The second messenger cAMP controls key biological processes in all mammalian cells (1–4). Its homeostasis is of critical functional importance and is regulated both temporally and spatially via plasma membrane-bound cAMP-generating adenylyl cyclases and cAMP-degrading phosphodiesterases tethered to various signaling complexes and membranes (5–8). In defining compartmentalized cAMP signaling, members of the phosphodiesterase-4 (PDE4) family (5, 9), which specifically hydrolyze cAMP, play a pivotal role in many cell types (10–13). Alternative mRNA splicing of transcripts from four genes (PDE4A/B/C/D) generates over 20 distinct PDE4 isosforms, each characterized by a unique N-terminal region involved in targeting the isoform to specific signaling complexes (7, 9, 14). PDE4-selective inhibitors provide potent anti-inflammatory therapeutics targeted to inflammatory pulmonary disease (9, 15) and depression (16), with the PDE4D gene being linked to stroke (17) and the PDE4B gene to schizophrenia (18).

Specific PDE4 isosforms and sub-families have distinct, non-redundant phenotypic roles identified by dominant negative approaches, small interference RNA-mediated knockdown (10–12, 19, 20) and genetic ablation (21, 22). Pivotal to this is the recruitment of particular PDE4 isosforms to specific signaling complexes and intracellular locales, which confers a spatial dimension on cAMP signaling (14). Indeed, particular PDE4 isoforms have been shown to interact with the signaling scaffold proteins β-arrestin (23), RACK1 (24), AKAPs (25–27), and DISC1 (28) as well as myomegalin (29), the p75 neurotrophin receptor (30), and SRC family kinases (31).

Adrenaline exerts many of its actions in regulating cellular signaling processes by increasing the levels of the ubiquitous second messenger, cAMP (32, 33). In this a pivotal role is performed by the β2-AR, which couples to the G-protein, Gαs, to activate adenylyl cyclase. This process undergoes rapid desensitization due to the phosphorylation of the β2-AR by G-protein receptor kinases, allowing the recruitment of cytosolic β-arrestin to the β2-AR, which interdicts its coupling to Gαs (34, 35).

Recently an additional facet of the desensitization process has been uncovered, namely that β-arrestin can sequester the cAMP-specific phosphodiesterase PDE4D5, thereby delivering an active cAMP-hydrolyzing enzyme to the site of cAMP synthesis at the plasma membrane (10, 19). The PDE4D5 isoform preferentially interacts with β-arrestin, with distinct binding sites in both its isoform-specific N-terminal region and its con-
served catalytic region (19, 23, 24, 36, 37). Thus PDE4D5 straddles β-arrestin, with its unique N-terminal domain interacting with the β-arrestin C-domain and its catalytic unit interacting with the β-arrestin N-domain (38). In cardiac myocytes as well as various other primary and transfected cell types, the cytosolic β-arrestin-sequestered PDE4D5 is delivered to plasma membrane localized β2-adrenoceptors (β2ARs) upon agonist challenge (10). The sequestered Mdm2 can also affect β-arrestin ubiquitination (47), influencing the stability of β-arrestin interaction with GPCRs, thereby regulating receptor trafficking (48, 49). Ubiquitination normally occurs through a three-step process involving Ub-activating (E1), Ub-conjugating (E2), and Ub ligase (E3) enzymes. One means of recognizing transferred ubiquitin moieties on a protein is the presence of the so-called ubiquitin interacting motif (UIM) (50). More recently, however, various studies have suggested that a UIM can direct ubiquitination in various proteins and may facilitate mono-ubiquitination (51–54).

It is now appreciated that β2AR, β-arrestin, and G-protein receptor kinase are all subject to transient, agonist-dependent ubiquitination (47, 55–57). This modification is critical for both the internalization of the β2AR and regulation of its levels by lysosomal degradation. Underpinning the ubiquitination of both β-arrestin and G-protein receptor kinase (47) is the oncoprotein Mdm2, a well-known suppressor of p53 activity (46).

Here we identify, for the first time, the ubiquitination of a cyclic nucleotide phosphodiesterase, namely PDE4D5. Critical for this is a requirement for the β-arrestin-sequestered E3 ligase, Mdm2; a functional UIM located in the C-terminal portion of PDE4D5 plus a priming ubiquitination of PDE4D5 that allows a cascade of ubiquitination within the unique N-terminal region of PDE4D5. This modification enhances the interaction of PDE4D5 with the signaling scaffold protein, β-arrestin, thereby facilitating the fidelity of interaction of β-arrestin with this particular PDE4 isofrom.

**EXPERIMENTAL PROCEDURES**

**Materials**—PolyFect transfection reagent and Mdm2 small interference RNA were from Qiagen. Proteases inhibitor mixture tablets were from Roche Applied Science. VSV affinity agarose, anti-VSV antibody, isoprenaline, and N-ethylmaleimide were from Sigma. Mouse monoclonal antibody against ubiquitin (UbP4D1) and anti-Mdm2 antibodies were from Santa Cruz Biotechnology. In vitro ubiquitination kit was from Biomol. Protein G beads were from Amersham Biosciences. ECL was from Pierce. Purified Mdm2 used in vitro ubiquitination assay was a kind gift from Prof. R. Hay (University of Dundee, UK). HEK293 cells stably expressing the V2 receptor were a gift from Dr. R. Lefkowitz (Duke University).

**Site-directed Mutagenesis**—PDE4D5 mutants were constructed using QuikChange (Stratagene) and verified by DNA sequencing.

**Cell Culture, Transfection, Immunoprecipitation, and Western Blotting**—HEK293B2 cells are a stable cell line overexpressing the green fluorescent protein-tagged β2AR (58), which were cultured and analyzed as described by us previously (10, 19). HEK293B2 cells were transiently transfected with VSV epitope-tagged forms of either wild-type PDE4D5, a β-arrestin binding-defective PDE4D5 (E27A), a RACK-1 binding-defective (L29/30A) PDE4D5, or a UIM-disrupted (E721A:E722A: E723A) PDE4D5. Cell lysis, immunopurification, and Western blotting were done as described before by us in some detail (10, 36, 37).

In Vivo Ubiquitination Assay—HEK293B2 cells seeded in 100-mm dishes were transfected with the indicated VSV-epitope-tagged PDE4D5 species. After 48 h, cells were challenged with 10 μM isoprenaline for the indicated time. Subsequently, cells were lysed in a 3T3 lysis buffer containing 20 mM HEPEs (pH 7.4), 50 mM NaCl, 50 mM NaF, 10% glycerol, 1% Triton X-100, 10 mM EGTA, 30 mM sodium pyrophosphate, 10 mM N-ethylmaleimide, and Roche Applied Science proteases inhibitor mixture. Ubiquitin conjugates were immunopurified on VSV-agarose beads. Ubiquitinated species were detected by immunoblotting using anti-Ubiquitin antibody. PDE4D5 expression levels were determined in cell lysates by Western blotting using specific antibodies. Endogenous ubiquitination analyses were done similarly except that immunopurification was done using a PDE4D5-specific antibody.

**In Vitro Ubiquitination Assay**—A Ubiquitination Kit (Biomol) was used, and the procedure was carried out according to the manufacturer’s instruction. PDE4D5 peptide array analyses were performed as described previously (36, 38). The membranes were washed with TBST (137 mM NaCl, 20 mM Tris HCl, pH 7.6, 0.1% Tween 20) to stop the ubiquitination reaction. E2-conjugating enzyme UbcH5b and E3 ligase glutathione S-transferease-Mdm2 were used in this assay. The ubiquitin moieties on the peptide array were detected by Western blotting using ubiquitin-specific antibody.

**SPOT Synthesis of Peptides and Overlay Experiments**—This was done as described by us in detail elsewhere (36, 38).

**RESULTS**

The PDE4D Family Uniquely Exhibits a Potential UIM—Individual PDE4 isofoms are characterized by their unique N-terminal regions. However, although all members of a particular PDE4 sub-family have identical C-terminal regions, these regions show no homology between each of the four different sub-families (5, 7). Sequence inspection (Table 1) shows that the sub-family specific C-terminal region of PDE4D contains a potential UIM, for which a 20-residue consensus sequence has been defined of the form: X-Ac-Ac-Ac-Ac-Φ-X-X-X-Ala-X-X-Ser-X-X-Ac-X-X-Φ, where Φ represents a hydrophobic residue, Ac represents an acidic residue, and X
represents residues that are less well conserved (59). There is no comparable sequence in PDE4 isoforms from the other three
represents residues that are less well conserved (59). There is no comparable sequence in PDE4 isoforms from the other three
represents residues that are less well conserved (59). There is no comparable sequence in PDE4 isoforms from the other three
represents residues that are less well conserved (59). There is no comparable sequence in PDE4 isoforms from the other three
represents residues that are less well conserved (59). There is no comparable sequence in PDE4 isoforms from the other three

Isoprenaline Selectively Triggers the Ubiquitination of PDE4D5—Because agonist stimulation of the β2-AR elicits the transient ubiquitination of β-arrestin (47) and β-arrestin preferentially sequesters PDE4D5 (37), we set out to determine whether PDE4D5 could be similarly ubiquitinated. To assess this we examined HEK293B2 cells, which are a HEK293 cell line that stably expresses the β2AR (58). We have used these cells, as well as primary cardiac myocytes and mouse embryonic fibroblasts (MEFs), to show that a pool of PDE4D5 is sequestered to β-arrestin and that this is transiently recruited to the β2-AR upon agonist stimulation (10, 19, 23).

HEK293B2 cells were transiently transfected with VSV-epitope-tagged PDE4D5 and, after 48 h, were challenged with the βAR agonist, isoprenaline, for the indicated times (Fig. 2a). VSV-PDE4D5 was immunopurified using VSV-agarose and then subjected to SDS-PAGE for immunoblotting using a specific Ub antibody (Fig. 2a). In doing this we identified the transient appearance of a distinctive smeared band that is typical of poly-ubiquitinated proteins (44, 61). This was evident 5 min after isoprenaline challenge, still apparent after 10 min, but absent after 12 min (Fig. 2a) and up to 180 min (supplemental Fig. S1a). We also observed a 105-kDa band indicative of mono-ubiquitinated PDE4D5 (Fig. 2a). This species was evident in resting cells, but its levels increased upon isoprenaline challenge (Fig. 2a).

Subsequently, we examined this for endogenous PDE4D5 in primary cells. We noted that isoprenaline caused the transient ubiquitination of PDE4D5 in cardiomyocytes, with a smeared band evident 5 min after isoprenaline challenge, increasing up until 10 min, and subsequently decreasing (Fig. 2b). These data are remarkably similar to those observed using transfected

represents residues that are less well conserved (59). There is no comparable sequence in PDE4 isoforms from the other three
represents residues that are less well conserved (59). There is no comparable sequence in PDE4 isoforms from the other three
represents residues that are less well conserved (59). There is no comparable sequence in PDE4 isoforms from the other three
represents residues that are less well conserved (59). There is no comparable sequence in PDE4 isoforms from the other three
represents residues that are less well conserved (59). There is no comparable sequence in PDE4 isoforms from the other three

Isoprenaline Selectively Triggers the Ubiquitination of PDE4D5—Because agonist stimulation of the β2-AR elicits the transient ubiquitination of β-arrestin (47) and β-arrestin preferentially sequesters PDE4D5 (37), we set out to determine whether PDE4D5 could be similarly ubiquitinated. To assess this we examined HEK293B2 cells, which are a HEK293 cell line that stably expresses the β2AR (58). We have used these cells, as well as primary cardiac myocytes and mouse embryonic fibroblasts (MEFs), to show that a pool of PDE4D5 is sequestered to β-arrestin and that this is transiently recruited to the β2-AR upon agonist stimulation (10, 19, 23).

HEK293B2 cells were transiently transfected with VSV-epitope-tagged PDE4D5 and, after 48 h, were challenged with the βAR agonist, isoprenaline, for the indicated times (Fig. 2a). VSV-PDE4D5 was immunopurified using VSV-agarose and then subjected to SDS-PAGE for immunoblotting using a specific Ub antibody (Fig. 2a). In doing this we identified the transient appearance of a distinctive smeared band that is typical of poly-ubiquitinated proteins (44, 61). This was evident 5 min after isoprenaline challenge, still apparent after 10 min, but absent after 12 min (Fig. 2a) and up to 180 min (supplemental Fig. S1a). We also observed a 105-kDa band indicative of mono-ubiquitinated PDE4D5 (Fig. 2a). This species was evident in resting cells, but its levels increased upon isoprenaline challenge (Fig. 2a).

Subsequently, we examined this for endogenous PDE4D5 in primary cells. We noted that isoprenaline caused the transient ubiquitination of PDE4D5 in cardiomyocytes, with a smeared band evident 5 min after isoprenaline challenge, increasing up until 10 min, and subsequently decreasing (Fig. 2b). These data are remarkably similar to those observed using transfected

represents residues that are less well conserved (59). There is no comparable sequence in PDE4 isoforms from the other three
represents residues that are less well conserved (59). There is no comparable sequence in PDE4 isoforms from the other three
represents residues that are less well conserved (59). There is no comparable sequence in PDE4 isoforms from the other three
represents residues that are less well conserved (59). There is no comparable sequence in PDE4 isoforms from the other three
represents residues that are less well conserved (59). There is no comparable sequence in PDE4 isoforms from the other three

Isoprenaline Selectively Triggers the Ubiquitination of PDE4D5—Because agonist stimulation of the β2-AR elicits the transient ubiquitination of β-arrestin (47) and β-arrestin preferentially sequesters PDE4D5 (37), we set out to determine whether PDE4D5 could be similarly ubiquitinated. To assess this we examined HEK293B2 cells, which are a HEK293 cell line that stably expresses the β2AR (58). We have used these cells, as well as primary cardiac myocytes and mouse embryonic fibroblasts (MEFs), to show that a pool of PDE4D5 is sequestered to β-arrestin and that this is transiently recruited to the β2-AR upon agonist stimulation (10, 19, 23).

HEK293B2 cells were transiently transfected with VSV-epitope-tagged PDE4D5 and, after 48 h, were challenged with the βAR agonist, isoprenaline, for the indicated times (Fig. 2a). VSV-PDE4D5 was immunopurified using VSV-agarose and then subjected to SDS-PAGE for immunoblotting using a specific Ub antibody (Fig. 2a). In doing this we identified the transient appearance of a distinctive smeared band that is typical of poly-ubiquitinated proteins (44, 61). This was evident 5 min after isoprenaline challenge, still apparent after 10 min, but absent after 12 min (Fig. 2a) and up to 180 min (supplemental Fig. S1a). We also observed a 105-kDa band indicative of mono-ubiquitinated PDE4D5 (Fig. 2a). This species was evident in resting cells, but its levels increased upon isoprenaline challenge (Fig. 2a).

Subsequently, we examined this for endogenous PDE4D5 in primary cells. We noted that isoprenaline caused the transient ubiquitination of PDE4D5 in cardiomyocytes, with a smeared band evident 5 min after isoprenaline challenge, increasing up until 10 min, and subsequently decreasing (Fig. 2b). These data are remarkably similar to those observed using transfected

represents residues that are less well conserved (59). There is no comparable sequence in PDE4 isoforms from the other three
represents residues that are less well conserved (59). There is no comparable sequence in PDE4 isoforms from the other three
represents residues that are less well conserved (59). There is no comparable sequence in PDE4 isoforms from the other three
represents residues that are less well conserved (59). There is no comparable sequence in PDE4 isoforms from the other three
represents residues that are less well conserved (59). There is no comparable sequence in PDE4 isoforms from the other three

Isoprenaline Selectively Triggers the Ubiquitination of PDE4D5—Because agonist stimulation of the β2-AR elicits the transient ubiquitination of β-arrestin (47) and β-arrestin preferentially sequesters PDE4D5 (37), we set out to determine whether PDE4D5 could be similarly ubiquitinated. To assess this we examined HEK293B2 cells, which are a HEK293 cell line that stably expresses the β2AR (58). We have used these cells, as well as primary cardiac myocytes and mouse embryonic fibroblasts (MEFs), to show that a pool of PDE4D5 is sequestered to β-arrestin and that this is transiently recruited to the β2-AR upon agonist stimulation (10, 19, 23).

HEK293B2 cells were transiently transfected with VSV-epitope-tagged PDE4D5 and, after 48 h, were challenged with the βAR agonist, isoprenaline, for the indicated times (Fig. 2a). VSV-PDE4D5 was immunopurified using VSV-agarose and then subjected to SDS-PAGE for immunoblotting using a specific Ub antibody (Fig. 2a). In doing this we identified the transient appearance of a distinctive smeared band that is typical of poly-ubiquitinated proteins (44, 61). This was evident 5 min after isoprenaline challenge, still apparent after 10 min, but absent after 12 min (Fig. 2a) and up to 180 min (supplemental Fig. S1a). We also observed a 105-kDa band indicative of mono-ubiquitinated PDE4D5 (Fig. 2a). This species was evident in resting cells, but its levels increased upon isoprenaline challenge (Fig. 2a).

Subsequently, we examined this for endogenous PDE4D5 in primary cells. We noted that isoprenaline caused the transient ubiquitination of PDE4D5 in cardiomyocytes, with a smeared band evident 5 min after isoprenaline challenge, increasing up until 10 min, and subsequently decreasing (Fig. 2b). These data are remarkably similar to those observed using transfected
However, the increased levels of mono-ubiquitinated species seen in transfected cells may reflect a rate-limiting step for polyubiquitination in these cells.

PDE4D5 is also endogenously expressed in both HEKB2 cells (Fig. 2a) and MEFs (Fig. 2d) (10, 38), where isoprenaline challenge caused the transient appearance of a smeared polyubiquitinated species 10 min after isoprenaline challenge (Fig. 2, c and d). Note that with MEFs no such smeared species was seen 12 min after isoprenaline challenge (data not shown). Interestingly, in neither of these cells did we observe a distinct mono-ubiquitinated species. The basis for lack of accumulation of mono-ubiquitinated species in these cells remains to be ascertained. It might reflect a very rapid polyubiquitination that prevents accumulation of mono-ubiquitinated species, for example.

The transient appearance of ubiquitinated PDE4D5 could be because ubiquitination can target proteins for degradation through the proteasomal system. To evaluate this we challenged cells (Fig. 2e) with the proteasome inhibitor, MG132 (42). Doing this we observed that MG132 enhanced the accumulation of ubiquitinated PDE4D5 observed after 10-min isoprenaline challenge, but still no ubiquitinated species were evident 20 min after isoprenaline challenge (Fig. 2e). Although proteasome inhibition did not lead to a sustained accumulation of ubiquitinated PDE4D5 species, it nearly doubled the amount of ubiquitinated species observed after 10 min of isoprenaline challenge (191 ± 18% with MG132 compared with that without, mean ± S.D., n = 3).

We also set out to determine if PDE4 isoforms from other sub-families could be similarly ubiquitinated. To do this we transiently transfected HEKB2 cells with VSV-tagged forms of arange of other long PDE4 isoforms, namely, PDE4A5, PDE4B1, PDE4B3, PDE4B4, PDE4C1, and PDE4D3, challenged with isoprenaline for the indicated times (Fig. 2f).

**FIGURE 2.** GPCR agonist-stimulated ubiquitination of PDE4D5. a, HEKB2 cells were transfected to express VSV-tagged PDE4D5 and then challenged for the indicated times with isoprenaline (10 μM). VSV-tagged PDE4D5 was immunopurified and immunoblotted with either a VSV antibody or an ubiquitin antibody. b, rat cardiomyocytes were similarly treated, PDE4D5-immunopurified, and then immunoblotted with either an antisera specific for ubiquitin or one specific for PDE4D5. c, untransfected HEKB2 cells were challenged for the indicated times with isoprenaline (10 μM) and endogenous PDE4D5-immunopurified and then immunoblotted with either an antisera specific for ubiquitin or one specific for PDE4D5. d, MEFs were treated or not for 10 min with isoprenaline (10 μM) and endogenous PDE4D5-immunopurified and then immunoblotted with either an antisera specific for ubiquitin or one specific for PDE4D5.

Additional notes on the text include references to experimental procedures, cell lines used, and the specific conditions under which the experiments were conducted. The figure (FIGURE 2) illustrates the transient appearance of polyubiquitinated PDE4D5, demonstrating the role of ubiquitination in proteasomal degradation of the enzyme.
**Mdm2 Directs cAMP PDE4D5 Ubiquitination**

**FIGURE 3. PDE4D5 ubiquitination requires Mdm2 and β-arrestin.** a, HEKB2 cells transfected to express VSV-PDE4D5 either were or were not subjected to small interference RNA-mediated knockdown of Mdm2 (Mdm2 KO) prior to isoprenaline (10 μM) challenge (10 min). Upper panel shows lysate immunoblots for endogenous Mdm2 and VSV-PDE4D5. Lower panel shows immunopurified VSV-PDE4D5 blotted for ubiquitin and VSV. b, HEKB2 cells transfected to express the indicated mutant forms of VSV-PDE4D5 were challenged with isoprenaline and analyzed as in a. HEKB2 cells were transfected to express either wild type or the indicated mutant forms of VSV-tagged PDE4D5. UIM is the UIM-disrupted E721A:E722A:E723A-PDE4D5-VSV mutant; E27A-VSV-PDE4D5 is a mutant that fails to bind β-arrestin; and L29A:V30A-VSV PDE4D5 is a mutant that can bind β-arrestin, but which fails to bind RACK1. Upper panel shows immunopurified endogenous Mdm2 blotted for Mdm2 and for VSV-PDE4D5. Lower panel shows immunopurified VSV-PDE4D5 blotted for Mdm2 and for VSV-PDE4D5. c, HEKB2 cells were transfected to express the E27A-VSV-PDE4D5, a mutant that fails to bind β-arrestin, then challenged for the indicated times with isoprenaline (10 μM) prior to immunopurification of the VSV-tagged PDE4D5 mutant, which was then immunoblotted with either a VSV antibody (upper panel) or an ubiquitin antibody (lower panel). PDE4D5 wild type also analyzed as a positive control. d, as in c but with L29A:V30A-VSV PDE4D5, a mutant that can bind β-arrestin, but which fails to bind RACK1. e, cells were transfected with VSV-tagged forms of either PDE4D5 or PDE4D3 and challenged for the indicated times with isoprenaline prior to analysis as in c. Data show experiments typical of ones performed three times.

Time, selectively immunopurified each isoform using VSV-agarose, and then probed them with anti-ubiquitin antibody. However, in no instance did we observe the appearance of any immunoreactive species (Data not shown and Fig. 3e), signifying lack of ubiquitination under such conditions.

The UIM of PDE4D5 Is Required for Its Isoprenaline-stimulated Ubiquitination—To determine whether a UIM was needed for the isoprenaline-induced ubiquitination of PDE4D5, we disrupted it by mutating the conserved N-terminal patch of acidic amino acids, E721EE (Table 1 and Fig. 2f). In studies on other proteins, cognate residues have been shown to be key in conferring Ub binding (59). Indeed, the (E721A:E722A:E723A)-PDE4D5-VSV showed no ability to be ubiquitinated upon isoprenaline challenge (Fig. 2f) but still bound β-arrestin (Fig. 2g). These data indicate that PDE4D5 has a functional UIM and that the glutamate patch within this UIM is critical for its functioning.

**PDE4D5 Undergoes Prolonged Ubiquitination in HEK293V2 Cells Subsequent to Vasopressin Challenge—**The β,AR is a Class A GPCR, because it interacts transiently with β-arrestins, whereas the vasopressin V₂ receptor is a Class B GPCR, showing a prolonged interaction with β-arrestins (62). To evaluate whether cell activation through these two GPCR classes exerted different effects on PDE4D5 ubiquitination, we evaluated HEK293V2 cells stably overexpressing the vasopressin V₂ receptor (63). These were transiently transfected with VSV-tagged PDE4D5 and challenged with vasopressin for the indicated times. Immunopurified VSV-PDE4D5 was immunoblotted with a specific ubiquitin antibody (Fig. 2h). Strikingly, unlike the transient ubiquitination of PDE4D5 in HEKB2 cells, VSV-PDE4D5 became rapidly ubiquitinated and remained stably modified over an extended time course (Fig. 2h). This phenomenon is reminiscent of the protracted ubiquitination of both the V₂ receptor and β-arrestin that has been observed in such cells (64). In contrast, the (E721A:E722A:E723A)-PDE4D5-VSV mutant with a disrupted UIM failed to become ubiquitinated in these cells (Fig. 2i). No ubiquitination signal was detected in the VSV-immunoprecipitates from HEK293V2 cells that had not been transfected with VSV-tagged PDE4D5 (data not shown).

A β-Arrestin-sequestered Pool of the E3 Ligase, Mdm2 Is Required for Poly-ubiquitination of PDE4D5—Because β-arrestin is known to sequester PDE4D5 (10, 19) and the E3 ligase, Mdm2 (47), we set out to determine if Mdm2 mediates PDE4D5 ubiquitination. Indeed, small interference RNA-mediated knockdown of endogenous Mdm2 ablated isoprenaline-stimulated ubiquitination of PDE4D5 in HEK293B2 cells (Fig. 3a). Furthermore, we found that Mdm2 co-immunoprecipitated with PDE4D5-VSV expressed in HEK293B2 cells (Fig. 3b). This interaction was clearly mediated via β-arrestin sequestering both Mdm2 and PDE4D5, because Mdm2 failed to co-immunoprecipitate (Fig. 3b) with (E27A)-PDE4D5-VSV, a mutant form of PDE4D5 that is unable to bind β-arrestin (36). Reverse immunoprecipitation experiments were performed where wild-type and the mutant forms of VSV-tagged PDE4D5

---

**FIGURE 3.** PDE4D5 ubiquitination requires Mdm2 and β-arrestin. a, HEKB2 cells transfected to express VSV-PDE4D5 either were or were not subjected to small interference RNA-mediated knockdown of Mdm2 (Mdm2 KO) prior to isoprenaline (10 μM) challenge (10 min). Upper panel shows lysate immunoblots for endogenous Mdm2 and VSV-PDE4D5. Lower panel shows immunopurified VSV-PDE4D5 blotted for ubiquitin and VSV. b, HEKB2 cells transfected to express the indicated mutant forms of VSV-PDE4D5 were challenged with isoprenaline and analyzed as in a. HEKB2 cells were transfected to express either wild type or the indicated mutant forms of VSV-tagged PDE4D5. UIM is the UIM-disrupted E721A:E722A:E723A-PDE4D5-VSV mutant; E27A-VSV-PDE4D5 is a mutant that fails to bind β-arrestin; and L29A:V30A-VSV PDE4D5 is a mutant that can bind β-arrestin, but which fails to bind RACK1. Upper panel shows immunopurified endogenous Mdm2 blotted for Mdm2 and for VSV-PDE4D5. Lower panel shows immunopurified VSV-PDE4D5 blotted for Mdm2 and for VSV-PDE4D5. c, HEKB2 cells were transfected to express the E27A-VSV-PDE4D5, a mutant that fails to bind β-arrestin, then challenged for the indicated times with isoprenaline (10 μM) prior to immunopurification of the VSV-tagged PDE4D5 mutant, which was then immunoblotted with either a VSV antibody (upper panel) or an ubiquitin antibody (lower panel). PDE4D5 wild type also analyzed as a positive control. d, as in c but with L29A:V30A-VSV PDE4D5, a mutant that can bind β-arrestin, but which fails to bind RACK1. e, cells were transfected with VSV-tagged forms of either PDE4D5 or PDE4D3 and challenged for the indicated times with isoprenaline prior to analysis as in c. Data show experiments typical of ones performed three times.
were isolated on the VSV-agarose, and their association with Mdm2 was analyzed by immunoblotting using a specific Mdm2 antibody. These results showed lack of co-immunoprecipitation of Mdm2 with E27A-PDE4D5-VSV, a mutant that is unable to bind β-arrestin (Fig. 3b).

PDE4D5 can also be sequestered by RACK1 (36). However, the binding of PDE4D5 to RACK1 and β-arrestin is mutually exclusive due to overlapping binding sites (36). Here we show that (L29A:V30A)-PDE4D5-VSV, a mutant of PDE4D5 that binds β-arrestin but not RACK1 (36), co-immunoprecipitates with Mdm2 (Fig. 3b). Note that the E27A-PDE4D5-VSV mutant is still able to bind RACK1 (36).

The (E27A)-PDE4D5-VSV, which fails to bind β-arrestin, does not become ubiquitinated upon isoprenaline challenge when transfected into HEK-B2 cells (Fig. 3c). In marked contrast to this, the (L29A:V30A)-PDE4D5-VSV mutant that binds β-arrestin but not RACK1, clearly does become ubiquitinated (Fig. 3d). In “head to head” comparisons of wild-type PDE4D5-VSV with the (L29A:V30A)-PDE4D5-VSV mutant we did not see any difference (<9%; n = 3) between them in their increased levels of ubiquitination that occurred 10 min after challenge of cells expressing them with isoprenaline.

We have shown previously that transfection of β-arrestin1/2 double knockout MEFs with β-arrestin2 allows co-immunoprecipitation of endogenous PDE4D5 with β-arrestin2 (10, 23, 38). Here we show that transfection of β-arrestin1/2 double knockout MEFs, which express similar levels of Mdm2 to native cells (Fig. 4a), with FLAG-tagged β-arrestin2, allows for the reconstitution of the isoprenaline-mediated ubiquitination of endogenous PDE4D5 in these cells (Fig. 4b). In marked contrast to this, if such cells are transfected with the R26A mutant form of FLAG-tagged β-arrestin2, which is unable to bind to PDE4D5 (38), then such reconstitution fails to occur (Fig. 4b). In transfected MEFs, Mdm2 co-immunoprecipitated with similar amounts (<10% difference) of either wild-type FLAG-tagged β-arrestin2 or the R26A FLAG-tagged β-arrestin2 mutant (Fig. 4c). Thus the discrete R26A mutation that ablates PDE4D5 interaction does not prevent Mdm2 association with β-arrestin2. These data are consistent with the critical importance of β-arrestin in scaffolding both PDE4D5 and Mdm2 to allow for the isoprenaline-mediated ubiquitination of PDE4D5.

The N-terminal Region of PDE4D5 Contains Target Lysines for Ubiquitin Conjugation—No clear consensus motif has been reported to predict accurately the sites of ubiquitin conjugation on proteins. Indeed, in many cases, multiple lysine residues found within different sequence motifs can function as ubiquitin acceptor sites (65–67). In the subset of ubiquitinated proteins evaluated fully to date, extensive mutation of multiple lysine residues has been done to determine ubiquitination sites. This is a considerable challenge in proteins with large numbers of lysine residues such as PDE4D5, which contains 39 lysines. To aid in addressing this problem we employ here a novel methodology involving the in vitro ubiquitination of spot immobilized peptide arrays. These have been used previously by us to define protein-protein interaction surfaces of specific PDE4 isoforms with various scaffolds including β-arrestin and RACK1 (36, 38). Here, we generated an array of overlapping 25-mer peptides, each sequentially shifted by 5 residues, which encompassed the entire PDE4D5 sequence. This was subjected to in vitro ubiquitination using purified glutathione S-transferase-Mdm2 as the E3 ligase before probing with an anti-ubiquitin antibody for positive signals noted as dark spots and negative signals as clear areas (Fig. 5a). This identified two clusters of peptides that became ubiquitinated (Fig. 5a). Within the core sequence of the first set of peptides were Lys-48, Lys-53, and Lys-78, which are located within the unique N-terminal region of PDE4D5, and in the second set the single lysine, Lys-140 located within UCR1 (Fig. 1a). Consistent with the unique N-terminal region of PDE4D5 being critical for isoprenaline-stimulated ubiquitination, we failed to observe ubiquitination of PDE4D3 in cells transfected to express this isoform (Fig. 3e). Only the N-terminal region of PDE4D3 distinguishes it from PDE4D5, and this, unlike that of PDE4D5, does not contain any lysine residues.

To evaluate their possible involvement in ubiquitination of PDE4D5 we first mutated all these residues to arginine, as in quad-PDE4D5 (K48R:K53R:K78R:K140R) mutant. This mutation did not compromise binding to β-arrestin compared with...
Mdm2 Directs cAMP PDE4D5 Ubiquitination

FIGURE 5. PDE4D5 ubiquitination sites. a, a scanning peptide array of the entire 745-amino acid sequence of PDE4D5 was generated using overlapping immobilized 25-mer peptides each displaced by 5 residues. This was subjected to in vitro ubiquitination with recombinant Mdm2 and subsequent detection of bound ubiquitin using an anti-ubiquitin antibody. The dark spots indicate positive interacting peptides, whose sequence incorporated the indicated lysine residues. b, HEK2B2 cells were transfected with either wild-type VSV-PDE4D5 or the Quad (K48R:K53R:K78R:K140R)-VSV-PDE4D5 or Triple (K48R:K53R:K78R)-VSV-PDE4D5 mutant forms. Lysates expressing equal amounts of each VSV-PDE4D5 species were taken, then precipitated with a specific antiserum and the immunoprecipitates blotted with a VSV-specific antiserum to detect PDE4D5.

d, in resting HeK2B2 cells, the single K:R mutants forms of the PDE4D5 mutant which does not become ubiquitinated in these cells (10, 36). We thus set out to determine if the isoprenaline-stimulated poly-ubiquitination of these constructs as compared with wild-type PDE4D5 (Fig. 5e). However, in no instance was either poly-ubiquitination (smear) ablated or mono-ubiquitination ablated, as indicated by the immunoreactive band at 105 kDa (Fig. 5e). Intriguingly, K140R mutation of PDE4D5 alone ablated poly-ubiquitination, while still allowing evident isoprenaline-stimulated mono-ubiquitination of PDE4D5 (Fig. 5d).

These various K:R mutants in ubiquitination sites on PDE4D5 did not exhibit altered ability to bind β-arrestin in resting cells as evidenced from co-immunoprecipitation analyses (Fig. 5f). In resting cells, the single K:R mutant forms of PDE4D5 did not show (<12% change) any evident differences in their basal ubiquitination levels.

Ubiquitination of PDE4D5 Promotes Its Interaction with β-Arrestin—HEK293B2 cells, expressing VSV-tagged PDE4D5, were challenged for the indicated time with isoprenaline and endogenous β-arrestin immunopurified with a specific β-arrestin antibody. When the amount of β-arrestin-sequestered PDE4D5-VSV was determined by immunoblotting, we noted a time-dependent increase in the amount of PDE4D5 associating with β-arrestin (Fig. 6, a and c).

In contrast to this, no such increase in association with β-arrestin was evident when we expressed the (E721A:E722A:E723A)-PDE4D5-VSV mutant, which does not become ubiquitinated (Fig. 6, b and c). Furthermore, the time course for PDE4D5 ubiquitination and increased ability to bind β-arrestin parallel each other (Fig. 6c), consistent with PDE4D5 ubiquitination facilitating sequestration to β-arrestin.

PDE4D5 is also sequestered to the scaffold protein, RACK1 in these cells (10, 36). We thus set out to determine if the isoprenaline-induced enhanced association of PDE4D5 with β-arrestin caused by PDE4D5 ubiquitination altered the level of PDE4D5 sequestered to RACK1. Indeed, we noted a reduction

wild-type PDE4D5 in unstimulated cells (Fig. 5b). However, in transfecting this mutant construct into HEK2B2 cells we now found that isoprenaline challenge failed to elicit either mono- or poly-ubiquitination of quad-PDE4D5 (Fig. 5c).

We then assessed the role of the lysines found within the unique N-terminal region of PDE4D5, namely Lys-48, Lys-53, and Lys-78 by mutation to arginine, as in the triple-PDE4D5 (K48R:K53R:K78R) mutant. Expressed in HEK2B2 cells, the triple-PDE4D5 mutant failed to undergo isoprenaline-stimulated poly-ubiquitination (Fig. 5d). However, unlike the quad-PDE4D5 mutant, we still observed isoprenaline-stimulated mono-ubiquitination of PDE4D5 (Fig. 5d). This mutation did not show compromised β-arrestin binding compared with wild-type PDE4D5 in unstimulated cells (Fig. 5b).

Expressed in HEK293B2 cells, mutation of any one of Lys-48, Lys-53, and Lys-78 in PDE4D5-VSV, to arginine, led to an evident reduction in the isoprenaline-stimulated poly-ubiquitination of these constructs as compared with wild-type PDE4D5 (Fig. 5e). However, in no instance was either poly-ubiquitination (smear) ablated or mono-ubiquitination ablated, as indicated by the immunoreactive band at 105 kDa (Fig. 5e). Intriguingly, K140R mutation of PDE4D5 alone ablated poly-ubiquitination, while still allowing evident isoprenaline-stimulated mono-ubiquitination of PDE4D5 (Fig. 5d).
in the amount of RACK1 associating with VSV-PDE4D5 upon isoprenaline challenge (Fig. 6d). Quantification showed the level of VSV-PDE4D5 associating with RACK1 some 10 min after isoprenaline challenge was 44 ± 14% (mean ± S.D.; n = 4) of that seen in unchallenged cells. This is, presumably, due to the isoprenaline-induced enhancement of PDE4D5 recruited to β-arrestin, because no such change was observed (<7% change; n = 4) if the UIM disrupted (E721A:E722A:E723A)-PDE4D5-VSV species was used (Fig. 6d). This mutant is neither ubiquitinated nor shows any enhanced association with β-arrestin.

Ubiquitination of β-Arrestin—Isoprenaline challenge elicits transient ubiquitination of β-arrestin (47, 55, 56). It is thus curious that immunopurification of mutant PDE4D5 species engineered to ablate their ubiquitination, either by disrupting the UIM or by mutation of lysine targets for ubiquitination, did not leave any species indicative of co-immunoprecipitating ubiquitinated β-arrestin (Fig. 2). This mutant is neither ubiquitinated nor shows any enhanced association with β-arrestin.

FIGURE 6. Isoprenaline increases β-arrestin-sequestered PDE4D5. HEK293 cells were transfected to express either wild-type VSV-PDE4D5 (a) or the UIM-disrupted E721A:E722A:E723A-VSV-PDE4D5 mutant (b) and then challenged with isoprenaline (10 μM) for the indicated times. After this, β-arrestin was immunopurified and blotted for VSV-PDE4D5 with lysates blotted for both β-arrestin and VSV-PDE4D5. c, quantification (mean ± S.D.; n = 3) quantifying isoprenaline-stimulated changes in β-arrestin-sequestered wild-type VSV-PDE4D5 and the UIM-disrupted E721A:E722A:E723A-PDE4D5 mutant and for the level of poly-ubiquitinated wild-type VSV-PDE4D5. d, cells were transfected as in a, and lysates were blotted as indicated for either VSV, to detect recombinant PDE4D5, or for RACK1 and VSV-immunoprecipitates blotted for either PDE4D5-VSV or RACK1. All immunoblotting data show experiments typical of ones performed at least three times.

does not itself undergo ubiquitination. This is perhaps because, in docking to β-arrestin, PDE4D5 obscures key lysines for β-arrestin ubiquitination (supplemental Fig. S2).

Consistent with divergence between ubiquitination of β-arrestin compared with PDE4D5, we noted a temporal difference in the isoprenaline-stimulated ubiquitination of these two proteins (Figs. 2a and 7a). Thus, analysis of β-arrestin immunopurified from HEK293 cells identified its transient ubiquitination some 15 min after isoprenaline challenge (Fig. 7a). In contrast with PDE4D5, ubiquitination occurred much earlier, being apparent some 5–10 min subsequent to isoprenaline challenge and, indeed, had disappeared by 15 min (Fig. 2a). Identical results were noted regarding the ubiquitination of β-arrestin in cells transfected to overexpress either wild-type PDE4D5 (Fig. 7a) or the K4R-PDE4D5 (quad) mutation (Fig. 7b), which binds to β-arrestin but is not ubiquitinated and also in non-transfected cells (data not shown). Thus, if ubiquitylated β-arrestin bound to PDE4D5 we would have expected to identify it in PDE4D5 immunoprecipitates 15-min post isoprenaline challenge.
Mdm2 Directs cAMP PDE4D5 Ubiquitination

FIGURE 7. Ubiquitination of β-arrestin. In a HEK293 cell were transfected with wild-type-PDE4D5 and then challenged with isoprenaline (10 μM) for the indicated times, whereupon endogenous β-arrestin was selectively immunopurified and gels blotted for ubiquitin (upper panel) or β-arrestin (lower panel). In b cells were transfected with the Quad (K4R) mutant (K48R/K53R/K78R/K140R)-PDE4D5, which does not become ubiquitin- nated, prior to immunopurification for the indicated times and subsequent analysis of β-arrestin ubiquitination, as in a. Scanning analyses of β-arrestin peptide arrays have previously identified five peptides that interact with recombinant PDE4D5-MBP fusion protein probe expressed in E. coli (38). Five such peptides were synthesized, immobilized, and probed here with recombinant PDE4D5-MBP prior to detection with both an antibody to PDE4D5 (c and d: upper panel) and one to ubiquitin (c and d: lower panel), as indicated, using Odyssey IR-tagged secondary antisera. a, native untreated arrays and d shows arrays subjected to prior in vitro ubiquitination with Mdm2 as the E3 ligase. Data show experiments typical of ones performed three times. Peptide region: 1–35, (1–35) MGEKPGRTRVFX(11)K(12)SPNCKLTLYGKGRDFVHLK; 42 and 43, (206–235) KELYHGPEFLNIVH7TVSNSTKVKIKV; and 58, (286–310) RGLALDGKLOHDPLASLLTVEKG. Data show experiments typical of ones performed three times.

Previously we have mapped the interaction sites for PDE4D5 on β-arrestin2 using a scanning peptide array procedure with confirmation using truncation and mutation in two-hybrid and pulldown studies (38). This showed that the PDE4D5 catalytic unit bound to a site in the N-domain and its unique N-terminal region bound to two sites in the β-arrestin C-domain (Figs. 1 and 8). We generated an array of six 25-mer peptides that encompassed these three sites, respectively, namely aa 1–35, aa 206–235, and aa 286–310 (Fig. 7, c and d). As before (38), we probed them with purified MBP-PDE4D5 fusion protein expressed in E. coli and identified a positive interaction (Fig. 7, c and d). In a separate experiment we subjected such arrays to in vitro ubiquitination prior to probing with an ubiquitin antibody to detect modification or with MBP-PDE4D5 to detect binding. Doing this we see that Regions 1 and 2 become ubiquitinated, but Region 3 does not (Fig. 7d). Ubiquitination of Region 1 peptides (aa 1–35) of β-arrestin, where the PDE4D5 catalytic unit interacts, clearly compromises interaction with MBP-PDE4D5, although such modification of Region 2 peptides does not (Fig. 7d).

DISCUSSION

We show here that, like the β2AR and β-arrestin (47, 55–57), PDE4D5 becomes transiently ubiquitinated by a β-arrestin-sequestered population of the E3 ligase, Mdm2 in response to challenge of cells with the β-agonist, isoprenaline (Fig. 2).

Ubiquitination appears likely to be restricted to specific PDE4 isoforms such as PDE4D5 as it requires a UIM, which is only found in the PDE4D sub-family. Furthermore, in the case of PDE4D5 then three of the four target lysines for modification are located within its isoform-specific N-terminal region. Although isoforms from all PDE4 sub-families have potential to interact with β-arrestin through a conserved binding site in their catalytic unit, the unique N-terminal region of PDE4D5 has an additional binding site for β-arrestin that favors its selective sequestration by β-arrestin (10, 24, 37). Here we show that the amount of PDE4D5 scaffolded to β-arrestin is increased by its ubiquitination and that ubiquitination of PDE4D5 can also reduce the amount of this isoform that associates with the scaffold protein, RACK (Fig. 6). Thus an important functional consequence of the specific, isoprenaline-stimulated ubiquitination of PDE4D5 is to ensure the fidelity of its preferential interaction with β-arrestin (Fig. 8).

Critical in allowing PDE4D5 ubiquitination is a UIM located within its sub-family-specific C-terminal region (Table 1). This is centered on Val-725 and Ser-731 and extends from Glu-721 to Glu-734 (Table 1). UIMs have an important role in directing the ubiquitination of many substrate proteins (68). This appears to be the case here as UIM disruption clearly ablates isoprenaline-stimulated ubiquitination of PDE4D5 (Fig. 2f). No such UIM is evident in the three other PDE4 sub-families, thus showing, for the first time, a functional role for a sub-family-specific C-terminal region, in this case PDE4D.

Of the variety of UIMs uncovered (Table 1) then, without exception, they all contain 3/4 negatively charged residues that are located 8/9 residues N-terminal to an invariant serine. However, there is clearly considerable variation of the amino acids found between and around these residues. Indeed, even within the UIM “consensus,” a number of UIMs lack the glutamate-located 3 residues C-terminal to the conserved serine (Table 1). PDE4D has a UIM consisting of 3 acidic glutamates together with the conserved serine. In addition it has a glutamate located 3 residues C-terminal to the invariant serine, which is a feature of 18 of the 30 UIMs listed. It also has a conserved hydrophobic residue abutting the C-terminal side of the core acidic patch; a feature that is shared by 29 of the 30 listed UIMs. However, it does not have an alanine found 4 residues N-terminal to the invariant serine, which is a feature of 27 of the listed 30 UIMs. Sequence heterogeneity around the core UIM motif is, likely, indicative of UIMs being “tailored” for specific functional roles. Indeed, depending on the protein, UIMs can be involved in protein-protein interaction, allowing binding of a ubiquitinated protein to one having an UIM, also in facilitating catalytic reactions of E3-ligase-mediated ubiquitination and in...
Given such diversity it seems likely that UIMs will be tailored in both the mode and affinity with which they bind ubiquitin, so as to facilitate particular functional modalities. Indeed binding studies suggest that UIMs show a wide range of affinities for ubiquitin that are influenced not only by sequence differences within this core motif but also determined by the three-dimensional protein structure (42, 70). From various structural studies (69, 71–74) it can be inferred that the conserved acidic cluster interacts with positively charged residues on ubiquitin, while the conserved serine H-bonds to ubiquitin, providing common core interaction sites. The acidic patch is critical as its mutation ablates ubiquitin binding (70), which is consistent with the loss of function we note here with PDE4D5. The highly conserved alanine lacking in PDE4D5 (Table 1) can contribute to ubiquitin association but, unlike the acidic patch, is not essential as in ubiquitinated in this system (Fig. 3). Two specific reasons underpin the selective ubiquitination of PDE4D5 occurring upon isoprenaline challenge of primary cardiomyocytes, MEFs, and HEK293B2 cells (Fig. 2). Firstly, the isoform-specific N-terminal region of PDE4D5 contains the target lysines for ubiquitination (Figs. 1 and 5), and, secondly, this region contains a binding site conferring preferential interaction with β-arrestin (Fig. 1), which delivers the E3 ligase, Mdm2 (Fig. 3). In contrast to this, the unique N-terminal region of PDE4D3 does not contain any lysines, so would not be able to undergo such polyubiquitination. Thus, if PDE4D3 was to undergo ubiquitination it would have to be through modification of lysines within the common PDE4 “core,” which, presumably, would require interaction with an E3 ligase distinct from Mdm2. However, certain other PDE4D long isoforms do have lysines in their unique N-terminal regions, which might indicate that they could undergo ubiquitination as seen with ataxin-3 (69).

Given such diversity it seems likely that UIMs will be tailored in both the mode and affinity with which they bind ubiquitin, so as to facilitate particular functional modalities. Indeed binding studies suggest that UIMs show a wide range of affinities for ubiquitin that are influenced not only by sequence differences within this core motif but also determined by the three-dimensional protein structure (42, 70). From various structural studies (69, 71–74) it can be inferred that the conserved acidic cluster interacts with positively charged residues on ubiquitin, while the conserved serine H-bonds to ubiquitin, providing common core interaction sites. The acidic patch is critical as its mutation ablates ubiquitin binding (70), which is consistent with the loss of function we note here with PDE4D5. The highly conserved alanine lacking in PDE4D5 (Table 1) can contribute to ubiquitin association but, unlike the acidic patch, is not essential as in ubiquitinated in this system (Fig. 3). Two specific reasons underpin the selective ubiquitination of PDE4D5 occurring upon isoprenaline challenge of primary cardiomyocytes, MEFs, and HEK293B2 cells (Fig. 2). Firstly, the isoform-specific N-terminal region of PDE4D5 contains the target lysines for ubiquitination (Figs. 1 and 5), and, secondly, this region contains a binding site conferring preferential interaction with β-arrestin (Fig. 1), which delivers the E3 ligase, Mdm2 (Fig. 3). In contrast to this, the unique N-terminal region of PDE4D3 does not contain any lysines, so would not be able to undergo such polyubiquitination. Thus, if PDE4D3 was to undergo ubiquitination it would have to be through modification of lysines within the common PDE4 “core,” which, presumably, would require interaction with an E3 ligase distinct from Mdm2. However, certain other PDE4D long isoforms do have lysines in their unique N-terminal regions, which might indicate that they could undergo ubiquitination as seen with ataxin-3 (69).
Mdm2 Directs cAMP PDE4D5 Ubiquitination

ubiquitination given both an appropriate E3 ligase and appropriate stimulus.

In vitro ubiquitination of a PDE4D5 scanning peptide array library proved a novel and invaluable pointer to the sites of its ubiquitination by Mdm2. Thus, mutation of all of lysine 48, 53, 78, and 140 residues to Arg, ablated isoprenaline-stimulated mono- and poly-ubiquitination of PDE4D5 (Fig. 5). In contrast, poly-ubiquitination, but not mono-ubiquitination, was ablated upon Arg mutation of lysines 48, 53, and 78, located in the 88-amino acid isoform-specific N-terminal region of PDE4D5. We suggest that the mono-ubiquitination of Lys-140 may serve to prime PDE4D5 for a subsequent cascade of polyubiquitination occurring at lysines 48, 53, and 78 within its unique N-terminal region (Fig. 8). Consistent with this, Lys-140 → Arg mutation sufficed to ablate poly-ubiquitination, but not mono-ubiquitination (Fig. 5). This indicates that while mono-ubiquitination may ensue within the unique N-terminal region of PDE4D5, chain elongation through the addition of further ubiquitin moieties requires ubiquitination at Lys-140, which gates the cascade of polyubiquitination occurring in the N-terminal region of PDE4D5 (Fig. 8). Indeed, there is a growing body of evidence that mono-ubiquitination can trigger functional changes in proteins (76–79), and phosphatase and tensin homolog (PTEN) mono-ubiquitination has been suggested to trigger subsequent poly-ubiquitination at other sites (80). Mdm2 can also cause mono-ubiquitination of proteins as seen for dihydrofolate reductase and p53 (81, 82). Furthermore, Lys-140 lies within an established regulatory domain, called UCR1, which uniquely characterizes all long PDE4 isoforms. This region transduces functional changes associated with phosphorylation by protein kinase A (83, 84), extracellular signal-regulated kinase (85), and a reactive oxygen-activated kinase (86), as well as showing dynamic interactions with the associated UCR2 domain (87).

While isoprenaline can trigger the ubiquitination of β-arrestin (47, 56), which we confirm here (Fig. 7, a and b), the fraction of β-arrestin co-immunopurifying with PDE4D5 appears not to become ubiquitinated. Thus in cells expressing PDE4D5 mutants that ablated its ubiquitination, but did not prevent β-arrestin binding, there was no evidence for co-immunopurifying ubiquitinated β-arrestin (Figs. 2e, 3c, 4c, 5c, and 5d). This was despite the fact that probing immunopurified β-arrestin from isoprenaline-challenged lysates with an anti-ubiquitin antibody clearly identified a fraction of ubiquitinated β-arrestin (Fig. 7, a and b). However, in β-arrestin immunoprecipitates probed with anti-ubiquitin antibody we noted no clear signal for ubiquitinated PDE4D5. This likely reflects preponderance in the amount of β-arrestin that is ubiquitinated compared with PDE4D5, no doubt because <10% of total cellular β-arrestin in these cells is sequestered to PDE4D5 (10, 36). Additionally, we noted a subtle difference in time courses for generation of ubiquitinated PDE4D5 (Fig. 2a) and β-arrestin (Fig. 7, a and b), implying differences in regulation. We suggest that, in β-arrestin/PDE4D5 complexes, only the PDE4D5 component becomes ubiquitinated in response to isoprenaline challenge. We propose that, as PDE4D5 straddles β-arrestin, with its catalytic domain binding to the β-arrestin N-domain and its N-terminal region binding the β-arrestin C-domain (36, 38), a consequence of this may be to obscure access of Mdm2 to target lysine residues on β-arrestin (supplemental Fig. S2 and Fig. 8). Although it has been suggested that a considerable number of the 31 lysines in β-arrestin may be ubiquitinated, it is believed that ubiquitination of Lys-11 and Lys-12 may regulate the ubiquitination of other lysines (55, 56). In this regard, key residues involved in PDE4 binding surround Lys-11/Lys-12 of β-arrestin (Fig. 1c) making it likely that access of Mdm2 to such residues will be obscured by the sequestered PDE4D5 catalytic unit (Fig. 1 and supplemental Fig. S2). Thus the three binding interfaces identified on β-arrestin for PDE4D5 either encompass or obscure lysine residues proposed as key targets for ubiquitination (Fig. 1 and supplemental Fig. S2). Indeed, the majority of lysines in β-arrestin appear to accumulate on the face that accommodates the PDE4D5 catalytic unit (supplemental Fig. S2). It seems probable that sequestered PDE4D5 will sterically hinder the ubiquitination of β-arrestin by Mdm2.

Correspondingly, peptide array analyses (Fig. 7, a and d) suggest that ubiquitination of β-arrestin is likely to prevent PDE4D5 sequestration. In particular we see here that in vitro ubiquitination of Region 1 peptides, representing amino acids 1–35 of β-arrestin, severely compromises PDE4D5 binding (Fig. 7d). This region contains Lys-18, identified as not only a site for ubiquitination in β-arrestin (55), but a residue that forms a key constituent of the PDE4D5 binding site on β-arrestin (38). Thus ubiquitination of lysines within this region appear to inhibit PDE4D5 binding to β-arrestin by disrupting the docking site for the PDE4D5 catalytic unit (Fig. 8).

Interestingly, MG132-mediated inhibition of proteasome activity enhanced the accumulation of ubiquitinated PDE4D5 species seen after 10 min of isoprenaline challenge (Fig. 2e). This suggests that another functional role of this modification is to target a fraction of PDE4D5 for ubiquitination. However, this degradation does not primarily underpin the transient nature of PDE4D5 ubiquitination, because MG132 does not engender a sustained ubiquitination of PDE4D5 in response to adrenaline. This suggests that an active de-ubiquitinating enzyme is acting upon PDE4D5, and the transient nature of the response may thus be either due to de-ubiquitinating enzyme activation or cessation of the action of isoprenaline in promoting PDE4D5 ubiquitination. In this regard, the ubiquitination of β-arrestin is also transient in nature, albeit with different kinetics (Fig. 7a).

Here we have identified a novel role for Mdm2 in its β-arrestin-sequestered state. This is in mediating the ubiquitination of a specific cAMP-degrading phosphodiesterase, namely PDE4D5. Ubiquitination of PDE4D5 appears to ensue by a priming process that involves mono-ubiquitination of Lys-140 with the UCR1 regulatory module of PDE4D5, followed by a subsequent cascade of poly-ubiquitination at three lysines within the unique N-terminal region of PDE4D5 (Fig. 1a). Ubiquitination by β-arrestin-sequestered Mdm2 thus provides a dual fidelity role in not only enhancing the selective sequestration of PDE4D5 to β-arrestin but also directing it to a non-ubiquitinated sub-population of this important signal scaffold protein (Fig. 8). Indeed, an important role for the Mdm2-catalyzed ubiquitination of β-arrestin may be to create functionally distinct β-arrestin sub-populations having distinct
Mdm2 Directs cAMP PDE4D5 Ubiquitination

(1996) Virology 217, 285–292
67. Miranda, M., Dionne, K. R., Sorkina, T., and Sorkin, A. (2007) Mol. Biol. Cell 18, 313–323
68. Hoeller, D., Hecker, C. M., Wagner, S., Rogov, V., Dötsch, V., and Dikic, I. (2007) Mol. Cell 26, 891–898
69. Mao, Y., Senic-Matuglia, F., Di Fiore, P. P., Polo, S., Hodsdon, M. E., and De Camilli, P. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 12700–12705
70. Fisher, R. D., Wang, B., Alam, S. L., Higginson, D. S., Robinson, H., Sundquist, W. I., and Hill, C. P. (2003) J. Biol. Chem. 278, 28976–28984
71. Hirano, S., Kawasaki, M., Ura, H., Kato, R., Raiborg, C., Stenmark, H., and Wakatsuki, S. (2006) Nat. Struct. Mol. Biol. 13, 272–277
72. Swanson, K. A., Kang, R. S., Stamenova, S. D., Hicke, L., and Radhakrishnan, I. (2003) EMBO J. 22, 4597–4606
73. Wang, Q., Young, P., and Walters, K. J. (2005) J. Mol. Biol. 348, 727–739
74. Shekhtman, A., and Cowburn, D. (2002) Biochem. Biophys. Res. Commun. 296, 1222–1227
75. Fujiwara, K., Teno, T., Sugawara, K., Lee, J. G., Ohki, I., Kojima, C., Tocchio, H., Hiroaki, H., Hanaoka, F., and Shirakawa, M. (2004) J. Biol. Chem. 279, 4760–4767
76. Sun, L., and Chen, Z. J. (2004) Curr. Opin. Cell Biol. 16, 119–126
77. Raiborg, C., Slagsvold, T., and Stenmark, H. (2006) Trends Biochem. Sci. 31, 541–544
78. Haglund, K., Di Fiore, P. P., and Dikic, I. (2003) Trends Biochem. Sci. 28, 598–603
79. Sigismund, S., Polo, S., and Di Fiore, P. P. (2004) Curr. Top. Microbiol. Immunol. 286, 149–185
80. Trotman, L. C., Wang, X., Alimonti, A., Chen, Z., Teruya-Feldstein, J., Yang, H., Pavletich, N. P., Carver, B. S., Cordon-Cardo, C., Erdjument-Bromage, H., Tempst, P., Chi, S. G., Kim, H. J., Misteli, T., Jiang, X., and Pandolfi, P. P. (2007) Cell 128, 141–156
81. Maguire, M., Nield, P. C., Devling, T., Jenkins, R. E., Park, B. K., Polanski, R., Vlatkovic, N., and Boyd, M. T. (2008) Cancer Res. 68, 3232–3242
82. Carter, S., Bischof, O., Dejean, A., and Vousden, K. H. (2007) Nat. Cell Biol. 9, 428–435
83. Sette, C., and Conti, M. (1996) J. Biol. Chem. 271, 16526–16534
84. Hoffmann, R., Wilkinson, I. R., McCallum, J. F., Engels, P., and Houslay, M. D. (1998) Biochem. J. 333, 139–149
85. Hoffmann, R., Baillie, G. S., MacKenzie, S. J., Yarwood, S. J., and Houslay, M. D. (1999) EMBO J. 18, 893–903
86. Hill, E. V., Sheppard, C. L., Cheung, Y. F., Gall, I., Krause, E., and Houslay, M. D. (2006) Cell Signal 18, 2056–2069
87. Beard, M. B., Olsen, A. E., Jones, R. E., Erdogan, S., Houslay, M. D., and Bolger, G. B. (2000) J. Biol. Chem. 275, 10349–10358