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Location, location, location: PDE4D5 function is directed by its unique N-terminal Region

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Compartmentalised cAMP Signalling

Characterisation of the cyclic AMP signalling pathway in glycogenolysis as the first "second messenger" system opened the door for study of "cellular signalling" as a field (reviewed in [1]). We now take for granted that G_s-coupled receptor activation results in the stimulation of adenylyl cyclase [2] to produce cAMP, and that cAMP exerts its effects through one of three effectors (Protein Kinase A [3], EPAC [4] or CNG[5]). Work by Brunton and colleagues[6] in the 1980's was pivotal to our understanding of compartmentalisation within the cAMP signalling axis, as many distinct G_s-coupled receptors drive receptor-specific responses via cAMP generation, so it figures that each receptor must couple to a defined set of signalling intermediates that relay signals in a spatially and temporally restricted manner. Key to this shaping of the receptor-specific cAMP response is the action of the only super family of enzymes that are known to degrade cAMP, the phosphodiesterases [7]. Depending on receptor type, cAMP produced at the cell membrane can reach far into the cell or stay localised to its site of production. The distance cAMP travels and its ability to form three-dimensional gradients, triggering activation of cAMP effectors, are determined by the phosphodiesterase (PDE) landscape [8]. Often, PDEs are expressed at low levels yet it is their localisation to demarcated positions within cells that underpins both their effectiveness and function. Indeed, reporter technology devised to visualise cAMP gradient formation following specific receptor activation [9] has confirmed that PDE positioning is crucial to the formation of multiple, simultaneous and spatially distinct cAMP gradients that drive defined physiological responses.

PDE families

PDEs are a vast super-family of distinct, highly regulated enzymes which can be classified based on primary structure into class I, II and III – the largest, class I,

consists of all known mammalian PDEs [10]. The mammalian PDE super-family contains 11 groups (PDE1-PDE11) that are classified based upon structural considerations such as protein domains, sequence homology, and enzymatic properties including substrate specificity, activity and sensitivity to endogenous regulators and inhibitors [11]. Reviews on the regulation, function and pharmacology of the different PDE families are many, however the current review will concentrate on one family that are particularly known for intracellular targetting, PDE4 [12]. The PDE4 gene family consist of 4 genes, PDE4A, B, C and D that encode over 20 different PDE4 isoforms due to alternative mRNA splicing. The genes from the PDE4 family are found at distinct locations on the chromosomes; PDE4A at Chr19p13.2, PDE4B at Chr1p31, PDE4C at Chr19p13.1 and PDE4D at Chr5q12. Each of these genes encodes approximately 20 exons that determine the nature of various domains including catalytic domain, regulatory regions and N-terminal regions responsible for intracellular targeting. Splice variants of the PDE4 gene family can be categorised into four groups; these are termed long form, short form, super short form and dead short form, differing in modular composition [13]. Long isoforms have regulatory regions UCR1 (Upstream Conserved Region 1) and UCR2 (Upstream Conserved Region 2), short isoforms lack UCR1 but contain a fully intact UCR2, super short isoforms have only a truncated form of UCR2 and finally dead short isoforms lack both regulatory regions UCR1 and UCR2, as well as having an inactive catalytic unit which is both N- and C- terminally truncated [14].

The modular structure of PDE4s is such that each and every isoform encoded by all 4 genes contain an almost identical catalytic unit that can be targeted to different cells and tissues by virtue of the unique N-terminal “postcode” that is distinct between isoforms [12]. Once in position, the regulation of activity depends on the nature of post-translational modification and whether it is a long or short form. Numerous combinations for activation, inhibition and feedback regulation exist and this allows PDE4 activity to be uniquely tailored to suit the biological situation. Our understanding of the different functions performed by individual PDE4 isoforms has been explored by many recent studies that have utilised modern biochemical techniques to silence or disrupt the localisation of individual enzymes. This review will use PDE4D5 as an exemplar of a single PDE4 isoform that has been linked to a number of cellular functions.

PDE4D5 isoform targeting.

The first example of a PDE4 being targeted to a distinct intracellular domain was from studies on PDE4A1. PDE4A1 is almost exclusively membrane bound [13] and in contrast to the rest of the protein-protein targeting mechanisms discussed here, the PDE4A1 N-terminal domain contains a localisation sequence that targets phosphatidic acid in cell membranes. Mutants of the PDE4 without a 25 amino acid stretch in its N-terminal region are completely cytosolic [15]. This unique protein-lipid interaction is gated by calcium and allows phosphatidic acid selectivity by negating inhibitory charges at the lipid–protein interface, promoting association of the PDE4A1 with the membrane [16]Whereas PDE4A1 is restricted to the membrane by lipid targeting, a long-form PDE4D isoform, PDE4D5, can be recruited to a variety of locations via a number of distinct protein-protein interactions with different scaffolding proteins in response to cellular stimuli. Each "pool" of PDE4D5 regulates a local cAMP pathway to orchestrate activation of a specific subset of cAMP effectors that drive diverse physiological events. Here we review distinct binding partners of PDE4D5, characterise the different protein-protein interactions and investigate the relationship between cellular location and function for each PDE4D5 subpopulation.

RACK1

RACK1, a multi-functional WD-repeat scaffold protein [17] was originally identified as a binding partner for PDE4D5 in a yeast two-hybrid screen in 1999 [18]. The interaction was noteworthy for two reasons; firstly the interaction did not change the activity of the enzyme suggesting that the major point of interaction was not within the catalytic unit and secondly, the interaction seemed to be PDE4D5 specific as other isoforms, including those from the PDE4D subfamily, did not co-purify with RACK1. Both of these observations suggested that RACK1 binding was dependent on interaction with the unique N-terminal of PDE4D5 and this notion was supported by further yeast-two hybrid studies that showed the unique 88 amino acid N-terminal alone was sufficient to form a complex with RACK1 [18]. A more detailed yeast-two hybrid investigation a number of years later delineated a region named RAID1 (RACK interacting domain 1) spanning the PDE4D5 N-terminal between amino acids 12 and 49 as the crucial domain that bound to RACK1 with a similar affinity to the full length PDE4 protein [19]. Subsequent analysis using peptide array technology, in which a library of surface immobilised peptides corresponding to the full sequence of PDE4D5 were probed with purified RACK1, further refined the RAID1 region to between residues 22 and 45 of the PDE4D5 N-terminal [20]. Alanine scanning of this

region highlighted many of the essential residues found during initial yeast two-hybrid experiments [19]. Surprisingly, another binding region for RACK1 was detected between residues 609 and 658 of the catalytic unit of PDE4D5 called RAID2 (RACK interacting domain 2) during the peptide array mapping, and this was confirmed using yeast two-hybrid experiments [20]. RAID2 was mapped onto the crystal structure of the PDE4 catalytic domain [21] to reveal a surface exposed patch on helices 15a, 15b and 16 as well as the flexible linker between helices 16 and 17 [20]. A binding conformation that could alter the inhibition by rolipram, a PDE4 inhibitor, but not substrate (cAMP) binding was proposed [20].

Although the interaction between PDE4D5 and RACK1 had been fully characterised by 2006, the function of the complex remained unknown until 2010 when it was observed that PDE4D5 in a complex with RACK1 and Focal Adhesion kinase (FAK) translocated to the leading edge of polarizing cancer cells [22]. Previous work utilising the information from peptide array analysis of the PDE4D5-RACK1 interaction [20] had informed the development of cell permeable peptides that acted to disrupt the complex [23]. PDE4D5-RACK1 disruptors were pivotal for functional studies that illustrated the importance of PDE4D5 localisation by RACK1 to the leading edge of cells to control cAMP signalling events that are needed for the formation of nascent integrin adhesions as cell spread [22]. In short, cancer cells treated with the PDE4D5-RACK1 disruptor peptides could not undergo directional responses to initiate wound healing or chemotactic invasion, whereas cells treated with a disruptor to another scaffold for PDE4D5, beta-arrestin, behaved normally. Follow up work proved that the PDE4D5-RACK1-FAK direction-sensing complex signalled via a spatially restricted pool of EPAC to Rap1 to ensure efficient focal adhesion stabilisation [24].

Beta-arrestin

The second functional binding partner for PDE4D5 to be discovered was the signalling scaffold protein beta-arrestin (review of arrestin [25]). Appreciation that PDE4D5 translocated to the ^{2} 2-adrenergic receptor as part of the desensitisation mechanism with similar kinetics to the well characterised beta-arrestin prompted investigations into a functional complex involving the two proteins [26]. Interestingly, translocation of PDE4D5 by arrestin to the cell membrane aided receptor desensitisation by destroying the intracellular message (cAMP) in this vicinity whilst simultaneously arrestin was shutting down the signal between the activated receptor and G-protein (G_s) [26]. This “double whammy” efficiently shut down ^{2} 2-receptor

signalling and prevented further phosphorylation of the receptor by PKA, an action that promotes a switch in coupling of the receptor from G_s to G_i [27]. The importance of targetting a small but active pool of PDE4D5 to the ² 2-AR immediately after receptor activation was highlighted by the use of siRNA [28] and dominant negative forms of catalytically inactive PDE4D5 that act to displace the endogenous active pool [27]. Disruption of the pool of PDE4D5 associated with ² -arrestin in this manner not only increased localized PKA phosphorylation of the ² 2-AR [28] but dampened the reprogramming of its coupling specificity from G_s to G_i [27] attenuating the mitogenic signal mediated by the tyrosine kinase Src [29]. Disruptor peptides directed specifically at the PDE4D5-arrestin interaction had a similar action [23].

Molecular characterisation of the PDE4D5-arrestin interaction by peptide array and yeast two-hybrid indicated that arrestin bound over the same stretch of the PDE4D5 N-terminal domain that had been shown to confer RACK1 binding [20, 30]. Unsurprisingly, the interaction with the phosphodiesterase by the scaffolds was mutually exclusive when examined using yeast two-hybrid or co-immunoprecipitation analysis. In fact, dual overlay experiments where equivalent amounts of arrestin and RACK1, where applied to alanine scanning peptide arrays of the PDE4D5 N-terminal region, illustrated that regions or single amino acids existed in the PDE4D5 N-terminus that bound arrestin, RACK1, both or neither [20]. This information was used in conjunction with the NMR structure of the PDE4D5 N-terminus to envisage the binding modes of both RACK1 and arrestin with PDE4D5, and to create cell permeable peptides that could specifically disrupt the PDE4D5-RACK1 and PDE4D5-arrestin complexes [23]. Surprisingly, RACK1 is not the only protein to compete with arrestin for PDE4D5. A recent report suggests that EPAC1, a cAMP effector, binds to arrestin and is recruited to ² 2-AR following adrenergic stimulation [31]. Disruption of the PDE4D5-arrestin complex using cell permeable peptides promoted EPAC-arrestin complex formation to drive hypertrophic signalling events.

As the many functions of beta-arrestin are conferred by its ability to associate with hundreds of binding partners [26], the PDE4 binding sites on arrestin were discovered using similar approaches as those described above [32]. In common with other client proteins of arrestin, PDE4 bound to sites within both the N- and C-domains of the scaffold [30]. Mutant forms of the scaffold could be designed that do not associate with PDE4s. These mutations did not hinder arrestin translocation to receptors, but did promote PKA phosphorylation of the receptor by virtue of the lack of PDE4 activity within the proximity of the activated receptor [32]. Cellular regulation

of the abundance of the PDE4D5-arrestin complex can be controlled by ubiquitination [33]. This post-translational modification occurs on three lysines within the unique N-terminal of PDE4D5 (and one lysine in the UCR1 common region) and is mediated by a pool of MDM2 that is tethered by arrestin itself. Ubiquitination of PDE4D5 contributes to the fidelity of the arrestin interaction, thereby decreasing occurrence of the PDE4D5-RACK1 complex [33].

The importance of PDE4D5 recruitment in the fine control of sub-plasmalemma cAMP dynamics following receptor activation has been illustrated using a variety of techniques in a range of different cells. Reporters of cAMP based upon the structure of mutant cyclic nucleotide gated ion channels (CNGC) have shown that localised cAMP responses to isoprenaline in HEK293 cells are regulated by PDE4D5 recruited by arrestin, whereas the Gs-coupled response to prostaglandin are controlled by a different static pool of arrestin-independent PDE4D5 anchored by an A-kinase Anchoring Protein (AKAP), AKAP250 [34]. The cAMP compartments maintained by these differentially localised pools of the same enzyme surprisingly allow heterologous desensitisation of the ² 2-AR by cAMP produced by the prostaglandin receptor but the converse is not true. In neonatal cardiac myocytes, PDE4D5 recruitment by arrestin has been shown to desensitise the ² 2-AR but not the ² 1-AR [35]. In the latter case, a pool of another PDE4D isoform, PDE4D8, is actually released from the ² 1-AR to allow more extensive diffusion of cAMP following ² 1-AR activation. Localised PDE4D5 has also been shown to associate with the 5-hydroxytryptamine receptor [36] and the transient receptor vanilloid 1 (TRPV1) [37] to regulate downstream signalling. Over-expression of dominant negative PDE4D5 constructs increased release of glucagon-like peptide 1 (GLP-1) to influence insulin secretion in enteroendocrine L cells [38]. Finally, a recent report links a pool of PDE4D5 with cAMP regulation in longitudinal muscle cells that had been treated with TNF-alpha or IL-1 beta [39]. In this study, PDE4D5 activity regulated smooth muscle relaxation and influenced colonic dysmotility during inflammation.

HSP20

HSP20 is a small heatshock chaperone protein that is induced by cell stress. It has been identified as a multi-functional protective protein (reviewed in [40]) due to its many actions in combatting the deleterious effects of diseases such as cancer, neurological conditions and cardiovascular disease. Intriguingly, HSP20 is known to lie dormant in cells until it is activated by cellular stress. One modification that is key

to the activation of HSP20 is phosphorylation on serine 16 by PKA [41]. The protective phosphorylation is aided by HSP20's ability to complex with AKAP Lbc, bringing it within close proximity to PKA [42] . Conversely, the protective phosphorylation of HSP20 is attenuated by the chaperone's capacity to bind to members of the PDE4 family, including PDE4D5 [43]. Inhibitors of PDE4 activity promote the phosphorylation of HSP20 [44] and the localised nature of the PDE4-HSP20 complex has been demonstrated using cAMP reporters that are bound to the chaperone or are ubiquitously expressed in the cytoplasm of cells. [43]. PDE4 inhibitors alone do not significantly affect global cellular cAMP but significantly alter the cAMP dynamics in the vicinity of HSP20. The small pool of PDE4D5 that is bound to HSP20 to gate phosphorylation by PKA on serine 16 was identified by co-immunoprecipitation and ELISA but in contrast to the other PDE4D5 partners and in keeping with the fact that HSP20 can bind other PDE4 isoforms, the HSP20 binding site on PDE4D5 is not within the N-terminal but rather in the conserved catalytic region [43]. Cell permeable peptides corresponding to the HSP20 binding region could not only attenuate the formation of the PDE4D5-HSP20 complex but also promote phosphorylation of HSP20 on serine 16, without prior artificial elevation of cAMP levels. The ability of the peptide to trigger HSP20 phosphorylation under conditions of basal adenylate cyclase stimulation highlights the importance of the compartmentalisation of PDE4 in maintaining HSP20 in its "activatable" state, primed to combat cell stress. Indeed, the HSP20-PDE4D5 disruptor peptide was shown to enhance HSP20-mediated protection against the hypertrophic response induced by chronic isoprenaline treatment in neonatal cardiac myocytes and to attenuate pathological cardiac remodelling in a mouse model of pressure overload [45]. In the future, the strategy of disrupting the PDE4D5-HSP20 complex to promote phosphorylation of HSP20 may also have potential as a therapeutic intervention to combat Alzheimer's disease, where phosphorylation of HSP20 promotes the chaperone's association with beta-amyloid [46] and ischemia/reperfusion injury where phospho-HSP20 prevents autophagy and cell death [47].

Conclusion

The compartmentalisation of proteins that generate, degrade and are stimulated by cAMP is vital for the shaping of cAMP driven cell signalling cues that are finely tailored to support normal cellular function. Studies identifying the multiple binding partners of PDE4D5 have pinpointed this particular phosphodiesterase as a paradigm for the compartmentalisation of these enzymes. Guidance by isoform-

specific N-terminal regions direct specific interactions with scaffold and PKA substrate proteins, with biased complex formation between scaffolds such as RACK1 and beta-arrestin exemplifying the specialised targeting mechanisms utilised to permit PDE4D5 to perform different biological functions in a spatially and temporally controlled manner. The use of disruptor peptides to attenuate PDE4D5 coupling to protein partners has not only allowed recognition of isoform specific functions but has identified novel potential therapeutic avenues for both cardiovascular hypertrophy and integrin adhesion in metastasis. It is clear, therefore, that future intervention of aberrant intracellular cAMP signalling should be directed not towards the regulation of PDE4 activity, but alteration of enzyme localisation via interdiction of intracellular targeting mechanisms.

Figure 1 – A schematic representation of the long form PDE4D5 structure. Interaction sites for RACK1, beta-arrestin and HSP20 are outlined. The unique 88 amino acid N-terminal possesses binding sites, shared by the RACK1 and beta-arrestin scaffold proteins. The catalytic domain hosts accessory binding regions to these scaffolds in addition to HSP20 interacting region.

Figure 2 – Consequences of PDE4D5 complex disruption. Ablation of PDE4D5 interactions using disruptor peptides promotes beneficial cellular effects with clinical significance.

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