. The immunoprecipitates and lysates were each probed with antisera specific for either PDE4B, PDE4C or PDE4D, as indicated. These identified the co-immunoprecipitation of the endogenous long PDE4 Isoforms, PDE4B1, PDE4C2 and PDE4D9. Also shown are the probing of the ‘bead only’ lanes where no MK2 specific antisera was employed as a control. These data show typical experiments of ones performed three times.

*Figure 2. The binding of MK2 to PDE4A5 peptide array.*

Peptide array technology was used to synthesise a full-length array of the entire 844 amino acids forming PDE4A5 on a Whatman 50 membrane (amino acids identified by numbering from sequence with accession number P54748). A schematic representation of the modular structure of PDE4A5 is shown in (a). The PDE4A5 array consisted of consecutive 25 amino acid peptide spots that each overlapped by 5 amino acids. The membrane was probed with purified recombinant GST-tagged MK2 (b). GST-specific anti-sera was then used to detect the areas of MK2 binding to the membrane. Highlighted are the four main regions of most intense binding and their sequences. All peptide arrays are representative arrays of at least three separate experiments.
Figure 3. Alanine scanning analyses of MK2 binding site(s) on PDE4A5

Peptide array technology was used to synthesise alanine scanning arrays of PDE4A5 on a Whatman 50 membrane. This was done using specific sequences indicated. These are amino acids (a) 131-156, (b) 582-606, (c) 647-671, (d) 682-702. A control spot was created with each sequence the each amino acid in turn was mutated to alanine. The membrane was probed with purified recombinant GST-tagged MK2. GST-specific anti-sera was then used to detect the areas of MK2 binding to the membrane. Highlighted in red are the main amino acids where substitution with alanine attenuates binding. All peptide arrays are representative arrays of at least three separate experiments.

Figure 4. Co-immunoprecipitation of PDE4A5 and mutant species with MK2

PDE4A5 binding sites for MK2 interaction were mutated as indicated and expressed in COS-1 cells. Immuno-experiments of endogenous MK2 were prepared from cellular lysates and the co-purification of PDE4A5 and PDE4A5 mutants assessed by western blotting using PDE4A5 specific antibody. The specific PDE4A5 mutants tested were (a) the FLY motif, (b) individual amino acids within the FLY motif and (c) other motifs that had been identified as being important for the MK2 association (Fig. 3). Quantification of the importance of the FLY (Phe141, Leu142, Tyr143 cluster), ILD (Le654, Leu655, Asp656 cluster), WHYS (Trp664, Tyr665, His666, Ser667 cluster), FQF (Phe693, Gln694, Phe695 cluster )and TLEE (Thr698, Leu699, Glu700, Glu701 cluster ) motifs are shown in (d). Significances of mutations evaluated using student’s t-test compared with wild type PDE4A5. p< 0.05 (*), p<0.01 (**), p<0.01 (***)

Figure 5. Mutants of PDE4A5 that are compromised in MK2 binding show reduced phosphorylation on serine 147 following anisomycin treatment.

(a) HEK293 cells were transiently transfected with plasmids encoding the indicated PDE4A5 variants or empty vector. p38 MAPK was activated by a 1 h stimulation using anisomycin. Cell lysates were probed using specific antisera for total and phosphorylated (Ser147) PDE4A5 and for total and phosphorylated p38 MAPK (Thr180, Tyr182). The blot shown is a representative result for n=4. (b) Quantification of PDE4A5 (Ser147) phosphorylation of PDE4A5 wt and mutants following anisomycin treatment. n=4

Figure 6. Delineation of a multi-functional docking site on long-form PDE4 enzymes.
Peptide array technology was used to synthesise peptide arrays of PDE4D and PDE4A5 regions encapsulating the FQF domain. This was done using specific sequences indicated. These are amino acids (a) 651-700 of PDE4D5, (b) 667-716. The PDE4D5 arrays were overlain with GST, GST-UBC9, GST-Arrestin and GST-ERK2 whereas the PDE4A5 arrays were overlain with GST, GST-Lyn and GST-MK2. GST-specific anti-sera was then used to detect the areas of protein binding to the membrane. (c) Peptide array technology was used to synthesise alanine scanning arrays of PDE4A5 and PDE4D5 regions encapsulating the FQF domain. This was done using specific sequences indicated. These are amino acids on upper panel PDE4A5, 687-706 and lower panel PDE4D5, 664-685. A control spot was created for each sequence and each amino acid in turn was mutated to alanine. All peptide arrays are representative arrays of at least three separate experiments. (d) Equal amounts (1uM) of GST-bArrestin 1 and GST-ERK1 were simultaneously applied to alanine scanning arrays of PDE4D5 region encapsulating the FQF domain. Arrestin and ERk were detected concomitantly using Licor Odyssey Scanner as previously described (Bolger et al, 2006).

Figure 7. Structure of the PDE4 multi-functional docking domain and candidate interaction site on MK2.

(a) The PDE4 core catalytic domain is shown from PDE4B co-crystal structure 3KKT with bound inhibitor (spheres) marking the catalytic pocket. The Region-3 MK2 interaction sequence of PDE4A5 is shown mapped onto the structure, with specific interaction residues implicated in the array study shown (stick). The Region-4 peptide is similarly shown, spanning the C-terminal linker (red) and CR3 helix (blue), as far as the currently available crystallographic limit. The FQF phenylalanines are shown (blue stick) with CR3 in a capped position across the catalytic pocket. (b) In the majority of PDE4 crystal structures the C-terminal linker and CR3 are disordered; an overlay of the CR3 peptides from those structures that exhibit some order reveals helical secondary structure but with degree of conformational variability, especially affecting the presentation of the second Phe in the FQF motif (which lies at or near the limit of ordered structure determined to date). (c) An orthogonal view of the CR3 overlay is shown to that in (b).
PDE4A5

(a)

N-terminal  UCR1  UCR2  Catalytic unit  C-terminal

(b)

Region 1 : Q121 - A155 : UCR1
Region 2 : D577 - E606 : Catalytic
Region 3 : H637 - P681 : Catalytic
Region 4 : L677 – T716 : C-terminal
(a) Region 1: A133 - R158: UCR1

Region 2: T582 – E606: catalytic

Region 3: V647 – Q671: catalytic

Region 4: G682 – D706: C-terminal
(a) Spot 1  2  3  4  5  6
GST
GST-UBC9
GST-Arrestin
GST-ERK
GST-RACK1

PDE4D5 : S651-D700
Spot
1:SPSPAPDDPEEGRQQGQTEKFQFELT
2:PDDEEGRQQGQTEKFQFELTLEEDG
3:EGRQQGQTEKFQFELTLEEDGESDT
4:QTEKFQFELTLEEDGESDTEKDGS
5:QFELTLEEDGESDTEKDGSQSVEED
6:LEEDGESDTEKDGSQSVEEDTSCD

(b) Spot 1  2  3  4  5  6
GST
GST-Lyn
GST-MK2
GST-RACK1

PDE4A5 : S667-T716
Spot
1:SAIRQSPSPPLEEEPGGLGHPSLPD
2:SPSPPLEEEPGGLGHPSLPDKFQFE
3:LEEEPGGLGHPSLPDKFQFELTLE
4:GGLGHPSLPDKFQFELTLEEEEED
5:QFELTLEEEEEEDSLEVP
6:KFQFELTLEEEEEEDSLEVPGLPTT

(c) PDE4A5 sequence P687-D706 alanine scan
Con P  S  L  P  D  K  F  Q  F  E  L  T  L  E  E  E  E  E  E  D
GST-Lyn

PDE4D5 sequence Q664-E685 alanine scan
Con Q  G  Q  T  E  K  F  Q  F  E  L  T  L  E  E  E  E  D  G  E  S  D  T  E
GST-UBC9
GST-Arrestin
GST-ERK
PDE4D5 sequence Q664-E685 alanine scan

ERK1 + βArrestin 1 (1 uM) Blotted for ERK and βArrestin