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Selective SUMO modification of cAMP-specific phosphodiesterase-4D5 (PDE4D5) regulates the functional consequences of phosphorylation by PKA and ERK.

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Abstract

Enzymes from the PDE4 cAMP-specific phosphodiesterase family are crucial for the maintenance of compartmentalized cAMP responses in many cell types. Regulation of phosphodiesterase (PDE) activity can be achieved via post-translational modification such as phosphorylation by ERK MAP kinases and Protein Kinase A. In the present study we report for the first time that PDE4 isoforms from the PDE4A and PDE4D sub-families can be selectively modified by the small ubiquitin related modifier, SUMO. We identify a single SUMO site within a consensus tetrapeptide motif, ψ -K-X-E (ψ represents a hydrophobic residue), which lies in the catalytic unit of these enzymes. SUMO modification of PDE4 at this site was observed upon overexpression of the SUMO E3 ligase, PIASy in HEK cells and we identify PIASy as a novel binding partner for long PDE4 isoforms. Site directed mutagenesis of the acceptor lysine ablated conjugation of PDE4 with SUMO, suggesting the presence of a single SUMO site in the first sub-domain of the conserved PDE4 catalytic unit. This observation was supported by both cell-free, *in vitro* SUMOylation assays and by analysis of SUMOylated spot-immobilised peptide arrays. SUMO modification of long PDE4 isoforms serves to augment their activation by PKA phosphorylation and repress their inhibition by ERK phosphorylation. Following ligation of β -adrenergic receptors, SUMOylation of PDE4 isoforms sufficiently amplified PKA-stimulated PDE4 activity to reduce markedly the PKA phosphorylation status of the β_2 -adrenergic receptor. These data highlight a new means whereby cells might achieve the selective regulation of the activity of cAMP specific PDE4 phosphodiesterases.

Abbreviations: PDE, cyclic nucleotide phosphodiesterase; PKA, cAMP-dependent protein kinase; cAMP, 3', 5' cyclic adenosine monophosphate; HEK cells, human embryonic kidney cells; ERK, extracellular-signal-regulated kinase; GST, glutathione S-transferase; EPAC, exchange protein activated by cAMP; UCR, upstream conserved region; siRNA, small interfering RNA; SUMO, Small ubiquitin-related modifier; VSV, vesicular stomatitis virus; IPTG, isopropyl-D-thiogalactopyranoside; DMEM, Dulbecco's Modified Eagles Medium; Ubc9, Ubiquitin like protein SUMO conjugating enzyme; β_2 AR, β_2 -adrenergic receptor; SIM, SUMO-interacting motif; GST, glutathione-S transferase.

INTRODUCTION

Signal transduction via the cyclic AMP (cAMP) pathway is known to be ubiquitous and represents a major line of communication between many organisms and their environment [1]. Messages from a plethora of cell surface receptors are translated into specific downstream biological processes through the dual action of adenylyl cyclases [2] that synthesise the second messenger cAMP and its various effector proteins, namely PKA [1], EPAC [3] and cyclic nucleotide gated ion channels [4]. Signal termination is mediated by a group of proteins, the cyclic nucleotide phosphodiesterases (PDEs), which represent the sole route of cAMP degradation [5]

Recent technological advances in development of genetically encoded cAMP reporters have provided a unique insight into the pivotal role of PDEs and, particularly, PDE4 cAMP specific phosphodiesterases in the spatial and temporal regulation of cAMP signals [6, 7]. Compartmentalisation of PDEs to discrete intracellular locations allows cAMP gradients to be formed and shaped with such spatial regulation being crucial for normal cell function [5, 6].

Cells can express a defined subset of PDEs from the 11 different families, of which 8 have the ability to hydrolyse cAMP [8]. Recently, the cAMP-specific PDE4 family has become of increased interest due to its implication in a number of disease states [9, 10] and its identification as a therapeutic target. Additionally, the discovery that these enzymes play a critical role determining compartmentalised cAMP signalling in cells due to their isoform-specific N-terminal regions that allow them to be targeted to distinct signalling complexes [5, 6, 11]. The activity of PDE4s is constitutive but can be altered by a number of post-translational events [5]. Long PDE4 isoforms contain a PKA phosphorylation motif in their regulatory, Upstream Conserved Region 1 (UCR1) and phosphorylation by PKA within this domain causes activation of catalytic activity and a change in enzyme conformation [12, 13]. Such an arrangement serves to produce an orchestrated “pulse” of cAMP that is temporally and spatially restricted by this feedback control mechanism coupled to intracellular targeting [14]. All PDE4s, except those in the PDE4A sub-family, also contain an ERK Map kinase consensus phosphorylation motif in their conserved catalytic region [15]. Phosphorylation at this target site is facilitated by the ERK docking and specificity sites, which straddle the target serine [15]. In contrast to the action of PKA phosphorylation, which activates long PDE4 isoforms [12], the functional consequences of ERK phosphorylation on long PDE4 isoforms is to cause inhibition [16].

One challenge that faces researchers in the field is to decipher which signals regulate the formation of specific signalling complexes that include PDE4s. The isoform, PDE4D5, for instance, can bind the scaffolding proteins RACK1 [17] and β arrestin [18] in a mutually exclusive fashion [19]. As PDE4D5 can be anchored at other locations outwith the influence of these scaffolds, these scaffold proteins sequester only a portion of the total PDE4D5 pool at any one time. During times of β -adrenergic stimulation, the translocation of the β arrestin/PDE4D5 complex from the cytosol to the β_2 -adrenoceptor receptor at the plasma membrane serves to co-ordinate a dual desensitization event that effectively prepares the receptor for another round of agonist challenge [19, 20]. The efficacy of receptor desensitisation depends on the β arrestin-mediated delivery of

sufficient, suitably activated, PDE4D5 to the site of cAMP synthesis at the plasma membrane [21]. This action can be facilitated by the agonist-dependent ubiquitination of PDE4D5 by the β arrestin-scaffolded E3 ligase, MDM2 [22]. In this, mono-ubiquitination of PDE4D5 at a single lysine in UCR1 triggers a cascade of polyubiquitination occurring on three lysine residues in the unique N-terminal region of PDE4D5. Such modification, whilst not affecting the activity of PDE4D5, serves to enhance its binding to β arrestin [22].

In the last decade, SUMOylation has emerged as a novel way in which proteins can be modified to change their intracellular localisation, binding partner profile and activity [23]. Small ubiquitin-related modifier (SUMO) proteins are conjugated onto substrates via a multi-step, energy requiring process involving E1 SUMO activators, E2 conjugation enzymes and E3 SUMO ligases [24]. SUMO conjugation is highly lysine specific with the SUMO conjugation site invariably residing within a consensus tetrapeptide, ψ -K-X-E (ψ represents a hydrophobic residue). It is thought that this sequence orientates the catalytic region of the E2 conjugating enzyme (UBC9) towards the target lysine [25]. Added fidelity of UBC9/substrate interaction can depend on a negative patch of amino acids locate some four residues upstream of the acceptor lysine. This can take the form of either D/E residues [26] or an SP motif that is phosphorylated prior to SUMOylation [27].

Following sequence analysis of PDE4 isoforms, we identify a putative SUMO consensus motif within the conserved PDE4 catalytic region. Intriguingly, enzymes from only PDE4A and PDE4D subfamilies contain the putative acceptor lysine at cognate positions 358 in PDE4A4 and 323 in PDE4D5. Here we report, for the first time, that SUMOylation may provide a novel means by which PDE4 isoforms can be modified and regulated. A functional output of PDE4 SUMOylation appears to be in amplifying PDE4 long form activation by PKA phosphorylation while, simultaneously, protecting PDE4 long forms from inhibitory input elicited through phosphorylation by ERK MAP kinase.

EXPERIMENTAL

Materials

PolyFect transfection reagent was from Qiagen (Crawley, U.K.),. Protease inhibitor tablets were from Roche. VSV affinity agarose beads and VSV monoclonal antibodies were purchased from Sigma. *In vitro* SUMOylation kits were purchased from Biomol (UW8955). Plasmid DNA was produced using QIAprep[®] Spin Miniprep Kit from Qiagen.

Site-directed mutagenesis

The PDE4D5 SUMO mutant (K323R), was constructed using the QuickChange site-directed mutagenesis kit (Stratagene). The 5'- and 3'-primers designed for PDE4D5 SUMO K mutant (PDE4D5K323R) were as follows with the mutated nucleotides underlined: 5'-primer- CCCAAGGTTTGGAGTTCGAACT GAACAAGAAGATGTCC
3'-primer- GGACATCTTCTTGTTTCAGTTCGAACTCCAAACCTTGGG. All constructs were verified by DNA sequencing before use.

Purification of Recombinant Fusion Proteins

Competent *E.coli* BL21 pLysS (DE3) cells transformed with the appropriate pMAL plasmid, were grown directly in 30 ml of sterilized LB media, supplemented with 100 ug/ml ampicillin, overnight in an orbital shaking incubator at 37°C. 450 ml of sterilised LB media, supplemented with 100ug/ml ampicillin, was inoculated with the overnight culture and grown in the orbital shaker at 37°C for 1-2h. A 1ml sample of culture was removed to a plastic cuvette and the optical density measured at 600 nm (OD₆₀₀) against an LB media control. Expression of the fusion protein was induced with 0.2mM isopropyl-D-thiogalactopyranoside (IPTG) when an OD₆₀₀ of 0.6-1 was achieved. Expression of the target protein was undertaken for 4h in an orbital shaker set at 30°C. Hourly 1 ml samples of LB media were removed to monitor protein expression. The samples were centrifuged at 10,000g and the pellet was re-suspended in SDS sample buffer for analysis by SDS-PAGE (NuPage, InVitrogen) and Coomassie staining. The cells were then harvested by centrifugation at 4000rpm for 10min. The cells were re-suspended in 12ml of 50mM Tris-HCl; pH 8.0, 10mM NaCl and 10mM β-mercaptoethanol containing a 1x solution of Roche Diagnostic's (Mannheim, Germany) protease inhibitor cocktail tablets. The re-suspended cells were then frozen at -80°C and thawed on ice. Lysozyme was added at a final concentration of 1mg/ml and the cell suspension subjected to sonication to achieve sufficient cell lysis. A final concentration of 0.05% NP-40 was added to aid cell lysis and the subsequent cell debris removed by centrifugation at 13000rpm for 15 min. 1ml of amylose resin (New England BioLabs, Hitchin, U.K.) was pre-equilibrated with re-suspension buffer containing 0.05% NP-40. The cell supernatant was applied to the pre-equilibrated resin and incubated end-over-end for 1h at 4°C to bind the expressed fusion protein. The resin was collected by centrifugation at 2000g for 2min and washed three times with 750μl of re-suspension buffer containing 0.05% NP-40. The fusion protein was eluted from the amylose resin with 500μl of 10mM maltose, 50mM Tris-HCl; pH 8.0 by incubating end-over-end for 20min at 4°C. The eluted fractions were pooled and dialysed using Pierce (Rockford, IL, U.S.A.) Slide-A-Lyser dialysis cassettes against three 650ml volumes of 100mM NaCl, 50mM Tris-HCl; pH 8.0, 5% glycerol, for 1h each at 4°C. The purified fusion protein was frozen on dry ice, and stored as aliquots, at -80°C.

Cell culture and transfection

COS1, HEK293 and HEK β2 cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 2mM glutamine, 10% Fetal calf serum (FCS) (Sigma), 1% penicillin/streptomycin (100units/ml) (Sigma) in an atmosphere of 5% CO₂ and 37°C. All cells were transiently transfected with PolyFect (Qiagen).

Immunoprecipitation and Western blotting

Cells were transiently transfected with VSV epitope-tagged forms of either wild type βarrestin1-FLAG, PDE4D5-VSV, or a SUMO defective PDE4D5-VSV (K323R). 48h after transfection, cells were lysed using a 3T3 lysis buffer containing 20mM HEPES (pH7.4), 50 mM NaCl, 50mM NaF, 10% glycerol, 1% Triton X-100, 10mM EGTA, 30mM sodium pyrophosphate and Roche proteases inhibitor cocktail. Cell lysates were pre-cleared with Protein G beads and then used for immunoprecipitation of VSV tagged proteins. Cell lysates were incubated with VSV affinity agarose beads for 2h at 4 °C.

After centrifugation at 18,000 x g for 3 min beads were washed three times with the lysis buffer. Laemmli sample buffer was used to elute the immunoprecipitated VSV-fusion proteins and the resulting material was resolved by Nu-PAGE (Invitrogen). Proteins were transferred to nitrocellulose membranes and subjected to western blotting with detection by ECL (Pierce).

Phosphodiesterase Activity Assay

PDE activity was measured using a radioactive cAMP hydrolysis assay that has been described previously [28]. [^3H] adenosine cyclic-3', 5'-mono-phosphate was sourced from Amersham Biosciences (Little Chalfont, U.K.) and cyclic-3', 5'-mono-phosphate from Sigma.

***In Vitro* PKA and ERK Phosphorylation assay**

Kinase phosphorylation assays were undertaken using purified PKA catalytic subunit (Promega) and purified ERK2 (Millipore). 1 μg of fusion protein and recombinant kinase were diluted in phosphorylation buffer (100 mM Tris-HCl pH 7.5, 0.2 mM ATP, 10 mM MgCl_2 , 30 mM β -mercaptoethanol, 20% (v/v) glycerol and 10mM Calyculin A). The reaction was incubated at 30°C for 30min. The reaction was stopped by the addition of 12.5 μl of 5x SDS sample buffer. The reaction tubes were mixed and heated for 10min at 70°C on a heating block. The tubes were then centrifuged at 13000g for 1min and the sample subjected to SDS-PAGE and transferred to a nitro-cellulose membrane. In all cases, the extent of PDE4D5 phosphorylation was corrected for by determining the immunoreactive amount of target protein.

Pull-down Assays

MBP-4D5 fusions proteins (500 μl of 2 μM) were mixed with 100 μl slurry of 50% (v/v) PBS-washed Amylose resin (Amersham Biosciences) for 2 h at 4°C. The beads were pelleted by centrifugation (14,000g, 1 min) and washed twice with PBS containing 1% Triton X-100 before the addition of equal molar amounts of β arrestin-GST fusion protein in a 1-ml solution of PBS and Triton X-100 containing 5 mM dithiothreitol. After a 2 h incubation at 4 °C, the beads were collected and washed three times with PBS and eluted in 100 μl of 2x Laemmli buffer [29]. Eluates were resolved by SDS-PAGE, and the GST fusion proteins were detected by immunoblotting

SPOT synthesis of Peptides

Peptide libraries were produced by automatic SPOT synthesis and 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) as previously described by us [30].

***In vitro* expression and SUMOylation of PDE4**

In vitro transcription/translation of PDE4 was performed using 1 μg of plasmid DNA and a wheat germ-coupled transcription/translation system according to the instructions provided by the manufacturer (Promega) and as described previously by us [31]. [^{35}S] methionine (Amersham Pharmacia Biotech) was used in the reactions to

generate radio-labelled PDE4 proteins. 1 μ l of 35 S-methionine PDE4 was incubated for 4 hours (37°C) in a 10 μ l reaction mix containing an ATP regenerating system (50mM Tris, pH 7.6, 5mM MgCl₂, 2mM ATP, 10mM creatine phosphate, 3.5U/ml creatine kinase, and 0.6U/ml inorganic pyrophosphatase), 0.1 μ M SAE1/SAE2, 4.4 μ M Ubc9, 9 μ M Sumo-1 or 2, protease inhibitors EDTA free (Roche) and 5mM DTT. Reactions were terminated with SDS sample buffer containing β -mercaptoethanol. Reaction products were fractionated by SDS-PAGE (10% or 8%). Dried gels were analysed by phosphorimager (Fuji BAS 1500, MacBAS software).

***In vitro* SUMOylation assay**

A SUMO Kit (Biomol) was used and the procedure carried out according to manufacturer's instruction for the SUMOylation of MBP or GST fusion proteins. Peptide arrays that included the putative SUMO site on PDE4A4, PDE4B2, PDE4C2 and PDE4D5 were synthesised and used in the same SUMOylation assay. The SUMO assay mix was incubated with array membranes for 30 mins at 30°C with shaking. Membranes were washed with TBST (137mM NaCl, 20mM TrisHCl, pH 7.6, 0.1% Tween20) before the SUMOylated moieties on the peptide array were probed using an antibody specific for SUMO. Control arrays were carried out with SUMO assay mixes that did not contain UBC9, the E2 ligase for SUMO conjugation.

Cyclic AMP Measurements

The concentration of cAMP in lysates extracted from COS1 cells was done using a cAMP competitive EIA assay kit (99-0050, Zymed).

RESULTS

PDE4A and PDE4D sub-families contain a SUMO consensus motif

Previous sequence analysis of PDE4s has uncovered regions of highly conserved sequence that exist in all PDE4 isoforms, namely the catalytic unit, UCR1 and UCR2 [4, 32]. Other regions, such as the C-terminal tail, are unique to specific PDE4 sub-families, and the extreme N-terminal region that, with one exception (where the N-terminus of PDE4B5 is identical to that of PDE4D6 [33]), is unique to each individual isoform [32].

Our analysis of the PDE4 catalytic region sequence indicated a putative SUMO consensus motif that is found in the PDE4A and PDE4D sub-families and not either of the PDE4B and PDE4C sub-families (Figs. 1a and 1b). This motif is akin to the so-called 'negatively charged amino acid-dependent SUMOylation motif' (NDSM) that has been defined as ψ -K-X-E-X-X-D/E-D/E, where ψ represents a hydrophobic residue and X represents residues that are less well conserved [26]. Using example long isoforms from each of these two PDE4 sub-families, then the putative SUMO octapeptide NDSM commences at Val357 in PDE4A4 and Val322 in PDE4D5 (Fig. 1a). An inspection of PDB entries revealed that the peptide is present at the N-terminus of the structurally defined core catalytic domain region in both of the currently available PDE4A structures and seven of the twenty-two PDE4D structures [34]. In each structure the proposed SUMO acceptor lysine is located on a region of prominently exposed coil immediately preceding helix-1 located in sub-domain-1. Moreover, in the majority of these structures the local conformation of the peptide backbone spanning the core ψ -K-X-D/E region closely resembles the corresponding region in the SUMOylated RanGAP1 protein, as

seen in its crystal structure (Fig. 1d) complexed with UBC9 and a ligase fragment from Nup358 [35]. The similarity between RanGAP1 and PDE4A/D in the presentation of this motif strongly suggested that these PDE4 isoforms should be able to dock UBC9 successfully. Indeed superimposition of the PDE4 structures onto the SUMO-RanGAP1-UBC9 complex (Fig. 1d) confirmed that the acidic residue at position-4 is well poised to form a salt bridge to Lys76 in UBC9 whilst the hydrophobic residue at position-1 fits into a shallow basin on the surface of UBC9 proximal to Ala129. There is a cluster of basic residues exposed on the surface of UBC9 close to Lys76 that might, in principle, interact favourably with the paired C-terminal acidic residues of the NDSM motif. The SUMOylation sequence in RanGAP1 lacks these acidic residues and does not make contact with this region of the SUMO-conjugating enzyme however. In contrast, the putative PDE4A and PDE4D SUMOylation motifs do possess a pair of adjacent acidic residues at positions -6 and -7. These are located at the N-terminal end of helix-1 and are also prominently surface-exposed.

Intriguingly, whilst isoforms from the PDE4B and PDE4C sub-families exhibit the hydrophobic and acidic residues of the core SUMO motif, plus both residues of the acidic patch and a similar conformational organisation, the lysine SUMO-acceptor residue at position-2 is substituted by either an N or a Q in these two sub-families, respectively (Fig. 1a).

This analysis indicates that only members of the PDE4A and PDE4D sub-families should have the potential to be subject to modification by SUMOylation.

PDE4A and PDE4D peptide arrays are selectively SUMOylated *in vitro*

We have used peptide arrays on solid support coupled with *in vitro* ubiquitinylation assays to define the sites on PDE4D5 where Mdm2-catalysed ubiquitination occurs [33]. Recently, such an approach has been used employing *in vitro* SUMOylation assays to gain insight into the selectivity and fidelity of SUMO conjugation, using RanGAP1 as a substrate [36] and we have used it to define sites for Mdm2-catalysed ubiquitination of PDE4D5 ([22]). Such studies indicated that SUMOylation of RanGAP1 depends upon the amino acids that make up the core and flanking regions of the consensus motif (NDSM).

We thus adopted this methodology to determine whether the differences in sequence seen between PDE4 subfamilies might result in differential SUMOylation of the cognate catalytic regions (Fig. 1c). Peptide arrays of overlapping 25-mer peptides, sequentially shifted by 5 amino acids and spanning the entire indicated PDE4 isoform sequence, were incubated with a SUMOylation assay mix before detection of SUMO conjugation using a SUMO antibody. Dark spots represent positive areas of SUMO conjugation whereas clear spots are negative for the modification by SUMO (Fig. 1c). Doing this we observed that whilst no signal was observed using the PDE4B2 and PDE4C2 arrays, positive signals were obtained using the PDE4A4 and PDE4D5 arrays (Fig. 1c). Clearly, the inclusion of a putative acceptor lysine residue within the SUMO core consensus NDSMs of both PDE4A4 and PDE4D5 allows SUMO modification of the immobilised peptides that contain it, whereas cognate peptides from PDE4B2 and PDE4C2 do not appear to allow for conjugation to SUMO.

PDE4A and PDE4D longforms are selectively SUMOylated in HEK cells.

To determine whether the differential SUMOylation of PDE4 isoforms could be observed in cells, we transiently transfected PDE4A4, PDE4B2, PDE4C2 and PDE4D5 into HEK293 cells. Under resting conditions, we could not detect (Fig. 2a) the 25 kDa “bandshift” that typically characterises the covalent addition of SUMO to an acceptor protein [37].

However, protein SUMOylation is dually controlled by action of the E2 conjugating enzyme, UBC9, and by SUMO specific proteases that rapidly cleave the covalent bond between SUMO and the substrate [23]. The dynamic nature of this relationship means that, under steady state conditions, only a small proportion of a protein is SUMOylated at any one time and this, invariably, seriously hampers biochemical detection of SUMO modified proteins (see e.g. [23]). Although endogenous levels of SUMO activating enzymes (SAE1/2) and the conjugating enzyme (UBC9) are usually sufficient to allow SUMOylation to occur in cells, it has been shown [23] that the detection of SUMOylated species in cells can be facilitated when an E3 ligase also forms part of the SUMOylation complex. This markedly increases both the specificity and fidelity of SUMOylation *in vivo*. Thus, to circumvent the universal difficulties encountered in monitoring endogenously SUMOylated proteins, investigators have routinely amplified SUMOylation by transiently co-expressing the SUMO E3 ligase, PIASy [38, 39]. Here we have utilised the same approach by co-expressing HA-tagged PIASy, together with representative PDE4 isoforms from all four sub-families. Using this method, we were able to visualise an additional, more slowly migrating immunoreactive PDE4 species that appeared to be approximately 25 kDa larger than that of the native forms of both PDE4A4 and PDE4D5 (Fig. 2a). In marked contrast to this, no such slower migrating species was evident upon co-transfection of HA-tagged PIASy with either PDE4B2 or PDE4C2 (Fig. 2a). This suggests that, in agreement with the peptide array data (Fig. 1c) and bioinformatics analysis (above), both PDE4A and PDE4D sub-families are able to become SUMOylated whilst PDE4B and PDE4C sub-families cannot. Consistent with the notion that the retarded mobility was due to SUMOylation, only the more slowly migrating species was recognised by antibodies to SUMO1 (Fig. 2a), showing that PDE4A4 and PDE4D5 can indeed be SUMOylated in a cellular context. Surprisingly, the short PDE4D1 isoform, could not be SUMOylated in the same manner as PDE4D5 even although it contains the putative SUMO octapeptide (Fig. 2b). However, in subsequent analyses, utilising immunopurified forms of PDE4D5 and PDE4D1, we found that the E3 ligase PIASy only associated with the long PDE4D5 isoform and not the short PDE4D1 isoform (Fig. 2c). In order to investigate the basis of this selective interaction, we probed GST-fusion proteins of distinct PDE4D regions (Fig. 2d) and full-length peptide arrays of PDE4D5 and PDE4D1 (Fig. 2e) for the ability to associate with PIASy-HA. Clearly, 2 areas of association exist in PDE4D5 (Fig. 2e) for the docking of PIASy, one in the long isoform specific UCR1 domain and one within the conserved catalytic region. As PDE4D1 and other short PDE4 isoforms do not contain UCR1 (diagram Fig. 2e), we suggest that the lack of one of the PIASy interaction sites may underpin the selective nature of SUMOylation for long PDE4 isoforms. This notion is supported by the GST-pull-down data (Fig. 2d), which are consistent with the notion that both sites are required for the efficient docking of the SUMO E3, PIASy.

PDE4D isoforms are selectively SUMOylated *in vitro*.

To reinforce the premise that long PDE4D sub-family isoforms are SUMO substrates, we incorporated PDE4D3 and PDE4D5 into an *in vitro* SUMOylation assay and contrasted their response with that of PDE4B2, which we expected on the basis of the above reported studies not to become SUMOylated. Proteins were transcribed *in vitro* and labelled with ³⁵S-Methionine before being incubated with recombinant SAE (E1), UBC9 (E2), SUMO and an ATP-generating system. For both PDE4D isoforms a single novel species of increased molecular weight species expected for SUMOylation (circa 25kDa) was detected only in the presence of all assay components (Fig. 3a). In contrast, no such novel retarded species was evident using PDE4B2 (Fig. 3b), further indicating that PDE4D, but not PDE4B isoforms, are targets for SUMOylation.

K323 is the sole SUMOylation site on PDE4D5

To determine whether SUMO modification of PDE4 isoforms is directed through the potential SUMO site within the conserved catalytic region, we repeated both the *in vivo* and *in vitro* SUMOylation assay protocols using a mutant form of PDE4D5 (K323R-PDE4D5) that had the putative acceptor Lys323 mutated to an arginine (K323R). The K323R-PDE4D5 mutant was generated as a ³⁵S-labelled species and subjected to an *in vitro* SUMOylation reaction mix (Fig 4a). A VSV-tagged K323R-PDE4D5 mutant form was expressed in HEK cells together with HA-tagged PIASy (Fig 4b). In neither instance did we observe a more slowly migrating species arising, signifying that SUMOylation of PDE4D5 was ablated by the single point substitution of Lys323 to Arg323. These data indicate that PDE4D5 is SUMOylated at a single site, namely Lys323.

In addition, *in vitro* SUMOylation of an alanine scanning peptide array (Fig. 4c) encompassing the putative SUMO site on PDE4D5 illustrated that K323 was crucial for SUMO conjugation. Furthermore, we found that alanine substitution of either the hydrophobic residue at position-1 (V322A) or the acidic amino acid at position-4 (E325A) in the SUMO consensus motif (NDSM) both severely attenuated SUMOylation (Fig. 4c). In contrast to this, mutations of the amino acids at positions -6 and -7, which make up an "acidic patch" (E327A/D328A) in the NDSM, appeared to have little effect on *in vitro* SUMOylation of this peptide derived from PDE4D5 (Fig. 4c).

SUMOylation enhances activation of PDE4D5 by PKA phosphorylation and attenuates PDE4D5 inhibition by ERK phosphorylation.

Previous work has illustrated that the constitutive activity of PDE4D5 can be regulated by phosphorylation through both PKA and ERK [15, 40-42]. PKA phosphorylation of PDE4D5 in UCR1 results in an activation of PDE catalytic activity, whereas phosphorylation by ERK, occurring within the catalytic region, causes inhibition [5, 15, 40, 42]. We thus set out to determine whether SUMOylation could alter the constitutive activity of PDE4D5 and, furthermore, whether modification by SUMO might reprogramme the activity changes triggered by PKA and by ERK phosphorylation. To do this, recombinant GST-PDE4D5 was preSUMOylated *in vitro* before incorporation into kinase assays containing active forms of either PKA or ERK. We first demonstrated that *in vitro* SUMOylated PDE4D5 migrated through the gel more slowly than native (untreated control) PDE4D5, suggesting that efficient SUMOylation of this species had taken place (Figs. 5a, 5c). After *in vitro* phosphorylation with PKA, samples were blotted

for phosphorylation by PKA using a phospho-PKA substrate antibody, while phosphorylation by ERK was monitored using a phospho-serine antibody. Both SUMOylated (upper band) and native PDE4D5 (lower band) appeared to be equally well phosphorylated by either PKA or ERK, suggesting that the conjugation of SUMO had neither enhanced nor hindered access of the kinases to their substrates or their action (Figs 5a and 5c). To reinforce these data, the phosphorylation kinetics of PDE4D5, K323R-PDE4D5 and SUMO-PDE4D5 were monitored using a phospho-antibody raised against the PKA site in UCR1 [40, 43] after treatment of HEK cells with the adenylyl cyclase activator forskolin and the non-selective PDE inhibitor, IBMX (Fig. 5d) to increase cAMP levels and activate PKA [40]. The phosphorylation of PDE4D5, K323R-PDE4D5 and SUMO-PDE4D5 species all followed an identical time-course suggesting that, once again, SUMOylation did not affect the phosphorylation kinetics of PDE4D5.

Next, we set out to determine the PDE catalytic activity of these various phosphorylated species using equal amounts of the various isoforms as determined immunologically with an anti-VSV antibody. Modification of PDE4D5 by SUMO had no effect (< 9 % change; n=3) on the constitutive activity of PDE4D5 (Fig. 5b). As previously reported, PKA phosphorylation produced a marked increase in PDE activity, while phosphorylation by ERK elicited a circa 50% inhibition [16, 44]. Surprisingly, however, SUMOylation acted in different ways whose net outcome was to maintain maximally activated PDE4D5. Firstly, activation of PDE4D5 by PKA phosphorylation was augmented by SUMOylation, amplifying activity from around 275 ± 12 % of control (100%) for the PKA phosphorylated species alone to around 400 ± 49 % of control (Fig. 5b) for the species that was both PKA phosphorylated and SUMOylated (mean \pm SD; n = 3). Secondly, ERK inhibition of PDE4D5 was markedly attenuated by SUMOylation, with PDE4D5 activity now little changed from that of basal levels (Fig 5b).

When using either PKA or ERK to phosphorylate PDE4D5, there appeared to be no observable difference in the phosphorylation status of SUMO-modified PDE4D5 compared to native PDE4D5. This suggests that SUMOylation acts to change the conformation of PDE4D5 so as to reprogram the functional output of these phosphorylation events in terms of altering the response of the catalytic unit to regulatory inputs.

SUMOylation of PDE4D5 reprograms cAMP signalling in COS1 and HEK- β 2 cells.

Post-translational modification of PDE4D5, by SUMO, dually attenuates ERK induced inhibition and enhances PKA mediated activation of this cAMP phosphodiesterase *in vitro* (Fig 5). To determine whether these effects had functional correlates in living cells, we firstly investigated the intracellular cAMP levels in COS1 cells that had been transfected to overexpress recombinant PDE4D5 such that, as before [16], its activity represented >98% of the total cAMP PDE activity in these cells. As previously noted by us [16], EGF treatment, in causing ERK phosphorylation and inhibition of the activity of the recombinant, predominating PDE4D5, engenders a transient increase in cAMP that peaks at 10 min (Fig. 6). Interestingly, however, the amplitude of the EGF-mediated increase in intracellular cAMP was significantly blunted upon co-transfection with PIASy to cause the SUMOylation of PDE4D5, which is consistent with the SUMOylated fraction of PDE4D5 being less prone to ERK directed

inhibition (Fig. 6a). The EGF-mediated increase in intracellular cAMP was also completely abolished by pre-treatment with the MEK inhibitor PD98059, being consistent with an effect mediated by ERK (fig. 6a). No effect of PIASy co-transfection was evident in cells transfected with the VSV-tagged K323R-PDE4D5 mutant form (data not shown).

One functional output arising from an enhanced PKA activation of PDE4D5 is the lowering of localised cAMP in the vicinity of the β_2 -adrenoceptor (β_2 AR) [18-20]. Studies from our laboratory, using both dominant negative and siRNA approaches, have shown that active PDE4D5 is essential for a component of the desensitisation process regulating the β_2 AR, following agonist stimulation [19]. This relates to the ability of β -arrestin recruited PDE4D5 to regulate the phosphorylation of the β_2 AR by a locally acting fraction of PKA that is tethered to the β_2 AR by AKAP79 [18-20]. Here we noted that co-transfection of PDE4D5 with PIASy into HEK β_2 cells, which stably overexpress the β_2 AR, caused isoprenaline to elicit less PKA phosphorylation of the receptor at peak activation (5 min) when compared with identical cells over-expressing PDE4D5 alone (Fig. 6b). No effect of PIASy co-transfection was evident in cells transfected with the VSV-tagged K323R-PDE4D5 mutant form (data not shown). Presumably, the dual action of SUMO in both enhancing the activation of the PDE4 by PKA while concomitantly preventing inactivation via ERK phosphorylation, results in greater hydrolysis of the cAMP pool near to the β_2 AR which, in turn, leads to an associated down-regulation of localised PKA activity.

DISCUSSION

The small modifier SUMO, has been shown to have an important role in many biological processes including DNA repair, transcription and the cell cycle [23] with dysregulated SUMOylation being linked to diseases such as cancer, diabetes and neurological disorders [24]. Here, we report, for the first time, a novel potential regulatory function for SUMO within the cAMP signalling pathway. This involves the SUMOylation of the cAMP degrading long PDE4D and PDE4A isoforms at a single site within their conserved catalytic unit.

Post-translational modification of PDE4 enzymes by PKA- and ERK-mediated phosphorylation can confer functional changes in terms of alterations in enzyme activity status, conformation and interaction with various partner proteins, for example [15, 16, 40, 44, 45] with PKA-mediated activation of PDE4 long isoforms having a pivotal role in the desensitization of cells to cAMP signalling [15]. Moreover, recent studies [6, 7, 46, 47] utilising novel, genetically encoded cAMP reporters have shown that much of the specificity of function achieved by cAMP produced by adenylyl cyclase in response to agonists for different Gs-coupled receptors is determined through the control of spatially discrete pools of cAMP achieved by differently sequestered PDE4 isoforms, thereby underpinning compartmentalised cAMP signalling.

Modification by SUMO does not alter the constitutive ability of PDE4 enzymes to hydrolyse cAMP. Instead, we show here that SUMOylation serves to both enhance PKA-directed activation (Fig. 5) and to negate ERK-induced inhibition (Fig. 5); processes that have in common an absolute requirement for the regulatory UCR1 domain [12, 16, 42, 44]. As Conti and colleagues showed [48], we confirm here that PKA-mediated phosphorylation provides a critical component for the cellular desensitization

mechanism to cAMP by activating PDE4 long isoforms and thereby conferring transience on the increase in cAMP caused by ligands that activate adenylyl cyclase. We show here, however, that such transience is markedly pronounced in cells where PDE4D5 SUMOylation is triggered, indicating that this modification has the ability to amplify the cellular desensitization process towards cAMP (Fig. 7). Intriguingly, SUMOylation is restricted to long isoforms from the PDE4A and PDE4D sub-families; PDE4B and PDE4C enzymes lack the key acceptor lysine residue central to the classical SUMO consensus motif (Fig. 1) and short isoforms lack a crucial docking site for the SUMO E3 ligase, PIASy. Thus, only in signalling complexes involving PDE4D and PDE4A long isoforms would SUMOylation amplify activation due to PKA phosphorylation. This might therefore confer specific negative regulation of cAMP levels in distinct microdomains due to the selective sequestration of long PDE4A and PDE4D5 isoforms in concert with active SUMOylation machinery.

In addition to amplifying the effect of PKA-mediated phosphorylation on PDE4A and PDE4D, we also show here that SUMO modification reprograms the functional outcome of phosphorylation by ERK. This effect is restricted to PDE4D long isoforms, however, because PDE4A enzymes do not contain the ERK consensus motif in the third sub-domain of their catalytic unit and therefore cannot be phosphorylated by this kinase [15]. Also, as stated above, short PDE4Ds cannot bind SUMO E3s in order to increase the requisite fidelity for SUMOylation to ensue. Thus, uniquely, it is PDE4D long isoforms where SUMOylation negates the inhibitory action of ERK phosphorylation (Fig. 4).

Further work is required in order to define the mechanistic basis underpinning the functional consequences of PDE4A and PDE4D SUMOylation. However, it is conceivable that the SUMO subunit interfaces with UCR2 to stabilise interactions with the phosphorylated UCR1 whilst destabilising interactions with the ERK phosphorylation site. The basis for this proposal is the current consensus that UCR2 folds onto the core catalytic domain to exert an auto-inhibitory action [49, 50]. PKA-mediated phosphorylation of UCR1 establishes an interaction between the two UCR modules that either removes UCR2 from the core catalytic unit or alters its folding so as to remove the inhibitory action [13]. Phosphorylation by ERK, on the hand, may stabilise UCR2 in its inhibitory conformation. Given that the SUMO subunit is tethered to a site on the core catalytic domain proximal to the connection to UCR2, a direct interaction between SUMO and the UCR modules would be reasonable. It is also possible that SUMOylated PDE4s become novel interacting partners or substrates for proteins that contain conserved SUMO-interacting motifs (SIMs) [51]. SIM directed interactions are known to modify the turnover, conformation and localisation of SUMOylated enzymes and may be the reason why only a small proportion of a particular protein is modified at any given time. To our knowledge, none of the known PDE4 interacting proteins contain SIMs, however, we cannot rule out this possibility for novel interactions that remain undiscovered.

In summary we have discovered that long PDE4D and PDE4A isoforms, but not isoforms from the PDE4B and PDE4C sub-families have the potential to become SUMOylated. A major functional outcome of this modification is to amplify PDE4 activation elicited by PKA phosphorylation, thereby contributing to the cellular desensitization system attributed to PKA phosphorylation of PDE4. Additionally,

SUMOylation negates inhibitory regulation of long PDE4 isoforms caused by ERK phosphorylation, thereby ensuring that SUMO modified PDE4 remains fully active or activated. Finally, we show that SUMOylation enhances the activation of PDE4D5 following activation of the β_2 -adrenergic receptor and that this results in less PKA phosphorylation of the receptor by PKA. In this regard SUMO modification may represent a novel means to increase desensitization efficiency of this receptor. It is worth noting that SUMO modification of PDE4 isoforms may have consequences outside of β -adrenergic signalling. For example, PDE4A isoforms have been shown to modulate cAMP signals in T-cells to potentiate their activation [52], to control functioning of the p75 neurotrophin receptor (p75NTR) [53] and to be involved in cognitive deficits associated with sleep deprivation [54].

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Figure Legends

Figure 1 Delineation of a SUMO consensus motif in PDE4A and PDE4D isoforms.

(a) Delineation of a known negatively charged amino acid-dependent SUMO consensus motif that appears in known SUMO substrate PML and PDE4 isoforms from the PDEA and PDE4D subfamilies but not in PDE4B and PDE4C isoforms. (b) Schematic diagram of PDE4D5 highlighting domain structure and the positions of protein binding sites, phospho-sites and the putative SUMOylation site. (c) *In vitro* SUMOylation of peptide arrays encompassing cognate regions in representative PDE4 isoforms from four different sub-families, namely PDE4A, PDE4B, PDE4C and PDE4D. Putative SUMOylation sites are in red and successful conjugation of recombinant SUMO to immobilised peptides using an *in vitro* SUMO assay kit is represented by dark spots. (d) Structural detail of RanGAP1 (magenta) conjugated to SUMO (orange) and complexed to UBC9 (green) from crystal structure 1Z5S [35]. The PDE4A and PDE4D SUMOylation peptides (respectively yellow and blue) are shown superimposed from crystal structures 2QYK & 2FM0 [34, 55]. The local backbone conformation of the PDE4 SUMOylation peptides closely resembles the RanGAP1 structure. A cluster of basic residues on the surface of UBC9 may facilitate interaction with paired C-terminal acidic residues in the more extended NDSM: ϕ -K-X-D/E-X-X-D/E-D/E. RanGAP1 lacks these residues whilst the PDE4A and PDE4D peptides possess paired EE and ED residues at positions-6 and -7. Though surface-exposed, the location of these residues on helix-1 of the PDE4 core catalytic domain suggested that they are unlikely to facilitate SUMOylation by contributing to a docking interface for UBC9.

Figure 2 Selective SUMOylation of long PDE4 isoforms is triggered by expression of the SUMO E3 ligase PIASy.

(a) HEK293 cells were transfected with representative PDE4s from four separate subfamilies in the presence of empty vector (Cont) or the SUMO E3 ligase PIASy-HA (PIASy). Cell extracts were then blotted with antibodies specific for SUMO, PDE4 subfamily, or HA (PIASy). (b) HEK293 cells were transfected with short form PDE4D1 in the presence of empty vector (Cont) or the SUMO E3 ligase PIASy-HA (PIASy). Cell extracts were then blotted with antibodies specific for SUMO, PDE4 subfamily, or HA (PIASy). (c) HEK293 cells were transfected with either PDE4D1 or PDE4D5 in the presence of empty vector (Cont) or the SUMO E3 ligase PIASy-HA (PIASy). Immunoprecipitations of HA-PIASy and control IPs (beads alone) were probed for PDE4D. (d) Purified GST-fusions of indicated regions of PDE4D3: UCR1 (upstream conserved region 1), UCR2 (upstream conserved region 2), Cat. (Catalytic unit) and FL (full length) and GST alone (upper panel) were mixed with cellular lysates from HEK293 cells overexpressing HA-PIASy. GST pull-downs were blotted for the presence of HA-PIASy (lower panel). (e) Peptide arrays of overlapping 25-mer peptides, each sequentially shifted by 5 residues, which encompassed the entire PDE4D5 sequence were probed with cellular lysates from HEK293 cells over-expressing HA-PIASy.

Figure 3 Selective *in vitro* SUMOylation of PDE4D but not PDE4B isoforms in a cell-free assay

(a) PDE4D5 protein was transcribed *in vitro* and labelled with ^{35}S before being incubated in a SUMOylation assay mix. Control lanes contained PDE4D5 alone (TNT alone) or assay mixes where one of the following constituents had been omitted: SAE (-E1), UBC9 (-E2), recombinant SUMO1 (-sumo). SUMOylated PDE4D5 bands are denoted by “*”. (b) PDE4B2, PDE4D3 and PDE4D5 were transcribed *in vitro* and labelled with ^{35}S before being incubated in a SUMOylation assay mix that contained either SUMO1 (S1), SUMO2 (S2) or no SUMO (Un). SUMOylation assay mixes containing SP100 were included as a positive control. Higher molecular weight bands, which represent SUMOylated PDE4D3 and PDE4D5 bands are denoted by “*”. SUMOylated positive control SP100 are denoted by “‡”

Figure 4 Substitution of lysine 323 to arginine ablates SUMO modification of PDE4D5.

(a) PDE4D5 Wt and mutant PDE4D5 K²³²A were transcribed *in vitro* and labelled with ^{35}S before being incubated in a SUMOylation assay mix that contained either SUMO1 (S1), SUMO2 (S2) or no SUMO (Un). SUMOylation assay mixes containing SP100 were included as a positive control. Higher molecular weight bands, which represent SUMOylated PDE4D5 Wt are denoted by “*”. SUMOylated positive control SP100 are denoted by “‡”. (b) HEK293 cells were transfected with PDE4D5 Wt or mutant PDE4D5 K²³²A in the presence of the SUMO E3 ligase PIASy-HA (PIASy). Cell extracts were then blotted with antibodies specific for SUMO, PDE4D5, or HA (PIASy). (c) Alanine scanning peptide arrays of the putative SUMO consensus site (K305 – V329) from PDE4D5 was overlain with SUMO conjugation assay mix. Successful conjugation of recombinant SUMO to immobilised peptides is represented by dark spots.

Figure 5 SUMO modification of PDE4 isoforms augments activation of PDE4 activity conferred by PKA phosphorylation and represses inactivation caused by ERK phosphorylation.

Bacterially expressed, recombinant PDE4D5-GST was purified and SUMOylated using the SUMOylation kit before incorporation into kinase assays containing active PKA (a) or ERK (b). Samples were then immuno-blotted for PDE4D5 (upper panels) and phosphorylation of PKA consensus (a) or phospho-Serine (b) (lower panels). The PDE4 activity of all samples was determined using an established PDE assay (c). The data represents a mean of three independent experiments with associated standard errors (SEM). Statistical analysis was done using Student's T-test * <0.05 , *** <0.001 . (d) HEK293 cells were transfected HA-PIASy and co-transfected with either PDE4D5 (lower set) or PDE4D5 K323R (upper set) and treated with IBMX (100 μM) and forskolin (10 μM) for the indicated times. Cell lysates were then blotted for PIASy (HA), PKA phosphorylated PDE4 (P-UCR1), PDE4D and SUMO. Experiments are typical of those repeated 3 times.

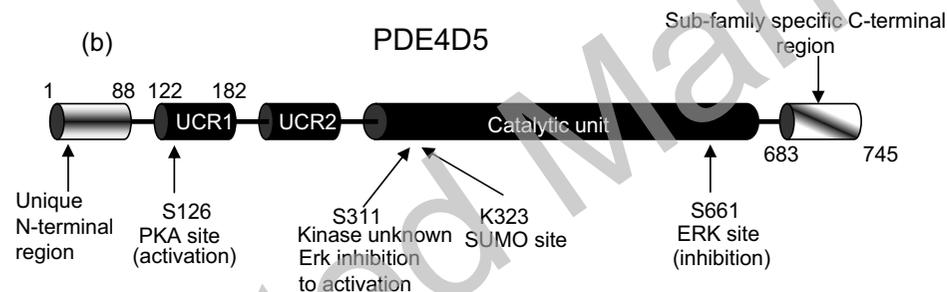
Figure 6 SUMO modification of PDE4D5 leads to an increase in cellular cAMP following EGF treatment and a reduction in PKA phosphorylation of the β 2-adrenergic receptor following isoprenaline treatment. (a) COS1 cells were transfected

with VSV-PDE4D5 or a combination of VSV-PDE4D5 and HA-PIASy. Cells were then treated with EGF for the indicated times. One set of cells, which had been transfected with a combination of VSV-PDE4D5 and HA-PIASy, was pre-treated with the MEK inhibitor (PD98059, 20 μ M) for ten minutes before EGF treatment. Cells were harvested and lysates analysed for cAMP concentrations as described in methods. Statistical analysis was done using Student's T-test, on data from three experiments, * <0.05 . (b) HEK- β 2 cells were transfected with VSV-PDE4D5 or a combination of VSV-PDE4D5 and HA-PIASy. Cells were then treated with isoprenaline (10 μ M) for the indicated times. Cell lysates were probed with antibodies raised against PKA phosphorylated β 2-AR (Ser345, Ser346), total β 2-AR, SUMO and VSV. The amount of relative phosphorylation of the β 2-AR was quantified using densitometry and plotted as a mean (n=3) with standard errors. Statistical analysis was done using Student's T-test, ** <0.01

(a)

	core	acidic patch
Consensus	ψ K x E x E E x	
PML (489-496)	I K t E s E E g	
PDE4A4 (357-364)	V K t D q E E I	
PDE4B2 (158-165)	V n t E n E D h	
PDE4C2 (206-213)	V q t D q E E q	
PDE4D5 (322-329)	V K t E q E D v	

(b)



(c)

PDE4A4	340-klmhsnslnnsniprfg VKtDqEEI -364
	345-nslnnsniprfg VKtDqEEI laqel -369
	350-sniprfg VKtDqEEI laqelenlnk -374
PDE4B2	141-klmhsslnntsisrfg VNtEnEDh -165
	146-sslnntsisrfg VNtEnEDh lakel -170
	151-tsisrfg VNtEnEDh lakeledlnk -175
PDE4C2	189-glchsaslsstpvprfg VQtDqEEq -213
	194-aslsstpvprfg VQtDqEEq lakel -218
	199-atvprfg VQtDqEEq lakeledtnk -223
PDE4D5	305-klmhsslnntssiprfg VKtEqEDv -329
	310-sslnntssiprfg VKtEqEDv lakel -334
	315-ssiprfg VKtEqEDv lakeledvnk -339

(d) Figure1

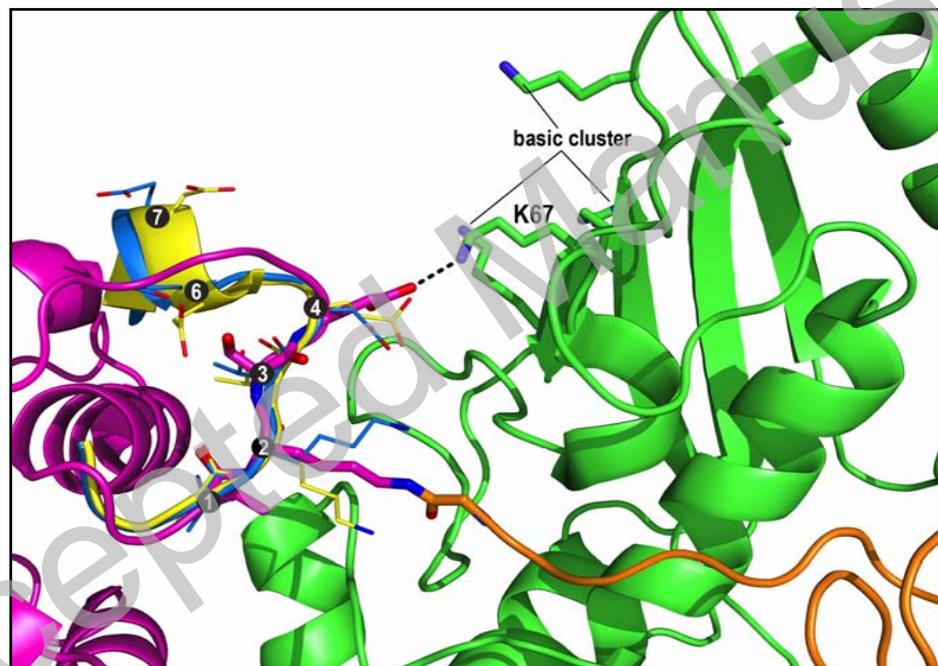
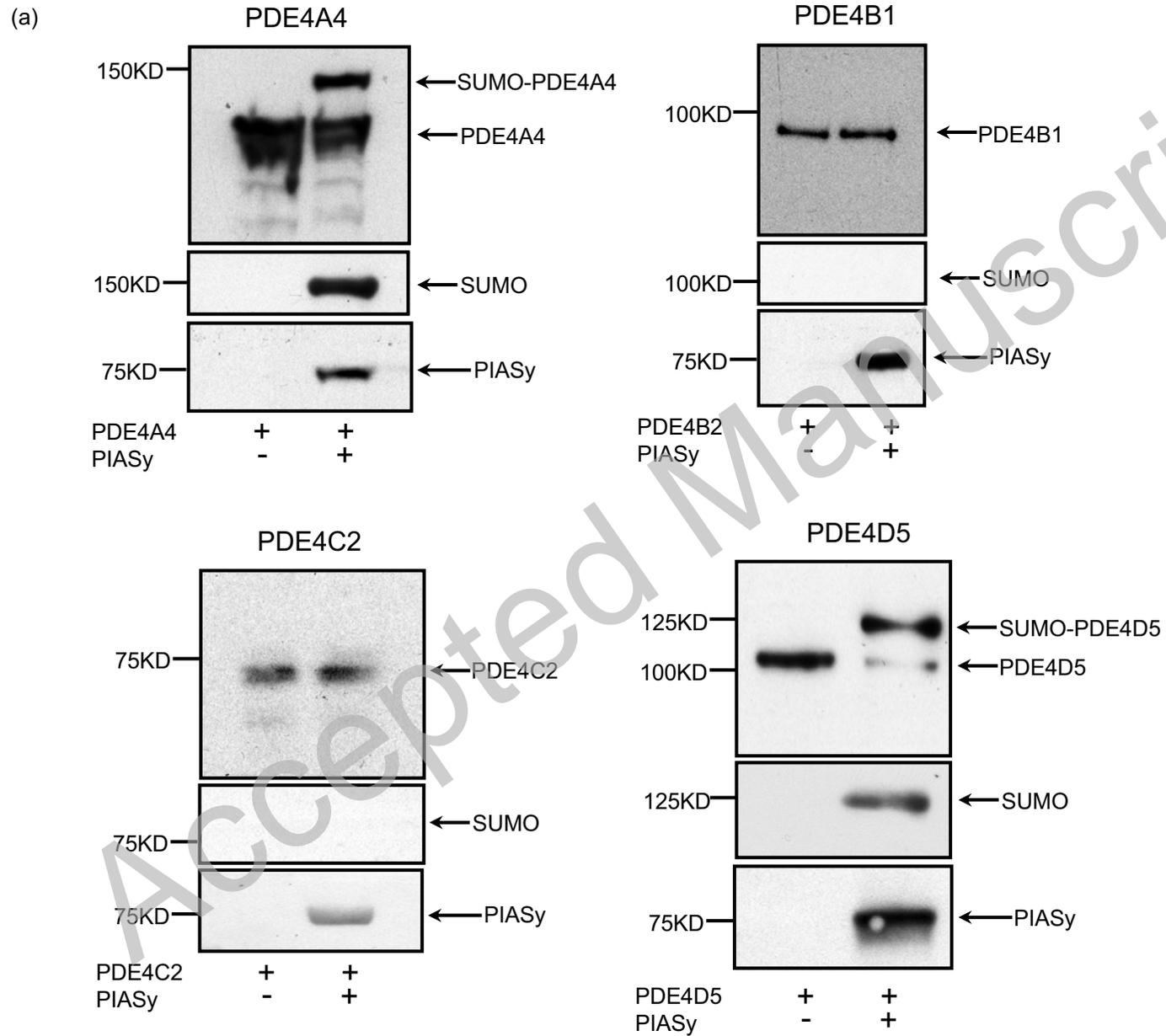
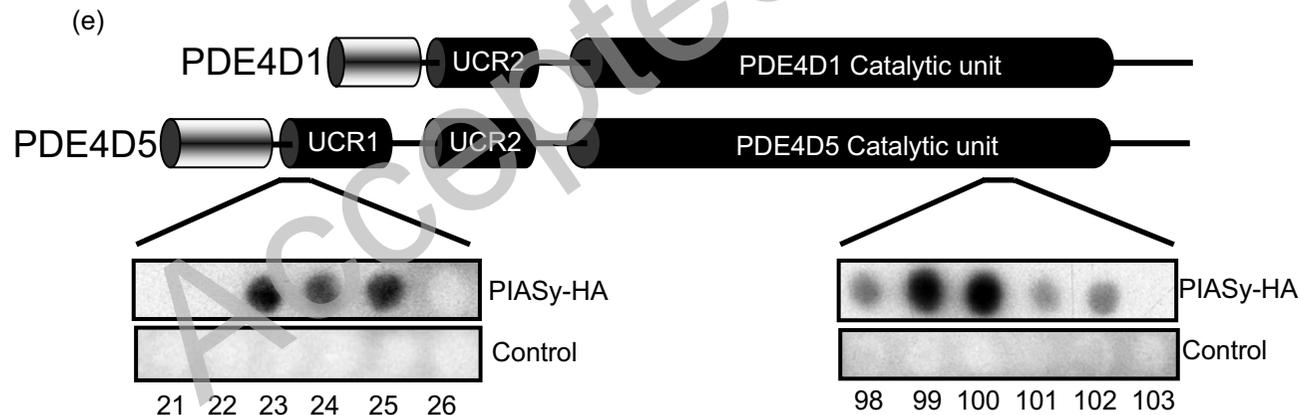
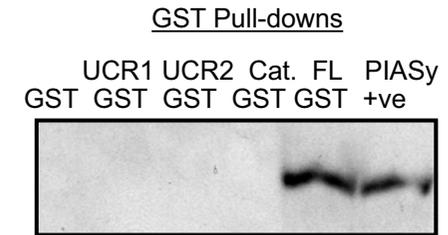
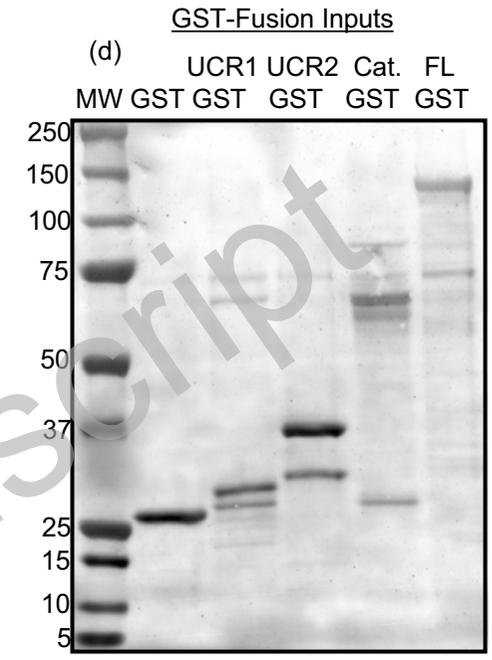
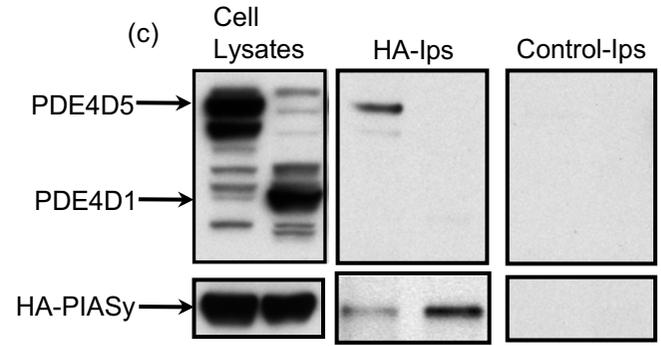
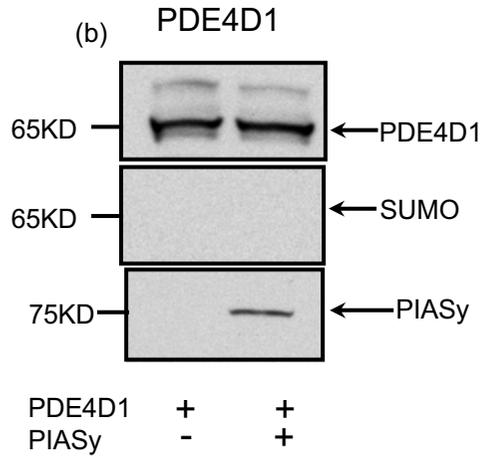


Figure2

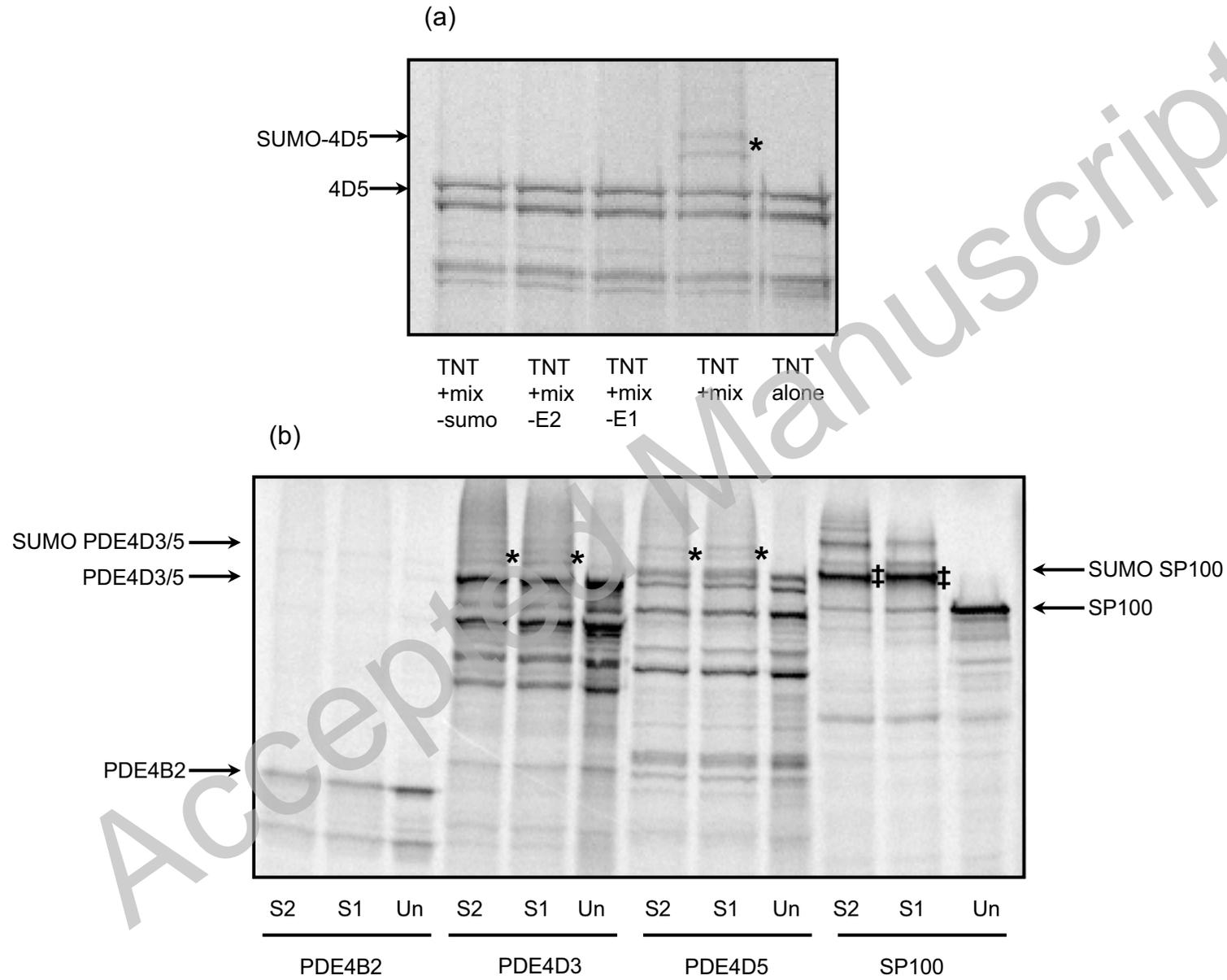




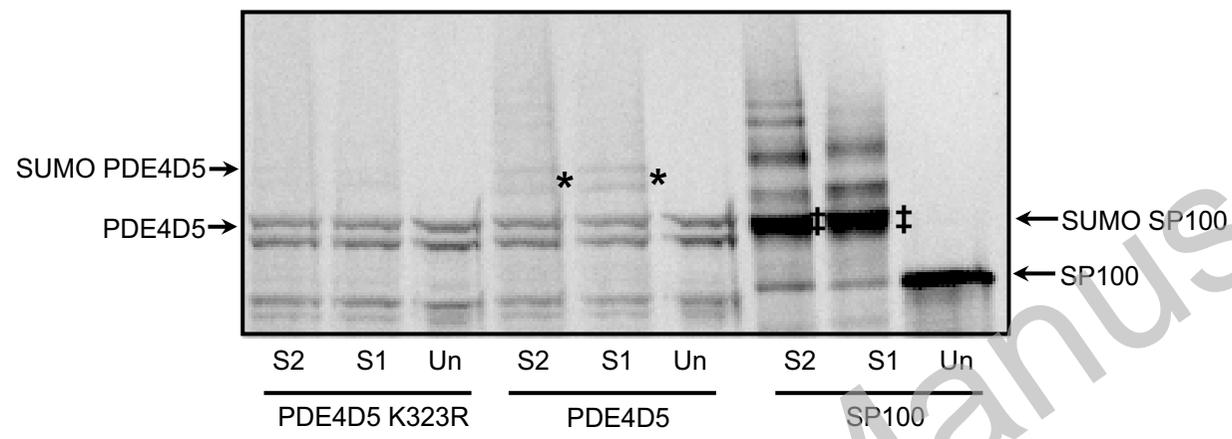
Spot 23: GLILQANFVHSQRRESFLYRSDSDY (111-135)
Spot 24: ANFVHSQRRESFLYRSDSDYDLSPK (116-140)
Spot 25: SQRRESFLYRSDSDYDLSPKMSRN (121-145)

Spot 98: IFQNLTKKQRQSLRKMVIDIVLATD (486-510)
Spot 99: TKKQRQSLRKMVIDIVLATDMSKHM (491-515)
Spot 100: QSLRKMVIDIVLATDMSKHMNLLAD (496-520)
Spot 101: MVIDIVLATDMSKHMNLLADLKTVM (501-525)
Spot 102: VLATDMSKHMNLLADLKTVMETKKV (506-530)

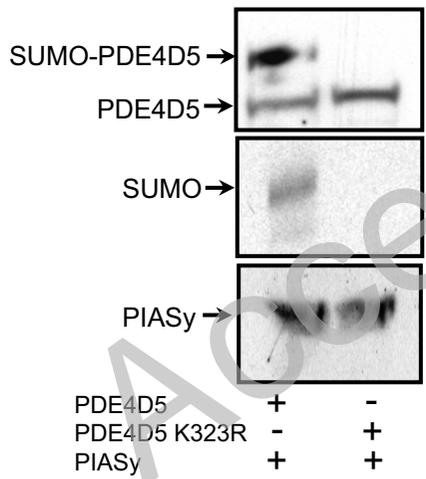
Figure3



(a) Figure4



(b)



(c)

