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Scanning peptide array analyses identify overlapping binding sites for the signaling scaffold proteins, β arrestin and RACK1 in the cAMP-specific phosphodiesterase, PDE4D5.

Graeme B. Bolger^{‡∞}, George S. Baillie^{#∞}, Xiang Li[#], Martin J. Lynch[#], Pawel Herzyk[†], Ahmed Mohamed[#], Lisa High Mitchell[‡], Angela McCahill[#], Christian Hundsrucker[¶], Enno Klussmann[¶], David R. Adams[¥] and Miles D. Houslay[#]

[‡]From the University of Alabama at Birmingham, Comprehensive Cancer Center, Birmingham, Alabama 35294-3300, USA; [#]Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland, UK; [†] Sir Henry Wellcome Functional Genomics Facility, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland, UK [¶]Forschungsinstitut für Molekulare Pharmakologie, Campus Berlin-Buch, Robert-Rössle-Str. 10, 13125 Berlin, Germany; [¥]Department of Chemistry, Heriot-Watt University, Riccarton Campus, Edinburgh, Scotland, UK.

[∞] These authors contributed equally to this study and should be considered as joint first authors.

[§] Address correspondence to:- Professor Miles Houslay, Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland, UK; tel +44(0)-141-330-4624; fax +44(0)-141-330-4365; email M.Houslay@bio.gla.ac.uk

Running title:- β arrestin and RACK1 interact with Phosphodiesterase-4D5

The cAMP-specific phosphodiesterase PDE4D5 can interact with the signaling scaffold proteins RACK1 and β arrestin. 2-hybrid and co-immunoprecipitation analyses showed that RACK1 and β arrestin interact with PDE4D5 in a mutually exclusive manner. Overlay studies with PDE4D5 scanning peptide array libraries showed that RACK1 and β arrestin interact at overlapping sites within the unique N-terminal region of PDE4D5 and at distinct sites within the conserved PDE4 catalytic domain. Screening scanning alanine substitution peptide arrays, coupled with mutagenesis and truncation studies, allowed definition of RACK1 and β arrestin interaction sites. Modelled on the PDE4D catalytic domain these form distinct, well-defined surface exposed patches on helices 15-16, for RACK1, and helix-17 for β arrestin. siRNA-mediated knockdown of RACK1 in HEK293B2 cells increased β arrestin-scaffolded PDE4D5 approximately 5-fold; increased PDE4D5 recruited to the β_2 AR upon isoproterenol challenge approximately 4-fold and severely attenuated (approximately 4-5 fold) both isoproterenol stimulated PKA phosphorylation of the β_2 AR and activation of ERK. The ability of a catalytically inactive form of PDE4D5 to exert a dominant negative effect in amplifying isoproterenol-stimulated ERK activation was ablated by a mutation that blocked the interaction of PDE4D5 with β arrestin. Here we show that the signaling scaffold proteins RACK1 and β arrestin compete to sequester distinct 'pools' of PDE4D5. In this fashion, alterations in the level of RACK1 expression may act to modulate signal transduction mediated by the β_2 -adrenergic receptor.

Abbreviations:- (β_2 AR, β_2 -adrenergic receptor; GPCR, G-protein coupled receptor; GRK2, G-protein receptor-associated kinase 2; PDE, cyclic 3', 5' AMP phosphodiesterase; PDE4, rolipram-inhibited, cAMP specific PDE; rolipram, 4-{3-(cyclopentoxyl)-4-methoxyphenyl}-2-pyrrolidone; PKA, protein kinase A; ERK, extracellular signal regulated kinase

Keywords:- scanning peptide array; rolipram; PDE4; cAMP phosphodiesterase; β arrestin; RACK1; PKA; cAMP dependent protein kinase; phosphorylation; β_2 -adrenergic receptor; Odyssey.

Introduction

G-protein coupled receptors (GPCRs), such as the β_2 -adrenergic receptor (β_2 AR), couple to the G-protein G_s and thereby activate adenylyl cyclase [1-3]. β_2 AR desensitization occurs upon its phosphorylation by G-protein receptor-associated kinase 2 (GRK2) and the consequential recruitment of cytosolic β arrestins [2, 4-6], which blocks β_2 AR coupling to G_s . However, the β_2 AR can also be phosphorylated by PKA, which switches its coupling to G_i , causing activation of ERK1/2 [5, 7-10].

The sole means of degrading cAMP in cells is through cAMP phosphodiesterase (PDE) action [11-13]. There is much interest in the cAMP-specific phosphodiesterase-4 (PDE4) [14-17] family as PDE4 selective inhibitors exert anti-depressant, memory-enhancing, immunomodulatory and smooth-muscle relaxant activity in humans and mammals [14, 16, 18-22]. Four genes generate around 18 PDE4 isoforms, each characterized by a unique amino-terminal region that determines its intracellular targeting [15, 23-29].

PDE4 enzymes contain an as yet undefined binding site within their conserved catalytic unit that allows them to interact functionally with β arrestins [5, 8, 30-33]. One functional consequence of this interaction is that β arrestins serve to recruit PDE4 isoforms to the agonist-occupied, GRK2-phosphorylated β_2 AR, where they lower cAMP levels in the local environment of the β_2 AR, reduce PKA activity, and thereby regulate PKA-phosphorylation of the β_2 AR. Through this action, the recruited PDE4 serves to regulate the ability of the β_2 AR to switch between the G_s -mediated activation of adenylyl cyclase and the G_i -mediated activation of ERK2 signaling.

The PDE4D5 isoform exhibits preferential interaction with β arrestins as its unique N-terminal region contains a β arrestin binding site in addition to that associated with the catalytic unit (Fig. 1) [30]. However, uniquely amongst PDE4 isoforms identified to date, PDE4D5 can also bind to the widely expressed signaling scaffold protein RACK1 [23, 26]. The isoform-specific 88 amino acid N-terminal region of PDE4D5 thus contains a RACK binding site (RAID1), extending from Asn22 to Leu38 [23] and, as deduced from truncation studies, a β arrestin binding site, extending from Met70 to Cys88 [30].

Since the PDE4D5 unique N-terminal region provides sites for both RACK1 and β arrestin binding we set out here to determine whether PDE4D5 could form a complex

with both of these signaling scaffolds or if their binding to PDE4D5 would be mutually exclusive and potentially regulatory. Although current studies indicate that these sites are discrete [23, 30] it is unclear whether steric interactions would prevent simultaneous binding of RACK1 and β arrestin to PDE4D5, especially as β arrestin, presumably, straddles PDE4D5 by binding to both its N-terminal region and catalytic unit [30]. Here we have used a novel peptide array approach, coupled with immunoprecipitation and 2-hybrid studies, to identify and characterize binding sites for RACK1 and β arrestin on PDE4D5 and demonstrate that RACK1 and β -arrestin bind in a mutually exclusive and regulatory fashion to PDE4D5.

Experimental procedures.

Materials—Polyclonal antisera specific to the PDE4D subfamily were used as described previously [8, 30, 31, 34]. Antibodies for detection of the native and phosphorylated forms of ERK1/2 and for detection of phosphoserine PKA substrates were from Cell Signaling Technologies (Beverly, MA, USA). A rabbit polyclonal antibody against the β_2 AR was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-FLAG M2 mouse monoclonal antibody, plus a conjugate of the same antibody to agarose, protein A beads, VSV antibody, IgM agarose and a tubulin antibody were from Sigma-Aldrich (Poole, Dorset, UK). An IgM monoclonal antibody against RACK1 was from BD Biosciences (San Jose, CA, USA). Antisera detecting β arrestin1/2 were from Dr R. Lefkowitz (Duke Univ., NC). HEK293B2 cells [35], which stably express a GFP- and Flag-tagged β_2 -adrenoceptor, were from Dr. G. Milligan (Univ Glasgow, UK).

Analysis of β arrestin-RACK1-PDE4D5 Interactions by Two hybrid Assay—This was performed using techniques described previously [26, 30, 36]. As “bait”, PDE4D5 cDNA (GenBank™ AF012073) was cloned into the NotI site of pLEXAN and for “prey” β -arrestin1 (GenBank™ BC003636), β arrestin2 (GenBank™ BC007427) or RACK1 (GenBank™ M24194) were cloned into the NotI site of pGADN. In ‘competition’ experiments, RACK1 was expressed with only an amino-terminal nuclear localization signal and, with the “bait”, cloned into a pBridge derivative [37]. Filter β -galactosidase assays were performed in *S. cerevisiae* strain L40.

Site-directed Mutagenesis—This was performed by the circular mutagenesis method, as described previously [23, 26]. All mutagenesis and deletion constructs were confirmed by DNA sequencing prior to use.

Mammalian Cell Expression Constructs — Human PDE4D5 cDNA [34] with a carboxyl-terminal VSV epitope tag, was cloned into pcDNA3 (Invitrogen, Paisley, Scotland, UK). pcDNA3NARB2FLAG, encoding wild-type, carboxyl-terminal FLAG-epitope tagged human β arrestin2, was described previously [30].

Cell Culture — HEK293 and HEK293B2 cells were cultured as before [8, 30, 31]. Transfections were performed using Polyfect (Qiagen), according to the manufacturer's instructions.

Immunoprecipitation Studies to Evaluate the Interaction between Recombinant β -Arrestin2 and PDE4D5 Constructs in Transfected Cells—This was performed as described previously [7, 8, 30, 31]. In some instances cells were transfected to express either VSV-tagged forms of PDE4D5 or a β arrestin2-GFP fusion protein.

Expression of Fusion Proteins in Escherichia coli—Full-length PDE4D5 was expressed as an amino-terminal maltose-binding protein (MBP) fusion and human β arrestin1 and β arrestin2 were expressed as amino-terminal glutathione *S*-transferase (GST) fusions as before [26, 30]. In some experiments, β -arrestin1 was expressed as a carboxyl-terminal hexahistidine fusion [30]. Proteins were expressed and purified as before [26, 30].

SDS-PAGE and Immunoblotting—This was performed as described previously by us [8, 26, 30].

SPOT synthesis of Peptides and Overlay Experiments — Peptide libraries were produced by automatic SPOT synthesis [38]. They were synthesized on continuous cellulose membrane supports on Whatman 50 cellulose membranes using Fmoc-chemistry with the AutoSpot-Robot ASS 222 (Intavis Bioanalytical Instruments AG, Köln, Germany) [38, 39]. The interaction of spotted peptides with GST and GST fusion proteins was determined by overlaying the cellulose membranes with 10 μ g/ml of recombinant protein. Bound recombinant proteins were detected with specific rabbit antisera and detection performed with secondary anti-rabbit horseradish peroxidase coupled antibody (1:2,500 dilution; Dianova) as for immunoblotting. Where indicated, dual detection was done at 680 and 800nm using a LI-COR Odyssey infrared imager (LI-COR Biosciences, Cambridge, UK). The anti- β arrestin antisera, raised in rabbit, was detected using goat anti rabbit IgG conjugated to Alexa Fluor 680 (Molecular Probes, Eugene, Oregon, USA) and the anti-RACK1 monoclonal antibody detected using rabbit anti-mouse IgG conjugated to IRDye 800 (Rockland, Gilbertsville, PA, USA).

Generation and Testing of siRNA RACK1 Knockdown Constructs — The RACK1 mRNA sequence was scanned for appropriate “target” regions for siRNA generation [40] and using the Ambion program (http://www.ambion.com/techlib/tb/tb_506.html). All “target” sequences were 21 nucleotides long and started with an AA dinucleotide, consistent with the observations of Elbashir et al. [41]. Sequences identified by this approach were then scanned against the GenBank™ nucleotide database and only those that were unique to RACK1 were considered further. DNA oligonucleotides from 4 candidate “target” sequences were synthesized and used to create siRNA expression cassettes (SECs) in which each of the “target” sequences was expressed under the control of either of the U6 or H1 RNA Pol II promoter. Each of the 8 SECs was then tested for its ability to knockdown RACK1 expression. For this purpose, COS7 cells were co-transfected, using Lipofectamine (Invitrogen), with the SEC and the plasmid pEGFP-RACK1, which encodes a fusion between enhanced green fluorescent protein and the amino-terminus of RACK1, and the effect of each SEC was scored as a reduction in GFP fluorescence. Four SECs consistently produced a greater than 90% reduction in GFP-RACK expression. These 4 SECs were then cloned into the vector pSecPuro (Ambion) and the resulting constructs were then tested for their ability to knockdown GFP-RACK1 in the same assay. All 4 cloned SECs were produced a greater than 90% reduction in RACK1 expression and one, called pRACKSEC-H1-36, which consistently produced greater than 95% reduction in RACK1 expression, was used in all subsequent experiments.

Homology Modeling – This was performed using Modeller software package version 7v7 [42]. Two models of C-terminal parts of PDE4D (amino acids 324-677) were generated using structures of corresponding C-terminal stretches of PDE4B, PDB codes 1F0J [43] and 1XM6 [44], respectively. In addition, both models used the structure of catalytic domain of PDE4D, namely 1XOM [44], as a second template. In case of 1F0J template, the position of C-terminal helix-17 was taken from the symmetry related molecule. This helix was then replaced by helix-17 from the 1XM6 structure (after structural superposition) as the latter was longer by five residues towards the C-terminus and contained a full stretch corresponding to PDE4D5 amino acids ⁶⁷⁰FQFELTL⁶⁷⁶. The modeling procedure was repeated ten times in each instance with the model having the lowest objective function being selected. Finally, the catalytic domain (amino acids 324-

647) was replaced by the corresponding one from the PDE4D structure (1XOM) and the C-terminal stretch 647-677 was energy minimized.

Results

RACK1 and β arrestin bind in a mutually exclusive fashion to PDE4D5 — RACK1 and β arrestin2 have previously been shown to interact with PDE4D5 (Fig. 1). Here we investigate whether they bind simultaneously to PDE4D5 or if their binding is mutually exclusive. Using a 2-hybrid assay with PDE4D5 expressed as “bait” and β arrestin2 as “prey”, we additionally expressed RACK1 as a “competitor” (Fig. 2a). This showed that expression of RACK1 ‘competitor’ completely blocked the interaction between PDE4D5 and β arrestin2 (Fig. 2a), indicating that RACK1 and β arrestin2 compete for binding to PDE4D5 rather than forming a heterotrimeric complex. Note that RACK1 is unable to interact directly with either β arrestin1 or β arrestin2 (Fig 2b).

We confirmed this result by examining immunopurified complexes from HEK293B2 cells. Immunoprecipitates of endogenous β arrestin contained endogenous PDE4D5, but not endogenous RACK1 (Fig. 2c). Attempting to identify β arrestin in RACK1 immunoprecipitates poses a fundamental difficulty as β arrestin migrates similarly to the Ig heavy chains from the immunoprecipitating antiserum. Thus we transfected HEK293B2 cells with a GFP- β arrestin2 chimera, which is of higher molecular weight. This allowed us to identify PDE4D5, but not GFP- β arrestin2 in immunoprecipitates of endogenous RACK1 (Fig. 2d).

In order to ascertain the distribution of RACK1- and β arrestin-sequestered PDE4D5 in HEK293B2 cells we undertook immunopurification of the endogenous pools of RACK1 and β arrestin and immunoblotted each pool for PDE4D5. Of total PDE4D5 in HEK293B2 cells some 31 ± 5 % is associated with RACK1 and 15 ± 3 % with β arrestin (means \pm SD; n=3).

Note that densitometric analysis of endogenous β arrestin immunoprecipitates did, as noted before by us [7], indicate a very small amount of associated PDE4D3 (data not shown). This comprised only some 6-11% of the total β arrestin-associated PDE4D (range; n=3). Our dominant negative and siRNA-mediated KO studies [7] clearly show is that it is PDE4D5 and not PDE4D3 that is functionally important in mediating actions consequent upon β arrestin recruitment to the β_2 AR. PDE4D3 appears to be sequestered

to other scaffolds in these cells and, with the exception of this residual pool, is essentially unavailable for interaction with β arrestin [11].

Peptide array analysis defines a binding site for RACK1 in the unique N-terminal region of PDE4D5 — Previous 2-hybrid, pull-down and co-immunoprecipitation studies showed that RACK1 interacts with the unique N-terminal region of PDE4D5 (Fig. 1) [23, 26]. Here explored this interaction site further using peptide array analysis, which provides a novel and powerful technology [38, 39]. A library of overlapping peptides (25 mers), each shifted by 5 amino acids across the entire sequence of PDE4D5 was immobilized on cellulose membranes and probed with recombinant RACK1-GST. Dark spots signified positive interactions (Fig. 3). Dramatically, the RACK1 probe interacted strongly with a single peptide (Fig. 3; peptide 5) that contains all of the sequence, extending from Asn22 to Leu38, constituting the proposed RACK1 binding site (RAID1) in PDE4D5 [23]. However, peptide 4 also contains this sequence while interacting considerably less effectively (Fig. 3). This implies that amino acids within the sequence novel to peptide 5, namely E⁴¹KSKT⁴⁵ are additionally important for RACK1 binding to PDE4D5.

We thus set out to define further the amino acids involved in forming RAID1. To do this we screened a family of peptides derived from a 25-mer parent peptide whose sequence reflected amino acids Asn22 - Thr45 of PDE4D5. The 25 progeny of this parent peptide each had a single substitution, to alanine, of successive amino acids in the sequence to form a scanning peptide array (Fig. 4a). This showed a striking absence/loss of binding upon substitution of Asn22, Pro23, Trp24, Asn26, Val30, Lys31, Leu33, Arg34, Glu35, Asn36, Leu37, Glu41, Lys42 and Lys44 (Fig. 4a). Loss of RACK1 binding upon substitution of the charged amino acids in E⁴¹KSKT⁴⁵ explains why peptide 4 bound weakly to RACK1 compared to peptide 5 (Fig. 3).

We set out to assess whether the various amino acids identified as being important in conferring RACK1 interaction upon PDE4D5 from our peptide array analysis might also be important in allowing interaction between full length PDE4D5 and RACK1. We have previously shown [23, 26] that deletion of the unique N-terminal region of PDE4D5 (Met1 to Cys88) ablates the interaction of PDE4D5 with RACK1 in 2-hybrid and co-immunoprecipitation analyses. Thus, using these biochemical assays, we can expect to identify single amino acid mutations in the unique N-terminal region that disrupt interaction with RACK1. In a 2-hybrid assay we see good correlation with the scanning peptide array analyses. Thus loss/reduced interaction was observed upon alanine

mutation of the core RAID1 [23] cluster of Asn22, Pro23, Trp24, Asn26, its hydrophobic extension of Val30, Leu33 and Leu37 plus the basic amino acid, Arg34 and Asn36 (Fig. 4a). Note that in 2-hybrid analyses we pairwise mutated Leu29:Val30 and Leu37:Leu38 to alanine. However, peptide array analysis indicated that Val30 and Leu37 are the species underpinning RACK1-PDE4D5 interaction (Fig. 4a). In contrast to the alanine substitution peptide array analyses, the following mutations in full-length PDE4D5, namely Lys31Ala, Glu35Ala, Glu41Ala, Lys42Ala and Lys44Ala, did not ablate PDE4D5-RACK1 interaction in the 2-hybrid assays (Fig. 4a). It would appear that in full-length PDE4D5 any potential disruption at such sites is compensated for in some way.

To evaluate PDE4D5-RACK1 interaction further we immunoprecipitated endogenous RACK1 from HEK293B2 cells transfected to express either wild-type or various mutant forms of epitope-tagged PDE4D5 (Fig. 4b). These experiments showed that PDE4D5 interaction with RACK1 was severely reduced/ablated using the Asn22Ala, Trp24Ala, Leu33Asp and Arg34Ala PDE4D5 mutants (Fig. 4b).

Peptide array analysis defines a binding site for β arrestin in the unique N-terminal region of PDE4D5 — In order to define where β arrestin2 binds in the 88 amino acid, unique N-terminal region of PDE4D5 we probed the PDE4D5 peptide library with recombinant β arrestin2-GST (Fig. 3). The clear interaction (Fig. 3) of β arrestin with peptide 13 (Arg61 – Ala85) is consistent with our previous progressive truncation analyses that led us to infer that the region from Met70 to Cys88 was involved in β arrestin binding [30]. However, peptide array analysis indicates that β arrestin is able to bind to amino acids covering a broad part of the PDE4D5 N-terminal region, extending from Thr11 through Ala85 (Fig. 3).

As this region encompasses RAID1 we set out to examine explicitly whether β arrestin can compete with RACK1 to bind to the N-terminal region of PDE4D5. To do this we simultaneously probed peptides 3 - 6, which encompass RAID1, with equimolar amounts of β arrestin and RACK1 (Fig. 4c). Simultaneous detection of β arrestin and RACK1 binding was done using the Odyssey® system using an anti-RACK1 mouse antibody and an anti- β arrestin rabbit antibody, each linked to distinct secondary antisera labeled with different wavelength probes. This independently identified (Fig. 4c) β arrestin2 (green) and RACK1 (red) associated with specific peptide spots in single channel analyses. We then combined signals from both channels (Overlay). Analysis using merged channels identified peptide spots with predominant or solely RACK1-

PDE4D5 complexes (red), those with predominant or solely β arrestin-PDE4D5 (green), those with mixed populations of RACK1-PDE4D5 and β arrestin-PDE4D5 (yellow) and those not interacting with either giving a null result (black). This showed that peptides 4 and 5, which each encompass RAID1, contain sub-populations of peptides that are complexed with either β arrestin or RACK1 (yellow), while peptides 3 and 6 contain solely β arrestin-PDE4D5 complexes, indicated as green spots in the overlay (combined) channel (Fig. 4c).

As the RACK1 binding site is fully contained within the Asn22-Thr45 peptide, we set out to identify the sites of interaction of β arrestin within this peptide (Fig. 4d). This we did by screening a library of peptide progeny generated by alanine scanning substitution. β arrestin binding was either ablated in the case of Leu33Ala and Arg34Ala or severely attenuated with Asn26Ala, Glu27Ala, Asp28Ala, Leu29Ala and Val30 (Fig. 4d). Thus, β arrestin and RACK1 both appear to require Leu33 and Arg34 for binding to this peptide (Fig4a, d, e).

Again we used Odyssey analysis to probe the Asn22-Thr45 alanine scanning peptide array simultaneously for the formation of both RACK1 and β arrestin complexes (Fig. 4e). Challenging this array with equimolar amounts of β arrestin and RACK1 showed that the control (native) peptide spot contained a mixed population of peptide complexes involving both RACK1 and β arrestin (Fig. 4e; yellow). This method of analysis clearly identifies both Leu33 and Arg34 as common sites of interaction for both RACK1 and β arrestin, as alanine substitution of either of these amino acids clearly ablated their binding (Fig. 4e; black). Alanine substitution of Glu27, Asp28 and Leu29 selectively compromised the binding of β arrestin, leaving the binding of RACK1 to predominate (Fig. 4e; red). Alanine substitution of Asn22, Pro23, Trp24, Asn26, Val30, Lys31, Asn36, Leu37, Glu41, Lys42 and Lys44 selectively compromised the binding of RACK1, leaving the binding of β arrestin to predominate (Fig. 4e; green). Alanine substitution of a further set of amino acids (Fig. 4e; yellow) did not appear to affect the binding of either β arrestin or RACK1 with complexes of both found associated with peptides in these spots as seen in the native peptide.

We then used immunoprecipitation studies to confirm that alanine mutation of Glu27, Leu33 and Arg34 ablated the interaction between PDE4D5 and β arrestin, while mutation of L25 did not (Fig. 4b).

The catalytic region of PDE4D5 contains an interaction site for β arrestin — Here we set out to define the site within the PDE4D catalytic unit that interacts with β arrestin2 by probing the PDE4D5 peptide library with recombinant β arrestin2-GST (Fig. 3). This probe interacts with four, sequentially located, peptides (Fig 3; peptides 130-133) in the PDE4D5 array whose sequence spans Ala655 - Glu694 of PDE4D5 (Fig. 3). These peptides all contain the E⁶⁶⁸KFQFELTLEE⁶⁷⁸ motif, which is conserved in all PDE4 proteins.

We also used 2-hybrid analysis to obtain independent confirmation that residues within the E⁶⁶⁸KFQFELTLEE⁶⁷⁸ motif contribute to PDE4D5- β arrestin2 interaction. Thus the Phe670Ala:Gln671Ala:Phe672Ala-PDE4D5 and Leu674Ala:Thr675Ala:Leu676Ala-PDE4D5 mutants failed to interact with β arrestin2, whilst the Glu668Ala:Lys669Ala-PDE4D5, Glu673Ala-PDE4D5 and Glu677Ala:Glu678Ala:Asp679Ala-PDE4D5 mutants still interacted with β arrestin2 (Fig 2e). However, all of these PDE4D5 mutants bound RACK1 (Fig 2e).

In order to gain further insight into the interaction of β arrestin with the PDE4D catalytic unit we used scanning alanine substitution of a peptide representing amino acids Glu660 to Glu685 in PDE4D5 (Fig. 5a). Substitution, with alanine, of any one of Phe670, Phe672, Leu674 or Leu676 led either to ablation or severe attenuation of the interaction of β arrestin with PDE4D5 (Fig. 5a). This shows remarkable agreement with the 2-hybrid analyses where various alanine mutants of PDE4D5 in this region were assessed for their ability to bind to β arrestin (Fig. 2e).

The catalytic region of PDE4D5 contains an interacting site for RACK1 — Probing the PDE4D5 peptide array library with RACK1-GST not only identified the established N-terminal binding site but, additionally, identified a novel RACK1 binding site associated with the PDE4D5 catalytic unit (Fig. 3). Indeed, RACK1 interacted with 10 sequential peptides in this region, namely 120 through 129 (Fig. 3). RACK1 did not appear to interact uniformly with all of these, as indicated by different intensities of the spots (Fig. 3). This suggests that RACK1 interacts at multiple sites within the PDE4D5 catalytic unit, extending over a region spanning Cys596 to Gly665.

Scanning alanine substitution analysis identified key amino acids involved in RACK1 binding to the PDE4D catalytic unit (Fig. 5b). Amino acids in 3 peptides, spanning the region from Ala601 to Gly665 in PDE4D5 and whose substitution led to either ablation or marked reduction in RACK1 interaction were Gly609, Phe610 and

Asp612 in helix-15a of the catalytic unit; His616, Pro617, Glu620, Thr621 and Trp622 in helix 15b; Ile633, Thr636, Arg641, Trp643 and Tyr644 in helix-16 and Asp657 and Asp658 in the flexible linker between helices 16 and 17 (Fig. 5b).

That the binding sites for RACK1 and β arrestin2 in the catalytic unit abut, rather than overlap, is clearly evident from Odyssey analysis of peptides 125 through 132, which span the region Thr621 – Gly689 in PDE4D5 (Fig. 5c). In both single channel and overlay analysis this shows exclusive RACK1 binding (red) to peptides 125 through 129 and exclusive β arrestin2 binding (green) to peptides 130 through 132 (Fig. 5c).

We used 2-hybrid analysis to obtain independent confirmation for a novel RACK1 interaction site in the PDE4D catalytic unit. Thus C-terminal truncation of PDE4D5, to G662, which removes a region that includes the E⁶⁶⁸KFQFELTLEE⁶⁷⁸ motif, ablated β arrestin binding but did not affect RACK1 binding (Fig. 2f). In contrast, C-terminal truncation to K513 also ablated RACK1 binding to PDE4D5 (Fig. 2f).

Consequences of the siRNA-mediated knockdown of RACK1 on PDE4D5- β arrestin complex functioning in HEK293B2 cells— Using a specific small interfering RNA (siRNA) we achieved the efficient (>90%) knockdown of RACK1 in HEK293B2 cells as detected by immunoblotting (Fig. 6a). This procedure had no discernible effect upon β arrestin, PDE4D3, PDE4D5 and tubulin expression (Fig. 6a). Similarly, knockdown of β arrestin had no discernible effect upon RACK1, PDE4D3, PDE4D5 and tubulin expression (Fig. 6a) and scrambled siRNA had no effect on expression of any of these proteins (<5%).

In order to determine if RACK1 knockdown affected the level of PDE4D5 associated with β arrestin we selectively immunoprecipitated β arrestin from HEK293B2 cell lysates and immunoblotted for PDE4D5 (Fig. 6b). RACK1 knockdown increased the level of β arrestin-complexed PDE4D5 approximately 5-fold (Fig. 6b, c). No PDE4D5 was seen in β arrestin immunoprecipitates from HEK293B2 cells subjected to β arrestin knockdown (Fig. 6b).

Treatment of HEK293B2 cells with isoproterenol elicits the rapid, time-dependent translocation of β arrestin-bound PDE4D5 to the β_2 AR [5, 8, 30, 31], as seen here in PDE4D5 immunoblots of β_2 AR immunoprecipitates (Fig. 6d). However, RACK1 knockdown markedly increased (around 4-fold) the amount of PDE4D5 associating with the β_2 AR after 5min of exposure to isoproterenol (Fig. 6d, e).

siRNA knockdown of RACK1 attenuates the ability of isoproterenol to elicit the PKA phosphorylation of the β_2 AR and activate ERK in HEK293 cells — Isoproterenol challenge of HEK293B2 cells causes the time-dependent phosphorylation of the β_2 AR by AKAP79-tethered PKA [7]. Here we show that siRNA-mediated RACK1 knockdown reduced (around 4-fold) isoproterenol-stimulated phosphorylation of the β_2 AR by PKA (Fig. 7a, b).

In HEK293B2 cells, PKA phosphorylation of the β_2 AR allows it to switch its coupling from G_s to G_i and thereby activate ERK [7, 8, 30]. This action is desensitized via the β arrestin-mediated delivery of PDE4D5, which attenuates PKA phosphorylation of the β_2 AR [7]. Here we show that RACK1 knockdown clearly attenuates (4-5 fold) isoproterenol activation of ERK (Fig. 7c, d). However, RACK1 knockdown did not cause any change in ERK activation by isoproterenol when cells were additionally challenged with the PDE4-selective inhibitor, rolipram (Fig. 7e). This argues against any PDE4-independent effect of RACK1 knockdown on ERK activation by isoproterenol in HEK293B2 cells.

PDE4D5 must bind to β arrestin for it to modulate isoproterenol-stimulated ERK activation – As observed previously [8] over-expression of a catalytically inactive PDE4D5 (Asp556Ala-PDE4D5), whilst still able to be co-immunoprecipitated with β arrestin (Fig. 7e) provides a dominant negative function in displacing active, endogenous, PDE4D5 from complex with β arrestin, thereby facilitating the PKA-mediated phosphorylation of the β_2 AR and profoundly increasing the ability of isoproterenol to activate ERK in HEK293B2 (Fig. 7f).

Using information derived from the analyses described above, we set out to determine whether the dominant negative action of Asp556Ala-PDE4D5 could be compromised if its ability to bind β arrestin was disrupted. To do this we generated the Glu27Ala mutation in Asp556Ala-PDE4D5 on the basis that Glu27Ala mutation would negate the binding of β arrestin but not that of RACK1 (Fig. 3, 4). Consistent with this, Glu27Ala:Asp556Ala-PDE4D5 did not co-immunoprecipitate with β arrestin (Fig. 7f) while it did with RACK1 (Fig 4d). Glu27Ala:Asp556Ala-PDE4D5 singularly failed to exert a dominant negative effect in promoting isoproterenol-stimulated ERK activation (Fig. 7g). Indeed, in cells over-expressing Glu27Ala:Asp556Ala-PDE4D5 the ability of isoproterenol to activate ERK was reduced compared to that observed in control cells (Fig. 7g). In these various experiments the degree of expression of both Asp556Ala-

PDE4D5 and Glu27Ala:Asp556Ala-PDE4D5 was similar, as detected by immunoblotting for their VSV-epitope tag, and had no discernible effect on ERK expression (Fig. 7g).

Discussion

PDE4D5 has a unique 88 amino acid N-terminal region that is able to interact with the signaling scaffold proteins RACK1 and β arrestin (Fig. 1) [5, 8, 23, 26, 30, 31]. Here we show that RACK1 and β arrestin bind to PDE4D5 in a mutually exclusive manner (Fig. 2). Using a novel peptide array approach we suggest that this is because RACK1 and β arrestin interact with overlapping sites within the unique N-terminal region of PDE4D5. Furthermore, they also bind to distinct sites within the third sub-domain of the PDE4D catalytic region. In so doing they can be expected to straddle PDE4D5, which may further serve to sterically interdict mutual interaction with PDE4D5.

Scanning substitution peptide arrays identified the motif 22 NPWxNxxxVxxLRxNLxxxEKxK⁴⁴ as providing the RACK1 interaction domain, RAID1 in the unique N-terminal region of PDE4D5 (Fig. 4a). These data were remarkably consistent with that from the 2-hybrid approach. Nevertheless, the underlined amino acids appeared not to be essential for RACK1 to bind full-length PDE4D5 in 2-hybrid studies (Fig. 4a). This may be due to conformational differences between the folding of the full-length protein and the 25-mer peptides folding and/or the presence of additional, compensating binding sites in the intact protein. In this regard, note that the sensitivity of assays used here to detect binding of full-length proteins requires that RACK1 binds both the N-terminal and catalytic sites of PDE4D5 to obtain a positive result [30]. Nevertheless, these data clearly indicate that scanning substitution peptide array analysis provides a rapid and effective means of highlighting amino acids that potentially are involved in protein-protein interaction.

The power of peptide array analysis, compared to simple truncation, is evident in analyses of β arrestin with the N-terminal portion of PDE4D5 (Fig. 3). Previously, 2-hybrid assay analysis of progressive N-terminal truncates [30] indicated that the last 18 or so amino acids of the unique N-terminal region of PDE4D5 were involved in β arrestin binding. However, peptide array studies, confirmed by point mutation analysis in pull-downs and 2-hybrid studies, show that β arrestin2 interacts over an extended surface of the PDE4D5 unique N-terminal region (Fig 1, 3). We might then expect that progressive N-terminal truncation of PDE4D5 will likely yield constructs with gradually diminishing

affinities for β arrestin2. Indeed, peptide array analysis clearly shows that RACK1 and β arrestin compete for binding to two peptides encompassing a region bounded by amino acids 16-45 in PDE4D5 (Fig. 4c). Scanning arrays of a peptide encompassing amino acids 22-45 in PDE4D5 clearly identified substitution of either Leu33 or Arg34 with alanine as ablating the interaction of both β arrestin2 and RACK1 (Fig. 4a, d, e), suggesting these two amino acids form part of the binding sites for both β arrestin2 and RACK1 on PDE4D5. Consistent with this, alanine mutation of these amino acids ablated PDE4D5- β arrestin2 interaction in both 2-hybrid and pull-down studies (Fig. 4a, b). Scanning array analysis of a peptide encompassing amino acids 22-45 in PDE4D5 indicated additional amino acids, namely those within a motif of 26 NEDLVxxLR 34 , as also being important for PDE4D5- β arrestin interaction (Fig. 4d). Interestingly, previous N-terminal truncation of this sub-region did not ablate PDE4D5- β arrestin interaction in either 2-hybrid or immunoprecipitation studies [30]. Thus mutation of certain amino acids in this sub-region may trigger extensive changes in the conformation of the N-terminal portion of PDE4D5 as a whole such that β arrestin binding site there is ablated, whilst its deletion does not.

Previous truncation analyses indicated [30] that β arrestin bound to a site within the C-terminal 83 amino acids of PDE4D5. As the catalytic unit is highly conserved between PDE4 sub-families, while the extreme C-terminus is not, we inferred the binding site for β arrestin might be located between amino acids 662-683 of PDE4D5 [30]. Our peptide array analyses indicate that β arrestin2 interacts with the catalytic unit of PDE4D5 in a sequence that spans Ala655-Glu694 (Fig. 3). This region contains the E 668 KFQFELTLEE 678 motif that is conserved in all PDE4 sub-families. Consistent with this region being involved in β arrestin binding, we noted that β arrestin failed to bind peptide 129, which lacks the LEE component of this motif and showed considerably reduced binding to peptide 133, which lacks the EK component of this motif (Fig. 5c). Scanning alanine substitution arrays (Fig. 5a), confirmed by 2-hybrid analysis (Fig. 2e), identified Phe670, Phe672, Leu674 and L676, which are located in sub-domain-3 of the PDE4 catalytic unit, as being critically involved in this interaction.

Peptide array analysis allowed us to identify an entirely novel interaction site for RACK1, which is located within the common PDE4D catalytic unit (Fig. 3). This site is crucial as its deletion by C-terminal truncation prevented PDE4D5 from interacting with RACK1 in a 2-hybrid assay (Fig. 2f). Unlike the highly localized interaction site for

RACK1 found within the PDE4D5 unique N-terminal region, that associated with the PDE4D5 catalytic unit is more extensive, spanning a region from around Gly609 in helix-15a through to Asp658 in the flexible linker region between helices 16-17 (Fig. 5b). Within this region, alanine substitution of a number of amino acids led to loss of RACK1 interaction. Thus RACK1, like β arrestin, binds the PDE4D catalytic unit, where these sites abut each other (Fig. 1, 5c). This contrasts with the unique N-terminal region of PDE4D5, where their binding sites overlap (Fig. 1, 4c).

The crystal structure of the isolated PDE4B and PDE4D catalytic regions is well known [43-49]. We show here that RACK1 binds to sub-domain 3 and involves helices-15a, -15b and -16 as well as the flexible linker region between helices-16 and -17. The majority of the amino acids identified as involved in RACK1 binding form a well-defined, surface exposed patch (Fig. 8a, b; red/blue surface). Also evident are two smaller surface exposed patches located to the side of this (Fig. 8a; red/blue surface). The larger surface patch stretches from inside the catalytic pocket over some 20 Å along the length of the combined helix-15/16 surface. Within the catalytic pocket Phe610 forms part of the purine-binding region, while Gly609 is located at the rim (Fig. 8b). Should RACK1 interact directly with the aromatic ring face of Phe610 this would block substrate binding. This is unlikely, as RACK1 does not alter PDE4D5 catalytic activity [26]. However, it may be that RACK1 binds across Gly609 and the edge of the Phe610 side chain without occupying or occluding the substrate-binding site. Such an intimate interaction of RACK1 close to the catalytic site might be expected to exert a functional effect. Indeed, this might explain why RACK1 alters PDE4D5 inhibition by rolipram [26]. If RACK1 binds across a surface extending from Thr621 to Arg641 and, simultaneously, wraps around Phe610, this would require a sharp bend in the RACK1 surface in the region that contacts Gly609. Thus the Gly609Ala substitution might be expected to compromise severely the ability of RACK1 to do this. Three of the surface exposed amino acids in this patch are also involved in contacts between helices 15-16 [43-49]. Asp612, on helix-15, forms a salt bridge with Arg641 on helix-16, while His616, on helix-15, forms a salt bridge with Glu638 on helix-16. Mutation of any of these could, potentially, perturb RACK1 binding by upsetting inter-helix alignments. While His616Ala substitution diminished RACK1 interaction, alanine substitution of Glu638, to which His616 ion pairs on helix-16 did not (Fig. 5b). This suggests that His616 may be directly implicated in the

association of PDE4D5 with RACK1 rather than simply serving a structural role in aligning helices-15 and -16 in order to allow RACK1 binding.

We also observed ablation of interaction upon Pro617Ala substitution (Fig. 5b). As Pro617 is surface exposed (Fig. 8a, b) and has no direct interactions with amino acids in the PDE4D catalytic unit it may be involved in direct surface contact between RACK1 and PDE4D5. Additionally, Pro617 is associated with a pronounced bend in helix-15 caused by the unique inability of Pro617 to hydrogen bond to the peptide carbonyl group in the P-4 position of the helix (Tyr613). Pro617Ala mutation might allow for a hydrogen bond between helix amino acids at positions 613 and 617, thereby straightening helix-15. This would affect its alignment with helix-16, inducing a wide-ranging conformational change.

Asp657 and Asp658, implicated (Fig. 5b) in the interaction of PDE4D5 with RACK1, lie beyond the C-terminal end of helix-16. Detailed structural information for this region of the enzyme is not available, but it is likely that they lie in a region of flexible loop (Fig. 8a, b) as noted below.

While there is no structural information available for the Phe670-Leu676 region of PDE4D, two crystal structures for a truncated PDE4B2 enzyme, namely (1F0J) [43] and 1XM6 [44], extend as far as the region corresponding to amino acids 672-677 in PDE4D5, respectively. As amino acids 324-677, in PDE4D5, show 85% sequence identity to the corresponding region in PDE4B we built a structural model of PDE4D that used the PDE4B structures as templates (Fig. 8). Both templates taken together indicated that the sequence corresponding to ⁶⁶⁰EEGRQGQTEKFQFELTLE⁶⁷⁷ in PDE4D5 formed an α -helix-containing structure (helix-17) that is separated from helix-16 by a flexible linker (Fig. 8; pink surface). However, the position of helix-17, with respect to the catalytic domain, is entirely different in these two templates. In the 1F0J structure helix-17 makes no direct packing contacts with the core catalytic domain of its parent protein molecule, but is packed against an adjacent symmetry-related molecule in the crystallographic unit cell [43]. In contrast, in the 1XM6 structure helix-17 is folded across the mouth of the catalytic pocket, trapping the PDE4 inhibitor, mesopram within [44]. Consequently, we constructed two models based upon each of the 1F0J (Fig. 8a,b) and 1XM6 (Fig. 8c) structures. In the model based upon 1F0J (Fig. 8a,b) helix-17 is conformationally mobile and does not interact with the catalytic domain. The amino acids involved in β arrestin binding, namely Phe670, Phe672, Leu674 and Leu676 (FxFxLxL

motif) form a coherent, semi-circular patch on an exposed surface located upon one side of the C-terminal part of helix-17 (Fig. 8a,b; yellow surface). It is therefore likely that this part of the surface of helix-17 physically interacts with β arrestin and any flexibility in the region between helix-16 and helix-17 may aid in accommodating the binding of β arrestin to both this location and within the unique N-terminal region of PDE4D5. On the other hand, the PDE4D model based upon the 1XM6 structure (Fig. 8c) presents, at least in principle, the ability of helix-17 to fold across the opening of the catalytic site. In this arrangement Leu674, which is key for β arrestin binding (Fig. 5a), is entirely packed against the catalytic domain (Fig. 8c). Additionally, helix-17, together with the flexible linker region that connects it to helix-16, partially occludes the surface implicated in RACK1 interaction (Fig. 8c).

It seems likely that helix-17 is conformationally mobile as it is connected to helix-16 via a 13 amino acid long flexible linker (Fig. 5b; Fig. 8a-c; pink surface) [43, 44]. Consequently, its positions in both models may not reflect exactly that adopted in either a β arrestin-PDE4D5 complex, where an additional β arrestin binding site is involved, nor in complexes of β arrestin with PDE4 isoforms that interact solely within the catalytic domain. Thus, these models represent two different and possible, but not exclusive scenarios. However, we consider that the PDE4D model based upon the 1XM6 structure may well be discounted as the folding of helix-17 across the opening of the catalytic site is likely to generate a catalytically inactive species that can neither bind to RACK1 nor to β arrestin. In contrast, the PDE4D model based upon 1F0J clearly shows surface exposed sites for both RACK1 and β arrestin binding (Fig. 8a, b). Whatever the precise folding of helix-17 it is apparent that PDE4D5 straddles each of these scaffolding proteins in a similar manner. Their association is mediated by interaction at overlapping sites within the unique N-terminal region of PDE4D5 supplemented by interactions with distinct, but proximal, surfaces of the core PDE4 catalytic unit. Both sets of interactions likely conspire to interdict simultaneous binding of RACK1 and β arrestin, explaining the competition for their binding to PDE4D5 as demonstrated here.

Immunoprecipitation of the entire pools of RACK1 and β arrestin identified around 31% of the total PDE4D5 associating with RACK1 and around 15% associating with β arrestin in HEK293B2 cells. This suggests that additional proteins act to sequester PDE4D5 in these cells. Indeed, PDE4D5 may effect multiple roles in cells each of which is associated with its ability to interact with distinct scaffolding proteins. Alterations in

the endogenous levels of particular scaffolds may redistribute PDE4D5 amongst other scaffolding proteins with consequential effect on their signalling. We demonstrate this through the targeted knockdown of RACK1 in HEK293B2 cells, which leads to a profound increase in PDE4D5 found associated with β arrestin (Fig. 6). Such an increase in β arrestin-associated PDE4D5 cannot simply be due to competing out residual PDE4D3-bound β arrestin, as the amount of PDE4D5 associating with β arrestin consequent upon RACK1 knockdown increases some 5-fold (Fig6b) whilst PDE4D3 forms some 6-11% of total PDE4D associated with β arrestin in these cells. Our observations indicate that in normal cells the endogenous pool of β arrestin is not saturated with PDE4D5.

Such a redistribution of PDE4D5 upon RACK1 knockdown has a functional consequence in that it increases the amount of PDE4D5 that is delivered to the β_2 AR, in complex with β arrestin, upon challenge of cells with isoproterenol (Fig. 6). The enlarged pool of PDE4D5 delivered to the β_2 AR acts to attenuate the PKA phosphorylation of the β_2 AR (Fig. 7). This has functional consequences for β_2 AR signaling as PKA phosphorylation of the β_2 AR causes it to switch its coupling from the G_s -mediated activation of adenylyl cyclase to the G_i -mediated activation of ERK [10]. Thus the attenuated PKA phosphorylation of the β_2 AR leads to a consequential, marked reduction in the ability of isoproterenol to activate ERK (Fig. 7). Consistent with this action being mediated by enhanced recruitment of PDE4D5 we noted that RACK1 knockdown failed to reduce the ability of isoproterenol to activate ERK in HEK293B2 cells treated with rolipram, which acts to inhibit PDE4D5 (Fig. 7e).

The ability of PDE4D5 to attenuate the PKA phosphorylation of the β_2 AR and the switching of its signaling to ERK is dependent upon PDE4D5 being recruited to the β_2 AR in complex with β arrestin [7, 8]. Such an inhibitory constraint supplied by endogenous PDE4D5 can be relieved by over-expressing a catalytically inactive Asp556Ala-PDE4D5 mutant [8]. This provides a dominant negative function in displacing active, endogenous, PDE4D5 from complex with β arrestin, thereby facilitating PKA phosphorylation of the β_2 AR [8]. However, if, based upon our peptide array data, we mutate catalytically inactive PDE4D5 (Glu27Ala:Asp556Ala-PDE4D5) such that it cannot bind β arrestin but can still bind to RACK1, then this species is now unable to exert a dominant negative effect on β_2 AR phosphorylation by PKA and signaling to ERK through G_i (Fig. 7). These data demonstrate that our peptide array and mutational

analyses can be successfully exploited to generate PDE4D5 mutants that identify functional consequences associated with selective PDE4D5 targeting.

This study exemplifies the power of analyzing protein-protein interaction through peptide arrays. It has allowed us to identify and define novel binding sites in PDE4D5 for both RACK1 and β arrestin and to generate mutants of PDE4D5 that selectively interact with these proteins. We show that the knockdown of a scaffolding protein for PDE4D5, namely RACK1, has functional consequences that relate to another PDE4D5 scaffolding protein, namely β arrestin. Thus changes in expression levels and binding properties of a particular PDE4D5 scaffolding protein may have far-reaching consequences. This can ensue through redistribution of released PDE4D5 between other proteins that sequester PDE4D5 provided that binding is not already saturated. Our data thus highlights an additional degree of functional complexity associated with the PDE4 enzyme family in instances where specific isoforms are tethered to arrays of functional scaffolds in cells. PDE4 isoforms are poised to play pivotal roles in underpinning the compartmentalization of cAMP signaling in cells. The identification and characterization of these partnerships may have potential for understanding the role of PDE4 isoforms in molecular pathologies as well as in providing novel means of therapeutic intervention.

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Table 1 Scanning peptide array analysis of PDE4D3 with RACK1 and β arrestin probes.

Amino acid sequence of sequential 25mer PDE4D5 peptides, as probed in Fig3, is given here with residue span in PDE4D5 sequence (residues) plus peptide number (peptide) in analysis. Identification of interaction with either RACK1 (R bind) or β arrestin (β A bind), as ascertained from overlay assays, is shown as no binding (N) and weak (+), medium (++) and strong (+++) based upon intensity of spots visualised as in Fig.3. All other peptides, which completed the scan of PDE4D5 sequence failed to interact with either RACK1 or β arrestin.

25-mer peptide sequence	residues	peptide	R bind	β A bind
MAQQTSPDTLTVPEVDNPHCPNPWL	(1-25)	peptide 1	N	N
SPDTLTVPEVDNPHCPNPWLNEDLV	(6-30)	peptide 2	N	N
TVPEVDNPHCPNPWLNEDLVKSLRE	(11-35)	peptide 3	N	+++
DNPHCPNPWLNEDLVKSLRENLLQH	(16-40)	peptide 4	+	+++
PNPWLNEDLVKSLRENLLQHEKSKT	(21-45)	peptide 5	+++	+++
NEDLVKSLRENLLQHEKSKTARKSV	(26-50)	peptide 6	N	+++
KSLRENLLQHEKSKTARKSVSPKLS	(31-55)	peptide 7	N	++
NLLQHEKSKTARKSVSPKLSPVISP	(36-60)	peptide 8	N	++
EKSKTARKSVSPKLSPVISPRNSPR	(41-65)	peptide 9	N	+++
ARKSVSPKLSPVISPRNSPRLRRM	(46-70)	peptide 10	N	+++
SPKLSPVISPRNSPRLRRMLLSSN	(51-75)	peptide 11	N	+
PVISPRNSPRLRRMLLSSNIPKQR	(56-80)	peptide 12	N	+
RNSPRLRRMLLSSNIPKQRRFTVA	(61-85)	peptide 13	N	+
LLRRMLLSSNIPKQRRFTVAHTCFD	(66-90)	peptide 14	N	N
LLSSNIPKQRRFTVAHTCFDVDNGT	(71-95)	peptide 15	N	N
RERGMEISPMCDKHNASVEKSQVGF	(586-610)	peptide 118	N	N
EISPMCDKHNASVEKSQVGFIDYIV	(591-615)	peptide 119	N	N
CDKHNASVEKSQVGFIDYIVHPLWE	(596-620)	peptide 120	+	N
ASVEKSQVGFIDYIVHPLWETWADL	(601-625)	peptide 121	++	N
SQVGFIDYIVHPLWETWADLVHPDA	(606-630)	peptide 122	++	N
IDYIVHPLWETWADLVHPDAQDILD	(611-635)	peptide 123	++	N
HPLWETWADLVHPDAQDILDTLEDN	(616-640)	peptide 124	+++	N
TWADLVHPDAQDILDTLEDNREWYQ	(621-645)	peptide 125	+++	N
VHPDAQDILDTLEDNREWYQSTIPQ	(626-650)	peptide 126	++	N
QDILDTLEDNREWYQSTIPQSPSPA	(631-655)	peptide 127	+	N
TLEDNREWYQSTIPQSPSPAPDDPE	(636-660)	peptide 128	++	N
REWYQSTIPQSPSPAPDDPEEGRQG	(641-665)	peptide 129	+++	N
APDDPEEGRQGQTEKFQFELTLEED	(655-680)	peptide 130	N	+++
EGRQGQTEKFQFELTLEEDGESDT	(660-685)	peptide 131	N	+++
GQTEKFQFELTLEEDGESDTEKDSG	(665-690)	peptide 132	N	+++
FQFELTLEEDGESDTEKDSGSQVEE	(670-695)	peptide 133	N	+
TLEEDGESDTEKDSGSQVEEDTSCS	(675-700)	peptide 134	N	N
GESDTEKDSGSQVEEDTSCSDSKTL	(680-705)	peptide 135	N	N

Figure Legends

Figure 1. Location of sites for RACK1 and β arrestin interaction on PDE4D5.

A schematic of PDE4D5 with its unique N-terminal region, upstream conserved region 1 (UCR1), upstream conserved region 2 (UCR2), catalytic region and extreme C-terminal region. Indicated above the schematic are the sites of β arrestin and RACK1 interaction identified prior to this study. Below it are interaction sites arising from this work.

Figure 2 RACK1 and β -arrestin interact with PDE4D5 in a mutually exclusive fashion.

(a) 2-hybrid analysis with PDE4D5 as bait and either β arrestin2 or RACK1, as prey. Where indicated RACK1 was additionally co-expressed as a 'competitor'. Blue indicates a positive interaction and pink a null interaction. Controls are vectors without inserts. (b) A two-hybrid assay was performed, as in Fig. 1. Also shown is the positive standard and negative ('empty' vector) control for this experiment ("standards"), which are both identical to those in Fig. 1. These data are typical of results obtained at least 3 times. (c) immunoblots of endogenous PDE4D3, PDE4D5 and RACK1 from HEK293B2 cell lysates (LY) and β arrestin immunoprecipitates (IP). (d) using a RACK1 specific antibody endogenous RACK1 was immunopurified (IP) from HEK293B2 cells transfected to express β arrestin-GFP. Lysates (Ly) and RACK1 immunoprecipitates (IP) were immunoblotted for endogenous PDE4D3, PDE4D5 and transfected β arrestin-GFP. No immunoreactive species were detected in control (Ctr) experiments using pre-immune antisera. These data are typical of experiments performed at least 3 times. (e) Various mutations in the EKFQFELTLEE motif of PDE4D5 (amino acids 668 to 678) were tested in 2-hybrid assays for their ability to block the PDE4D5's interaction with RACK1 and β arrestin2. A two-hybrid assay was performed, as in Fig. 1. Standards (not shown) were identical to those in Fig. 1 (f) Various carboxyl-terminal deletions of PDE4D5 were tested for their ability to interact with either RACK1 or β -arrestin2 (β arr2). A two-hybrid assay was performed, as in Fig. 1. Standards (not shown) were identical to those in Fig. 1.

Figure 3 Probing PDE4D5 peptide arrays for RACK1 and β arrestin interaction sites.

PDE4D5 is shown schematically as in Fig 1. Data shows immobilized peptide 'spots' of overlapping 25-mer peptides each shifted along by 5 amino acids in the entire PDE4D5 sequence probed for interaction with either β arrestin2-GST or RACK1-GST and detection by immunoblotting. Positively interacting peptides generate dark spots while none-interacting peptide leave white (blank) spots. In all other sections of the array spots were blank with either probe. Spot numbers relate to peptides in the scanned array and whose sequence is given in Table 1. The disposition of the peptides in PDE4D5 sequence are shown relative to identified domains in a schematic of PDE4D5, which indicates its isoform-specific N-terminal region, Upstream Conserved Regions 1 and 2 (UCR1, UCR2), catalytic unit and PDE4D C-terminal region.

Figure 4 The binding of RACK1 and β arrestin to sequential alanine substituted versions of a RAID1-containing peptide.

(a) The row of spots in the upper portion shows a peptide array based upon a 25mer 'parent' peptide of sequence Asn22-Thr45 in PDE4D5. Ctr refers to the unmutated peptide and all other spots reflect peptide 'progeny' where indicated amino acids were sequentially and individually substituted with alanine. This array was probed with RACK1-GST and interaction determined by immunoblotting with an anti-RACK1 antibody. The lower panel of colored patches shows various mutant forms of PDE4D5, with the indicated amino acids substituted with alanine, probed for interaction with RACK1 in a 2-hybrid assay (positive = blue and null = pink). For substitution/mutation at specific residues then red indicates abolition of binding using both assay procedures, whilst black arrows indicate binding is still apparent. Blue arrows indicate differences in the two assays procedures; (b) HEK293B2 cells were transfected with either wild-type or the indicted mutant forms of VSV epitope-tagged PDE4D5. Lysates and immunoprecipitates were immunoblotted as indicated. As β arrestin migrates on SDS-PAGE identically to Ig heavy chain we were unable to immunoblot for β arrestin in immunoprecipitates. Equal protein loading was used in each track. Lysates contained 1/20th protein loading compared to the immunoprecipitates; (c) Odyssey analysis of PDE4D5 peptides 3-6 (see Fig. 3) challenged with equimolar (200nM) β arrestin2-GST and RACK1-GST. β arrestin2 (green) and RACK1 (red) binding were detected simultaneously with dual; binding (yellow) shown in the combined channels (Overlay). Null binding is black. Data are typical of those obtained using 3 separately synthesized

arrays; (d) peptide array and 2-hybrid assays performed as in (a), above, but β arrestin2 was used in the two screens instead of RACK1; (e) performed as in (c) to probe β arrestin2-GST and RACK1-GST binding simultaneously but with the alanine scanning peptide arrays used in both (a) and (d). Data are typical of experiments performed at least 3 times.

Figure 5 Defining the binding of RACK1 and β arrestin to the PDE4D catalytic unit using scanning peptide arrays.

(a) Scanning Glu660 to Glu685 in PDE4D5 for interaction with β arrestin-GST. Anti- β arrestin antisera was used for detection; all other conditions were as in Fig 4a. Native peptide (Ctr) plus progeny with the indicated residue substituted for alanine. (b) scanning of Ala601 to Glu665 in PDE4D5 for interaction with RACK1-GST. Three peptides were used to cover this region (Ctr) with conditions as in Fig. 4a. The indicated residues are substituted for alanine, unless the indicated amino acid was alanine in native PDE4D5, when it was substituted with aspartate. Mutation of amino acids colored red indicate null interaction (<10%), while those colored blue indicate reductions >50% based upon densitometric analysis of spots for n=3 experiments. (c) Odyssey analysis of PDE4D5 peptides 125-132 (see Fig. 3), incubated with equimolar (200nM) β arrestin2-GST and RACK1-GST. Detection was as in Fig. 4c. Data are typical of experiments performed at least 3 times.

Figure 6 Small interfering RNA-mediated knockdown of RACK1 and β arrestin on PDE4D5 recruitment to the β_2 -adrenoceptor.

(a) HEK293B2 cells were subject to siRNA knockdown of either β arrestin or RACK1 or with scrambled siRNA. Lysates were immunoblotted for endogenous RACK1, β arrestin, tubulin, PDE4D3 and PDE4D5 as indicated. (b) Cells were siRNA treated as above and immunoprecipitates generated from lysates (Ly) using either a β arrestin-specific antiserum (IP) or with non-specific serum (Ctr). Note that loading blots cannot be performed for β arrestin as it co-migrates with Ig H-chain. Upper panel shows immunoblots and lower panel shows a quantitative analysis of 3 independent experiments. (c) As in (b) except that RACK1 immunoprecipitates were probed for PDE4D. (d) Cells were siRNA treated as in (a) and then treated with isoproterenol (10uM) for the indicated times. The β_2 -AR was selectively immunoprecipitated (IP) and

probed for PDE4D5 (e) A quantitative analysis of 3 independent experiments performed as in (d). Data are mean \pm S.D.

Figure 7 Small interfering RNA-mediated knockdown of RACK1 on β_2 -adrenoceptor phosphorylation by PKA-mediated and activation of ERK in HEK293B2 cells.

In (a) cells were transfected with either scrambled siRNA or siRNA specific for RACK1 prior to challenge with isoproterenol (10 μ M for 5min). Equal amounts of cell lysate were immunoblotted for RACK1, total β_2 -AR, PKA phosphorylated form of the β_2 -AR, β arrestin and PDE4D. (b) Quantification of the PKA phosphorylation status of the β_2 -AR upon isoproterenol (10 μ M) challenge (5min) of cells after prior transfection with either scrambled siRNA or that specific for RACK1 in 3 separate experiments performed as in (a). (c) performed as in (a) but lysates were immunoblotted for total ERK and phospho-ERK. (d) Quantification of ERK phosphorylation (n=3). (e) lysates were immunoblotted for total ERK and phospho-ERK with cells treated either with RACK1 siRNA or scrambled siRNA with subsequent challenge using isoproterenol plus the PDE4 selective inhibitor, rolipram (10 μ M). (f) cells were transfected with vsv epitope-tagged versions of either wild-type PDE4D5 or D556A-PDE4D5 or E26A:D556A-PDE4D5. β arrestin immunoprecipitates (IP) were generated from cell lysates (Ly), both were immunoblotted with a vsv-specific antibody, to detect recombinant PDE4D5. (g) cells were transfected as (f) and then challenged for the indicated times with isoproterenol (10 μ M). Equal amounts of lysate protein were immunoblotted to identify vsv-tagged PDE4D, total ERK and phospho-ERK. The data is typical of experiments performed at least 3 times. Data are mean \pm S.D.

Figure 8 Structural representation of binding sites for RACK1 and β arrestin on the PDE4D structural models.

(a-b) Two different perspectives of the structural model of the 324-677 stretch of the PDE4D generated as described in the "Experimental procedures" using 1F0J PDE4B structure as a template. Amino acids whose substitution with alanine leads to loss of RACK1 interaction signal are shown in red and those where substitution leads to a clear reduction in interaction signal are shown in blue. Amino acids whose substitution with alanine leads to loss of β arrestin interaction signal are shown in yellow, the flexible linker between helices 16 and 17 is shown in pink whilst the 1XOM inhibitor cilomilast

is shown in spacefill representation in green. (c) The structural model of the 324-677 stretch of the PDE4D generated using 1XM6 PDE4B structure as a template. Color-coding as above. These structural figures were created using PyMOL (<http://pymol.sourceforge.net/>) program.

Fig. 1

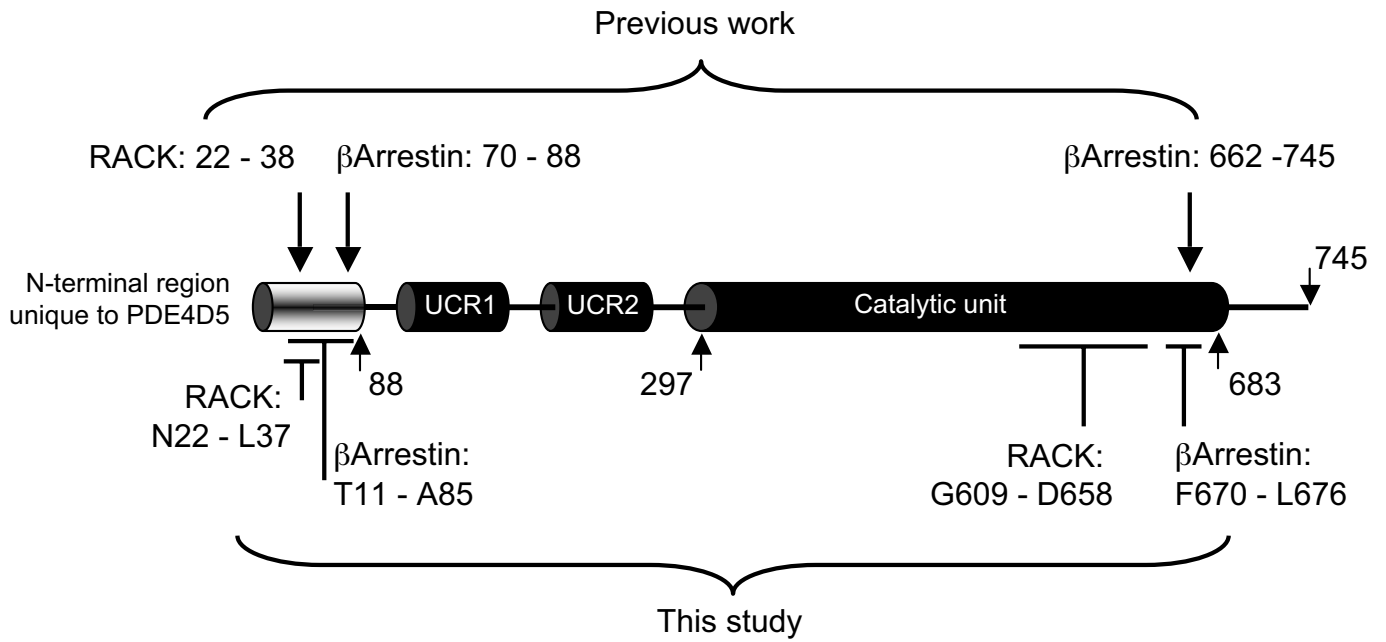


Fig. 2

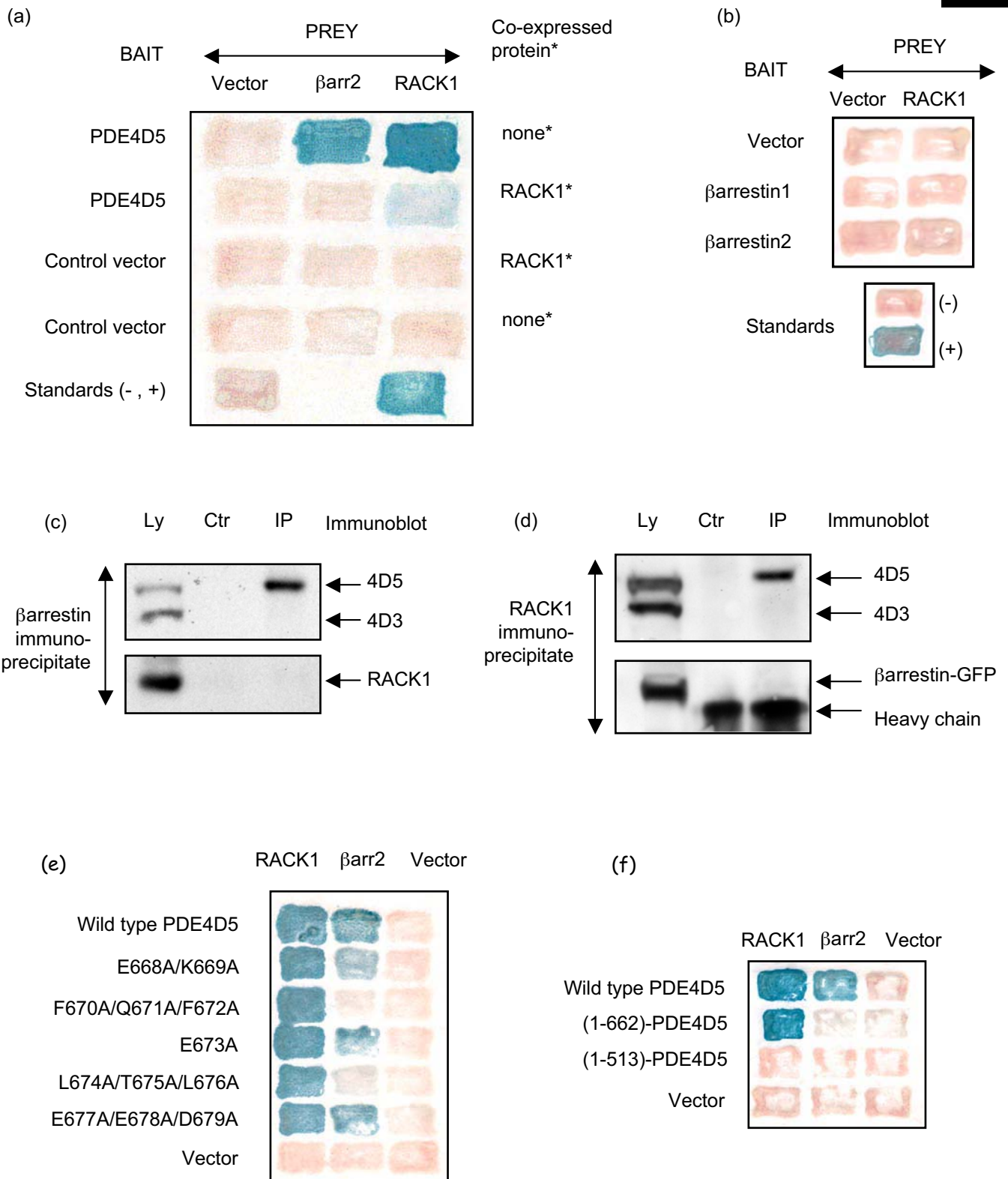


Fig. 3

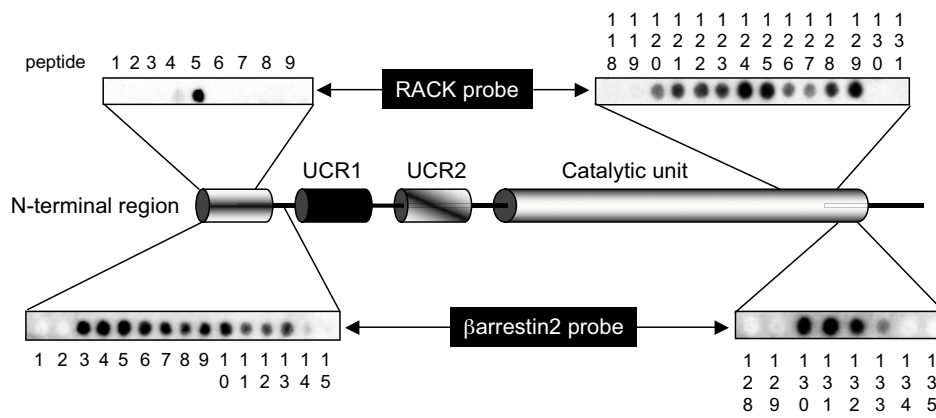


Fig. 4a

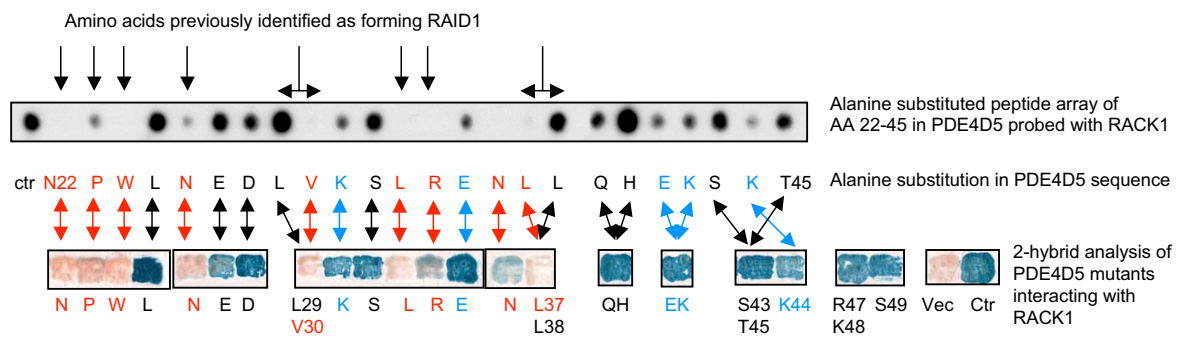


Fig. 4b

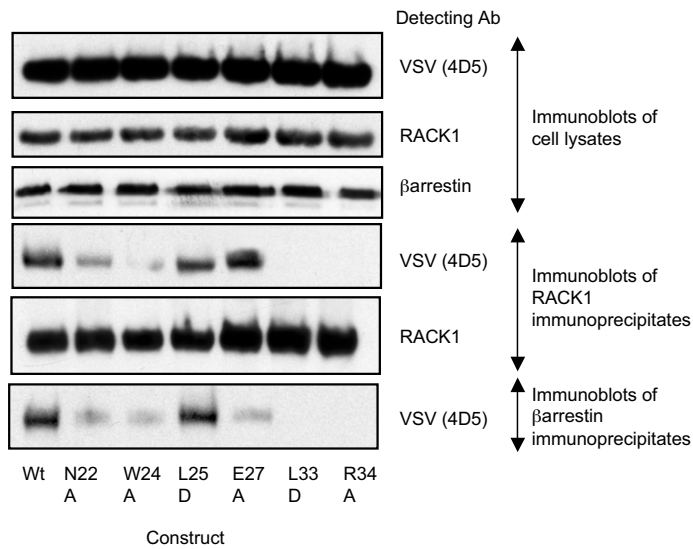


Fig. 4c

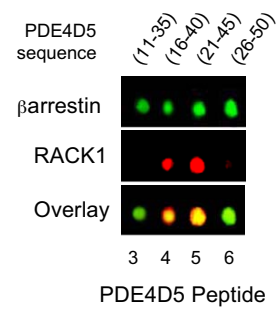


Fig. 4d

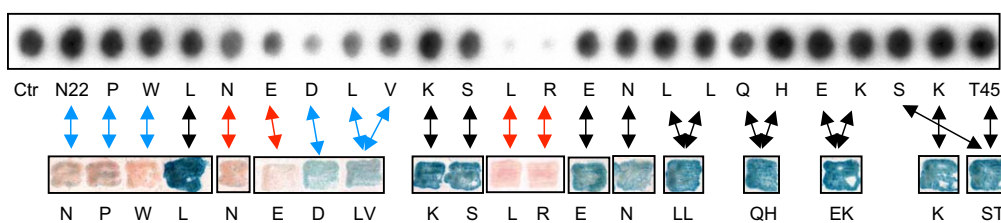
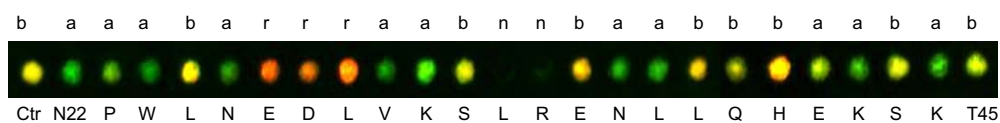


Fig. 4e



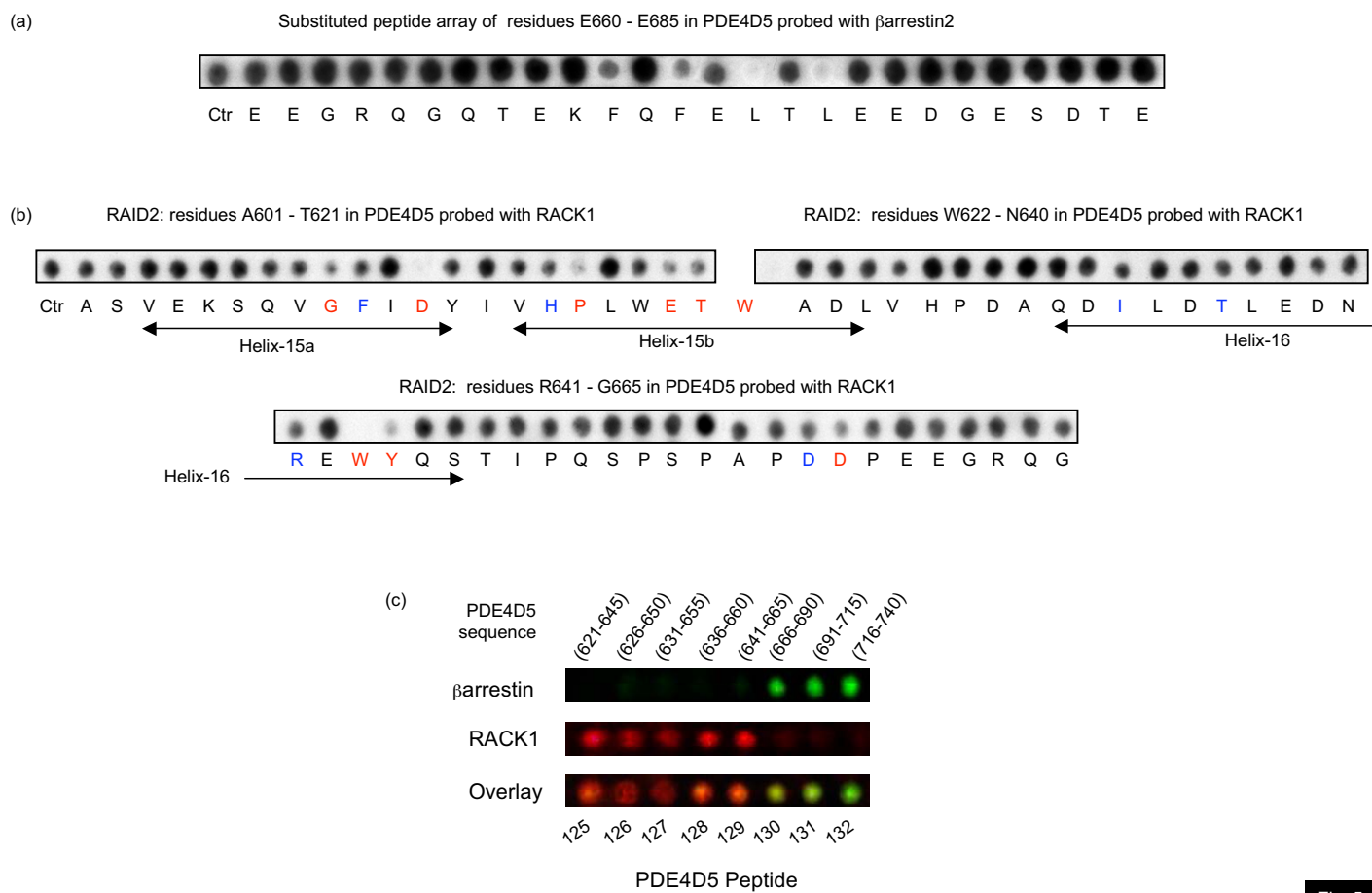


Fig. 5

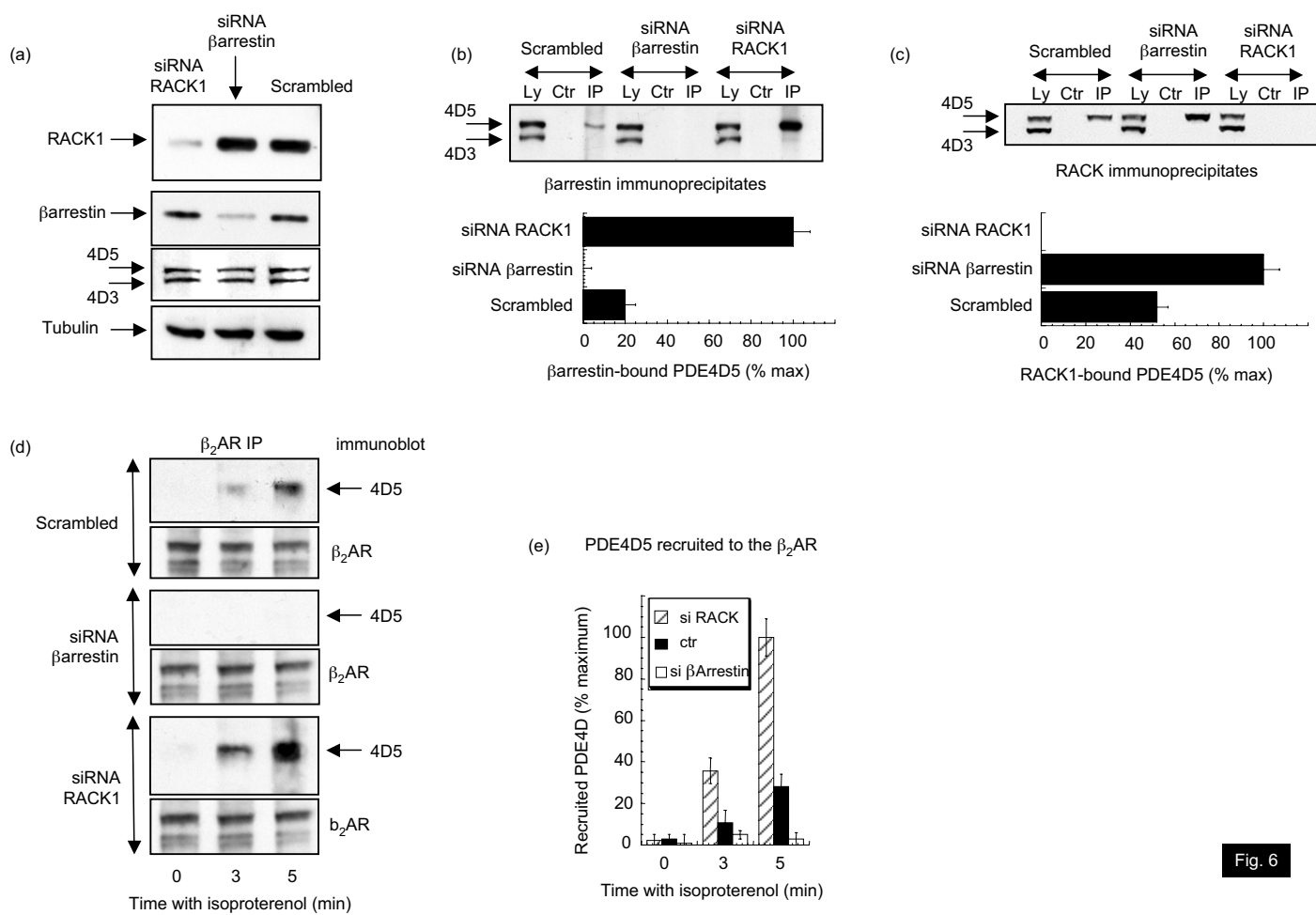


Fig. 6

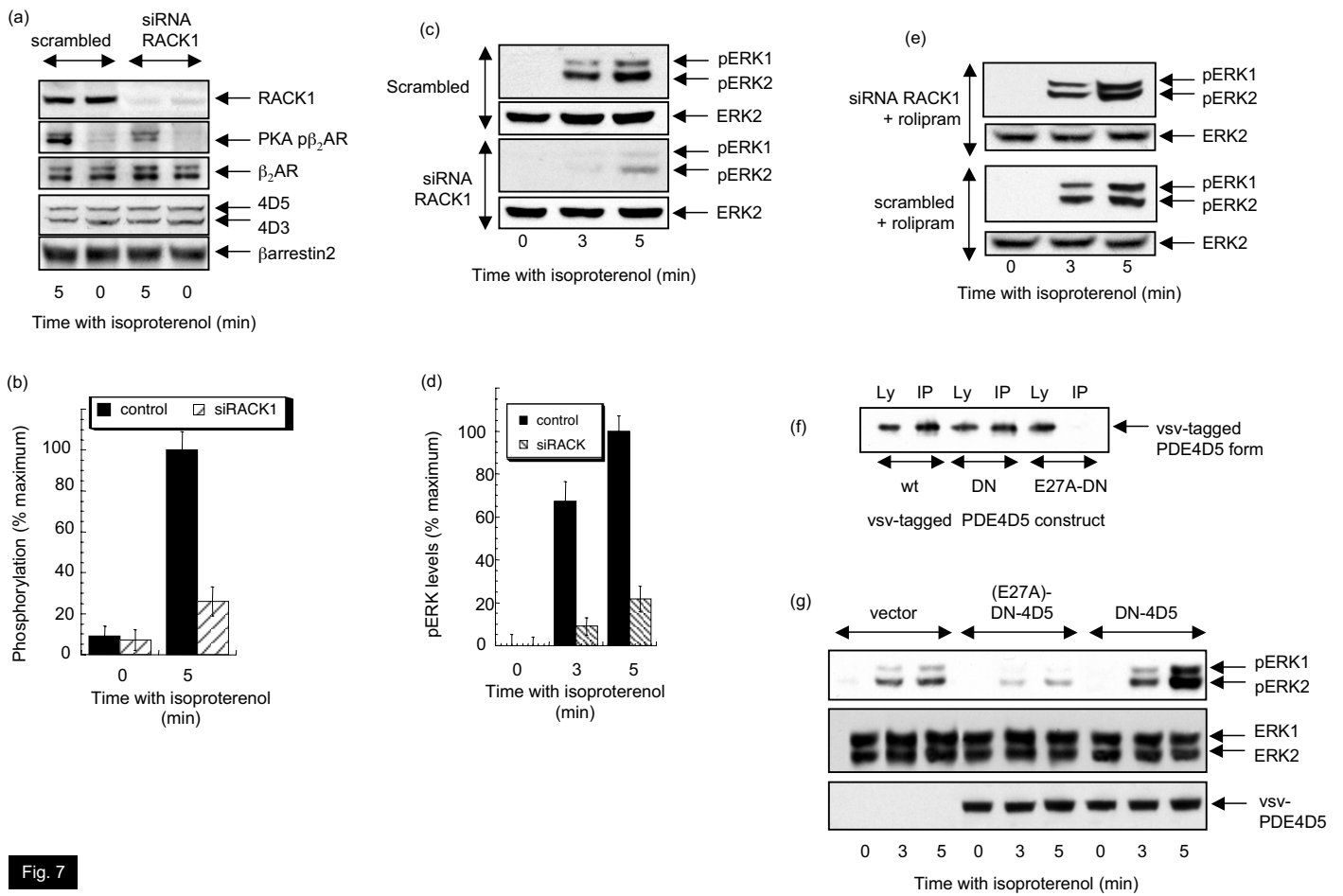


Fig. 7

