Dimerization of the Type 4 cAMP-specific Phosphodiesterases Is Mediated by the Upstream Conserved Regions (UCRs)*

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The cAMP-specific PDE4 family consists of four genes, each expressed as several splice variants. These variants are termed long and short forms depending on the presence or absence of two unique N-terminal domains called upstream conserved regions 1 and 2 (UCR1 and 2). UCR1 and UCR2 have been shown to form a module necessary for the activation of PDE4 upon phosphorylation by the cAMP-dependent kinase (PKA). Here we have uncovered PDE4 oligomerization as a novel function for the UCR1/UCR2 module. Using several different approaches including gel filtration, sucrose density gradient centrifugation, pull-down of differentially tagged PDE constructs, and yeast two-hybrid assay, we show that the long PDE4 splice variant PDE4D3 behaves as a dimer, whereas the short splice variant PDE4D2 is a monomer. Internal deletions of either the C-terminal portion of UCR1 or the N-terminal portion of UCR2 abolishes dimerization of PDE4D3 indicating that both domains are involved in this intermolecular interaction. The dimerization, however, is structurally distinguishable from a previously described intramolecular interaction involving the same domains. PKA phosphorylation and site-directed mutagenesis shown to ablate the latter do not interfere with dimerization. Therefore, dimerization of the long PDE4 forms may be an additional function of the UCR domains that further explains differences in the regulatory properties between the long and short PDE4 splice variants.

The second messengers cAMP and cGMP are key molecules for transducing the action of extracellular signals such as hormones, neurotransmitters, and light into the most diverse cell functions, thus playing an important role in a wide array of physiological processes that include vision, cell growth and division, memory, and immune response (1–5). Intracellular cyclic nucleotide levels are determined by the rates of their synthesis by adenylyl cyclases and of their degradation through phosphodiesterases, enzymes that hydrolyze the phosphodiester bond and generate the corresponding 5’-nucleoside monophosphates. Cyclic nucleotide phosphodiesterases (PDEs)† compose a superfAMILY of isoenzymes that are divided into 11 PDE families on the basis of their sequence homology and enzymatic properties (2). They all share a highly conserved catalytic domain of about 270 amino acids fused to additional N- and/or C-terminal sequences that contain distinct domains unique to the members of a PDE family. These terminal domains determine several properties of the PDEs such as the regulation of enzyme activity by post-translational modifications (e.g. phosphorylation by PKA, PKB, ERK2, CaMK, and PKG, Refs. 6–10) and binding of other messenger molecules (e.g. cGMP, Ca2+-calmodulin, and phosphatidic acid, Refs. 11–13), or by specifying the subcellular localization of the enzymes by protein-protein-interactions and membrane insertion (14–16). The terminal domains of the PDEs, therefore, provide diverse modules for coordinating PDE activity with the overall signaling network specific to a cell.

A structural feature unique to the cAMP-specific PDE4 family is the presence of two domains N-terminal to the catalytic domain called upstream conserved regions 1 and 2 (UCR1 and UCR2). The four genes included in the PDE4 family are each expressed as various splice variants that are distinguished as long and short forms depending on the presence of the UCRs (17–20). Long forms contain both UCR1 and UCR2, whereas short forms lack UCR1 but still possess all, or at least a portion, of UCR2. In the long PDE4 splice variants where both domains are present, UCR1 and UCR2 have been shown to interact with each other, and this interaction was thought to be intramolecular (21, 22). Site-directed mutagenesis further indicated that this interaction may involve positively charged residues in UCR1 (Arg-98 and Arg-101 in PDE4D3) and several negatively charged residues in UCR2 (Glu-146, Glu-147, and Asp-149 in PDE4D3, Ref. 22). In these studies, it was demonstrated that the interaction between the two domains constitutes an important module involved in the regulation of the enzyme activity in several different ways. Phosphorylation of a serine residue at the N terminus of UCR1 (Ser-54 in PDE4D3) by the cAMP-dependent protein kinase (PKA, Ref. 6), which causes enzyme activation, alters the interaction between UCR1 and UCR2. As UCR2 may function as an autoinhibitory domain of the catalytic center, a probable mechanism for PDE4 activation is that the PKA phosphorylation leads to an altered UCR1-UCR2 interaction that removes the autoinhibitory effect of UCR2 on the catalytic site (6, 21, 22). In addition, phosphorylation by the extracellular signal-regulated kinase 2 (ERK2) at the PDE4 C terminus affects the PDE4 enzyme activity in different ways that depend on the presence of the UCRs. Long PDE4 isoforms containing both a complete UCR1 and UCR2 were inhibited by ERK2 phosphorylation whereas the short splice form PDE4D1,
The single point mutations R98A, R101A, E147A, and D149A were inserted into the PDE4D3- \( p \) vector or the PDE4D3- \( \text{CAT} \) vector. To generate PDE4D3- \( \text{ΔCAT} \) and PDE4D3-\( \text{ΔCAT} \) constructs, respectively. The single point mutations R98A, R101A, E147A, and D149A were inserted into the PDE4D3-\( \text{ΔCAT} \) and PDE4D3-\( \text{ΔCAT} \) vectors using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

**EXPERIMENTAL PROCEDURES**

**PCr Primers**—The oligonucleotides used for PCR amplifications are numbered consecutively, restriction enzyme sites and exchanged nucleotides are underlined, and ATG-start and termination codons are shown in italics within the nucleotide sequences. P1, 5'-GCCGATTAATGAATGTCAGGCTCGAACATTCCCTCATGGTG-3'; P2, 5'-GCCGGAATTCGCTCGAGGAATGATACGGAGATGACTTGCGAGAAGCCGATC-3'; P3, 5'-GCCGCGATATTGAGAATGATACGGAGATGACTTGCGAGAAGCCGATC-3'; P4, 5'-GCCGGAATTCGCTCGAGGAATGATACGGAGATGACTTGCGAGAAGCCGATC-3'; P5, 5'-GCCGCGATATTGAGAATGATACGGAGATGACTTGCGAGAAGCCGATC-3'; P6, 5'-GCCGCGATATTGAGAATGATACGGAGATGACTTGCGAGAAGCCGATC-3'; P7, 5'-GCCGGCCGAGCTCGAACATTCCCTCATGGTG-3'; P8, 5'-GCCGGCCGAGCTCGAACATTCCCTCATGGTG-3'; P9, 5'-GCCGAGAACATTGCTAGTCTGACAGGAGATGACTTGCGAGAAGCCGATC-3'; P10, 5'-GCCGAGAACATTGCTAGTCTGACAGGAGATGACTTGCGAGAAGCCGATC-3'; P11, 5'-GCCGAGAACATTGCTAGTCTGACAGGAGATGACTTGCGAGAAGCCGATC-3'; P12, 5'-GCCGAGAACATTGCTAGTCTGACAGGAGATGACTTGCGAGAAGCCGATC-3'; P13, 5'-GCCGAGAACATTGCTAGTCTGACAGGAGATGACTTGCGAGAAGCCGATC-3'; P14, 5'-GCCGAGAACATTGCTAGTCTGACAGGAGATGACTTGCGAGAAGCCGATC-3'; P15, 5'-GCCGAGAACATTGCTAGTCTGACAGGAGATGACTTGCGAGAAGCCGATC-3'; P16, 5'-GCCGAGAACATTGCTAGTCTGACAGGAGATGACTTGCGAGAAGCCGATC-3'; P17, 5'-GCCGAGAACATTGCTAGTCTGACAGGAGATGACTTGCGAGAAGCCGATC-3'; P18, 5'-GCCGAGAACATTGCTAGTCTGACAGGAGATGACTTGCGAGAAGCCGATC-3'; P19, 5'-GCCGAGAACATTGCTAGTCTGACAGGAGATGACTTGCGAGAAGCCGATC-3'; P20, 5'-GCCGAGAACATTGCTAGTCTGACAGGAGATGACTTGCGAGAAGCCGATC-3'; P21, 5'-GCCGAGAACATTGCTAGTCTGACAGGAGATGACTTGCGAGAAGCCGATC-3'; P22, 5'-GCCGAGAACATTGCTAGTCTGACAGGAGATGACTTGCGAGAAGCCGATC-3'; P23, 5'-GCCGAGAACATTGCTAGTCTGACAGGAGATGACTTGCGAGAAGCCGATC-3'; P24, 5'-GCCGAGAACATTGCTAGTCTGACAGGAGATGACTTGCGAGAAGCCGATC-3'; P25, 5'-GCCGAGAACATTGCTAGTCTGACAGGAGATGACTTGCGAGAAGCCGATC-3'; P26, 5'-GCCGAGAACATTGCTAGTCTGACAGGAGATGACTTGCGAGAAGCCGATC-3'; P27, 5'-GCCGAGAACATTGCTAGTCTGACAGGAGATGACTTGCGAGAAGCCGATC-3'; P28, 5'-GCCGAGAACATTGCTAGTCTGACAGGAGATGACTTGCGAGAAGCCGATC-3'; P29, 5'-GCCGAGAACATTGCTAGTCTGACAGGAGATGACTTGCGAGAAGCCGATC-3'; P30, 5'-GCCGAGAACATTGCTAGTCTGACAGGAGATGACTTGCGAGAAGCCGATC-3'; P31, 5'-GCCGAGAACATTGCTAGTCTGACAGGAGATGACTTGCGAGAAGCCGATC-3'; P32, 5'-GCCGAGAACATTGCTAGTCTGACAGGAGATGACTTGCGAGAAGCCGATC-3'; P33, 5'-GCCGAGAACATTGCTAGTCTGACAGGAGATGACTTGCGAGAAGCCGATC-3'.
that recognize the artificial V5-tag; 2) the monoclonal α-Myc antibody that recognizes the artificial Myc-tag; 3) the monoclonal antibody M3S1 raised against the C-terminal region of PDE4D; and 4) the polyclonal antibody K116 that recognizes the N-terminal domain of all PDE4 subtypes. Immunoreactive bands were detected by using either peroxidase-conjugated goat anti-mouse/anti-rabbit antibodies and the ECL detection reagents (Amersham Biosciences) or the alkaline phosphatase-conjugated goat anti-mouse/anti-rabbit antibodies and the corresponding detection system (BioRad, Hercules, CA).

**PDE Assay—** PDE activity was measured according to the method of Thompson and Apelman (26). In brief, samples were assayed in a reaction mixture of 200 μl containing 40 mM Tris- HCl (pH 8.0), 10 mM MgCl₂, 5 mM β-mercaptoethanol, 1 μM Ca²⁺, 0.75 mg/ml bovine serum albumin, and [γ-³²P]ATP (New England Nuclear) at 12 °C. The reaction was terminated by adding 200 μl of 10 mM EDTA in 40 mM Tris- HCl (pH 8.0) followed by heat inactivation in a boiling water bath for 1 min. The PDE reaction product 5'-AMP was then hydrolyzed by incubation of the assay mixture with 50 μg of *Crotalus atrox* snake venom (Sigma) for 20 min at 33 °C, and the resulting adenosine was separated by anion exchange chromatography using 1 ml of AG1-X8 resin and quantitated by scintillation counting.

**HPLC Size Exclusion Chromatography—** For gel filtration experiments, cells were harvested in a 10 mM sodium acetate buffer (pH 6.5) containing 1 mM EDTA, 0.2 mM EGTA, 5 mM β-mercaptoethanol, 10 mM NaF, 150 mM NaCl, 20% ethylene glycol, 1 mM AEDSBF, and a protease inhibitor mix (Roche Diagnostics). The cell homogenates were centrifuged at 14,000 × g for 20 min followed by a 30-min centrifugation at 100,000 × g, and 500 μl of the high speed supernatants were then loaded on a TSK-3000 analytical gel filtration column. The samples were eluted with a 10 mM sodium acetate buffer (pH 6.5) containing 1 mM EDTA, 0.2 mM EGTA, 5 mM β-mercaptoethanol, 10 mM NaF, 150 mM NaCl, and 20% ethylene glycol at a flow rate of 0.5 ml/min. Fractions of 500 μl were collected and assayed for PDE activity. Bovine thyroglobulin (670 kDa; 85 Å), horse ferritin (440 kDa; 41 Å), bovine gamma globulin (158 kDa), rabbit aldolase (158 kDa; 48.1 Å), bovine serum albumin (67 kDa; 35.5 Å), chicken ovalbumin (44 kDa; 30.5 Å), horse myoglobin (17 kDa), and coelalin (1.35 kDa) were used as molecular mass markers.

**Density Gradient Centrifugation—** Density gradients from 5 to 33% sucrose in 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.2 mM EGTA, 10 mM NaF, 150 mM NaCl, and 5 mM β-mercaptoethanol were prepared in 14 × 89-mm centrifugation tubes (Beckman, Fullerton, CA) using the Jule gradient former (Jule Inc., New Haven, CT). The gradients were stored overnight at 4 °C before they were loaded with 200 μl of the soluble cell extracts (100,000 × g supernatant) or marker proteins and centrifuged for 36 h at 28,000 rpm (Fig. 3) or 24 h at 40,000 rpm (Fig. 6B) in a Beckman SW41 rotor. Fractions of 200 μl were then collected starting from the bottom of the tube using a peristaltic pump and analyzed for PDE activity or protein concentration (molecular mass markers).

**Co-immunoprecipitation of Differentially Tagged PDE4D Constructs—** As a first approach to investigate PDE4 oligomerization, we performed pull-down experiments utilizing differentially tagged PDE4D constructs. For this purpose, two constructs C-terminally tagged with either a V5/His- or Myc/His tag were generated for both PDE4D3 and PDE4D2 as representatives of the PDE4 long and short splice variants, respectively. These differentially tagged constructs were then overexpressed in COS7 cells either separately or together. After harvesting the cells, the V5/His-tagged construct was immunoprecipitated from the cell lysates using an α-V5 antibody, and the immunoprecipitated pellets were probed for the presence of the Myc/His-tagged proteins. As shown in Fig. 1A, the V5/His-tagged PDE4D3 was specifically immunoprecipitated by the α-V5 antibody (lanes 4 and 6) but not by the IgG control (lanes 3 and 5) whereas the PDE4D3-Myc/His construct was not recognized and immunoprecipitated by either the α-V5 antibody or by IgG (lanes 1 and 2). Therefore, the pull-down of the Myc/His-tagged PDE4D3, when co-transfected with the PDE4D3-V5/V5/His construct (Fig. 1A, lane 6), is indirect and indicates an intermolecular interaction between the PDE4D3-Myc/His and the co-expressed PDE4D3-V5/His construct.

When the experiment was carried out under the same conditions using the differentially tagged constructs of the short splice form PDE4D2 (Fig. 1B), a co-IP of the Myc/His-tagged PDE construct with the co-transfected PDE4D2-V5/His (Fig. 1B, lane 6) was not observed. Moreover, the Myc-tagged PDE4D2 could not be co-immunoprecipitated when co-transfected with the long splice variant PDE4D3-V5/His (data not shown). These results provided a first indication that the PDE4D3 forms oligomers whereas the short splice form PDE4D2 does not, and that the oligomerization domain is located within the N-terminal region of PDE4D3, which is absent in PDE4D2.

In order to exclude the possibility that the co-IP of the differentially tagged PDE4D3 constructs is caused by the fusion peptides (i.e. an interaction between the N-terminal domain of PDE4D3 with either the Myc/His- or the V5/His tag), the PDE4D3 co-IP was repeated with two untagged PDE forms, PDE4D3, and PDE4D3-ΔCAT. In this PDE4D3-ΔCAT construct, the C-terminal epitope recognized by the M3S1 antibody (which was used for the pull-down of the full-length PDE4D3) has been removed. As shown in Fig. 1C, the full-length PDE4D3 could be selectively immunoprecipitated by the M3S1 antibody (lanes 2 and 6) whereas the PDE4D3-ΔCAT construct was not immunoprecipitated (lane 4). Conversely, PDE4D3-ΔCAT could be co-immunoprecipitated when co-expressed with the full-length PDE4D3 (lane 6). This finding, therefore, excludes the possibility of an artificial co-immunoprecipitation caused by the tags used and confirms that the potential intermolecular interaction between the PDE4D3 constructs is mediated by the domains present in the PDE sequence.

**Determination of Apparent Molecular Weights of PDE4D3 and PDE4D2 by Size Exclusion Chromatography and Density Gradient Centrifugation—** In order to determine the oligomerization of the PDE4D3 splice form by an independent approach, both native and recombinant PDE4D3 were analyzed by size exclusion chromatography. FRTL5 cells were chosen as the source of endogenous PDE4, as PDE4D3 is the only PDE4...
splice form in these cells and the most abundant PDE expressed. When fractionated on an analytical gel filtration column, the full-length recombinant PDE4D3 expressed in either MA-10 cells (Fig. 2C), insect cells, or E. coli (data not shown), as well as the endogenous PDE4D3 from FRTL5 cells (Fig. 2A) all eluted in the same fractions that correspond to an apparent molecular mass of ~388 kDa when compared with molecular size standards. The PDE4D3 (M<sub>th</sub> = 76,000) therefore, behaved as an oligomer. The short splice variant PDE4D2 (M<sub>th</sub> = 58,000) overexpressed in either MA-10 cells (Fig. 2C), insect cells, or E. coli (data not shown) eluted from the column in fractions corresponding to an apparent molecular weight of ~167,000. Thus, the PDE4D2 eluted at a higher apparent molecular weight than that predicted by the co-IP experiments. This behavior was dependent on the C terminus of the protein because the deletion mutants PDE4D2-ΔCAT and PDE4D3-ΔCAT, each lacking 74 amino acids at the PDE4D C terminus (see Fig. 1C for a scheme of the PDE4D3-ΔCAT), eluted in fractions corresponding to apparent molecular weights of ~65,000 (4D2-ΔCAT, Fig. 2C) and ~152,000 (4D3-ΔCAT, Fig. 2B), respectively.

The behavior of PDE4D3 and PDE4D2 in gel filtration may be explained by either an asymmetry of the PDE protein, the presence of the highly negatively charged C-terminal residues, or an additional oligomerization domain at the PDE4D C terminus. To distinguish among these possibilities, density gradient centrifugations of both proteins were performed as an independent assessment of molecular weights. As shown in Fig. 3, the migration of full-length recombinant PDE4D3, as well as endogenous PDE4D3 from FRTL5 cells (data not shown), corresponded to an S value of 6.1 (apparent molecular weight = 108,000) whereas PDE4D2 had an S value of 3.7 (apparent molecular weight = 50,000). These values are consistent with dimer and monomer, respectively, as the apparent molecular weight of PDE4D3 is twice the apparent molecular weight of PDE4D2. The removal of the C-terminal residues in the PDE4D3-ΔCAT construct did not appreciably change the mobility of the protein in comparison to the full-length PDE4D3 (Fig. 3). This finding lends support to the hypothesis that the elution of full-length PDE4D3 and PDE4D2 at a much higher apparent molecular weight than the corresponding ΔCAT proteins is caused by an anomalous behavior of the full-length proteins on the gel filtration column and not due to an additional oligomerization domain at the C terminus. Finally, the molecular weights of PDE4D3 and PDE4D2 correspond to dimer and monomer, respectively, based on calculations according to Siegel and Moncay (28) shown in Table I. These calculations utilize Stokes radii obtained from gel filtrations and sedimentation coefficients obtained from density gradient centrifugations in order to take both size and shape of the proteins into account.

Reversibility of PDE4D3 Dimerization—In order to analyze the stability of PDE4D3 dimerization, the pull-down of differentially tagged PDE4D3 constructs, either co-expressed or expressed separately, was compared. Differentially tagged PDE4D3 constructs fused C-terminally to either a V5/His tag or a Myc/His tag (see Fig. 1A for the design of these constructs) were transfected in COS7 cells either on the same or separate plates. In the latter case, the cell pellets from both plates were mixed together before the cells were lysed. After homogenization, cytosolic fractions of the cell lysates were prepared as input for the pull-down experiments (shown in the two upper panels), and the corresponding cell lysates were used for pull-down assays with either mIgG or an additional oligomerization domain at the C terminus. Finally, the molecular weights of PDE4D3 and PDE4D2 correspond to dimer and monomer, respectively, based on calculations according to Siegel and Moncay (28) shown in Table I. These calculations utilize Stokes radii obtained from gel filtrations and sedimentation coefficients obtained from density gradient centrifugations in order to take both size and shape of the proteins into account.
cating that the dimerization is stable and not subject to disso-
ciuation/reassociation. This result is consistent with gel
filtration and density gradient centrifugation profiles that did
not show the presence of a monomeric PDE4D3 species. It also
demonstrates that the co-IPs of the differentially tagged con-
structs shown in Fig. 1, A and C are not due to aggregation of
the PDEs during the immunoprecipitation procedure.

Mapping of the PDE4D3 Dimerization Domain—To confirm
the dimerization of PDE4D3 and to further narrow the location
of the dimerization domain within the N terminus, the co-IP of
several N-terminal-truncated constructs with the full-length
enzyme was investigated. As shown in Fig. 4, the deletion of 79
residues at the N terminus of the protein, which removes the
N-terminal PDE4D3-specific sequences as well as the N-termi-
nal part of the UCR1 (termed UCR1N, aa 50–80 of PDE4D3),
did not affect the co-IP. Further truncation of the UCR1 do-
main, however, prevented co-immunoprecipitation indicating
that these sequences are part of the dimerization domain.

induce quiescence. Under these conditions, PDE4D3 is the only PDE4D
splice form in these cells and the most abundant PDE expressed. The
cells were then harvested and lysed as described under “Experimental
Procedures,” and the high-speed supernatant was separated on a TSK-
3000 gel filtration column. The resulting fractions were analyzed for
PDE-activity (•). Three hundred microliters of the fractions containing
enzyme activity were used for pull-down assays using the PDE4D-
specific M3S1 mAb. After several washings, the final IP-pellets were
resuspended in 300 μl of homogenization buffer and used for the deter-
mination of PDE-activity (•). B and C, MA-10 cells were transfected
with vectors containing either wild type PDE4D3 or PDE4D2 cDNAs
(●) or the corresponding △CAT constructs encoding for C-terminally
truncated PDE4D3 splice variants (○). After cell harvest and lysis, the
corresponding high speed supernatants were applied to size exclusion
chromatography. All data are representative of experiments performed
at least three times. The elution profiles of the PDE constructs were
confirmed by Western blotting. The marker proteins reported were
separated on the gel filtration column under identical conditions.
could be co-immunoprecipitated in pull-down experiments, whereas the PDE4D2 (encoding aa 167–672 of PDE4D3) could not, the region between amino acids 80 and 167 including the subdomains UCR1C, LR1, and UCR2N (see Fig. 5A for domain organization) probably contains the putative dimerization domain.

This region has been the object of a comprehensive yeast two-hybrid analysis that identified an intramolecular but not an intermolecular interaction. The yeast two-hybrid system was, therefore, also employed in the present study to further characterize the putative PDE dimerization domain. Confirming previous reports (21, 22), UCR1C (aa 80–116), and UCR2N (aa 132–185) specifically interact with each other (Fig. 5B; see co-transformant AD-UCR1C:BD-UCR2N) whereas neither of them (AD-UCR1C and AD-UCR2N) interacts with a construct containing both the UCR1C and the UCR2N domain (BD-UCR1C-UCR2N). However, in further experiments it could be demonstrated that although not interacting with the separate UCR2N or UCR1C, the fragment spanning UCR1C-LR1-UCR2N did interact with itself (co-transformant AD-UCR1C:UCR2N: BD-UCR1C-UCR2N). Therefore, an intermolecular interaction between amino acid positions 80–185 also could be detected using the yeast two-hybrid system, and both UCR1C and UCR2N are essential for this interaction.
In agreement with the above results, the nested deletion of UCR1C and UCR2N ablated the oligomerization detected in co-IP assays whereas the mutation of the charged residues within these domains did not (Fig. 7). A summary of the properties of all the constructs is reported in Table I.

Fig. 5. Characterization of the dimerization domain using the yeast two-hybrid system. A, design of the deletion mutants used in the yeast two-hybrid experiments shown in B. Both the UCR1 and the UCR2 domains are shown as boxes and are separated into an N-terminal and C-terminal part referred to as UCR1N (UCR2N) and UCR1C (UCR2C), respectively. The linker region 1 (LR1) connecting UCR1C with UCR2N, and the linker region 2 (LR2) connecting UCR2C with the catalytic domain, are shown as a line. Amino acid positions are given according to the human PDE4D3 sequence. The activation domain (AD) (shown as a filled ellipse) represents the GAL4 activation domain or, in the case of the BD-fused constructs, the GAL4 DNA binding domain. B, vectors encoding fusion proteins of the indicated PDE4D3 fragments with the GAL4 activation domain (AD) and the GAL4 DNA binding domain (BD; see A for design of constructs) were co-transformed in competent yeast cells that were then grown on selection media agar plates. Resulting yeast colonies were restreaked on a single plate, incubated further overnight, and the two-hybrid interaction was then detected using a filter-lift assay as described under “Experimental Procedures.” The data are representative of experiments performed three times.

Impact of PKA Phosphorylation on PDE4D3 Dimerization—Because the intramolecular UCR1C-UCR2N interaction is ablated by PKA phosphorylation at the PDE4D3 N terminus (Ser-54; Ref. 22), the impact of PKA phosphorylation on enzyme dimerization was determined. However, in vitro phosphorylation of PDE4D3 by PKA did not shift the elution of the enzyme to a lower apparent molecular weight indicating that phosphorylation does not abrogate PDE4D3 dimerization. On the contrary, the PKA-phosphorylated enzyme consistently eluted approximately one fraction prior to the unphosphorylated form from the gel filtration column. Similar shifts in elution were observed whether native enzyme (FRTL5 cells treated with forskolin to induce PKA phosphorylation; data not shown), recombinant PDE4D3 (data not shown), or the PDE4D3-ΔCAT construct was used (Fig. 8A). The shift in elution of the phosphorylated PDE4D3 underscores the major conformational changes of the holoenzyme induced by this post-translational modification. Moreover, it is likely that the UCR domains are required for mediating the PKA-dependent regulation of enzyme activity as the internal deletion of either UCR1C or UCR2N prevents the activation of PDE4D3 upon PKA phosphorylation (Fig. 8B).

DISCUSSION

Two decades of investigation on the role of PDEs in cyclic nucleotide signaling have established that the function of these
enzymes extends well beyond termination of signals from the G protein-coupled receptor/adenylate cyclase system. Indeed, PDEs play an important role in fine-tuning cAMP levels in the cell, establishing cross-talk between different signaling pathways (7, 8, 13, 29) and controlling cAMP compartmentalization (30–35). Given the recent elucidation of the crystal structure of PDE4B (36), much is known about the atomic organization and function of the catalytic domain of PDEs. Though important, considerably less is known about the structural organization of the N- and C-terminal PDE domains. This is particularly true for PDE4 as the function of the conserved N-terminal domains (UCR1 and UCR2) unique to these enzymes is far from established. Using several different experimental approaches, we provide evidence here that these conserved regions constitute the dimerization domain of these proteins.

Four distinct lines of evidence indicate that the UCR1/UCR2 module is responsible for PDE4 oligomerization. Firstly, co-IP assays indicate that PDE4D3 is able to form oligomers whereas PDE4D2, which does not contain UCR1 and UCR2N, behaves as a monomer. Removal of the C terminus does not affect oligomerization of PDE4D3 assessed in the co-IP assay. Secondly, studies with a combination of gel filtration and sucrose density gradient centrifugation support the hypothesis that PDE4D3 behaves as a dimer whereas PDE4D2 does not. Thirdly, internal deletion of the UCR domains converts the full-length PDE4D3 from an oligomer to one with the properties of a monomer. Finally, yeast two-hybrid experiments further confirm that a domain encompassing the C terminus of UCR1 and the N terminus of UCR2 is capable of oligomerization. All these findings strongly support the hypothesis that the N terminus is critical for oligomerization of PDE4D3. We should emphasize that our data do not exclude the possibility that other domains, perhaps at the C terminus or within the catalytic domain, contribute to the stabilization of the quaternary structure of PDE4.

It is generally accepted that most PDEs exist as dimers or oligomers. This property has been most extensively studied for PDE5 and PDE6, and both physical characterization and three-dimensional molecular organization have been reported (37, 38). The dimerization domain is located at the N terminus of the protein, possibly overlapping with the cGMP binding sites/GAF domains (37, 38). Our findings support the idea that the dimerization of PDE4 depends, as for PDE5 and PDE6, on domains present at the N terminus. Thus, the overall structural organization of a PDE, with a catalytic domain flanked by regulatory and dimerization domains at the N terminus, is likely conserved across different PDE families. Further exper-
domains are, however, required to confirm this possibility as PDE3A/B catalytic domains appear to behave as dimers (39).

At odds with the data reported on PDE5, it was thought that PDE4 oligomerization is mediated by several different domains localized within the C terminus or the catalytic domain (40–44). On the basis of deletion mutants expressed in bacteria, a previous report had reached the conclusion that the C terminus mediates dimerization of a short PDE4D splice form (41). It should be pointed out that these conclusions were derived only from gel filtration chromatography data. We believe that the elution of PDE4 from gel filtration columns does not closely follow the mass of the native enzyme because of an anomalous migration of the PDE4 due to either an asymmetric shape and/or the highly charged C-terminal domain. Indeed, when the C-terminal portion was removed, PDE4D3 and PDE4D2 behave as dimers and monomers, respectively. Sucrose density gradient centrifugation does not appear to be affected by the presence of the C terminus as no major change in mobility was observed in the truncated constructs. Indeed, several studies have cautioned the exclusive use of gel filtration as the means to determine the molecular weight of a protein (28, 45). Anomalous behavior of PDE4 also has been observed by SDS-PAGE with the protein migrating with an apparent molecular weight higher than the theoretical molecular weight. In addition, several other reports have attempted to investigate the state of oligomerization of PDE4 with mixed results due to the extensive aggregation experienced with purified recombinant proteins (40, 42).

Although our data strongly support the view that the major dimerization domain coincides with UCR1/UCR2, it is possible that additional inter-subunit interactions stabilize the PDE4 dimer. Previous studies, including our own, showed by gel filtration and density gradient centrifugation that the catalytic domains of PDE4A and PDE4B behave as oligomers (40, 42–44). As the catalytic domains of the PDE4 subtypes are highly conserved, it is unlikely that this discrepancy is due to sequence differences between PDE4A/B and the PDE4D used in our present study. It is more likely that, although the UCR domains are the crucial dimerization domains and essential for dimerization in vivo, several other contacts between the two molecules that stabilize the dimer are present, for example, within the catalytic domain. Whereas these interactions might not be sufficient for dimerization in vivo, they might be sufficient for oligomerization in purified and/or concentrated preparations of overexpressed PDE4 constructs. This conclusion is consistent with data reported on the molecular organization of PDE5 and PDE6 (38) where the dimerization and tightest interaction between the two interacting monomers is dependent on the N-terminal domain. Nevertheless, the three-dimensional organization of those proteins also indicates that the catalytic domains of both monomers are in close proximity. These contacts are not sufficient to allow dimerization of a purified N-terminally truncated PDE5 construct (37). Similar contacts in the catalytic domain of PDE4 cannot be detected by co-IP or yeast two-hybrid assay, but may cause recombinant purified PDE4 constructs to form oligomers. Indeed, a point of contact within the catalytic domain has been implied by the analysis of the crystal structure of PDE4B (36).

Previous reports have shown that UCR1 and UCR2 are involved in protein interactions (21, 22) whereas other interacting domains within the PDE4 were not observed in yeast two-hybrid assays. Our present findings are consistent with, and expand, these previous reports. Several positively charged residues within the C-terminal part of UCR1 and several negatively charged residues within the N-terminal part of UCR2 were previously identified as critical for the UCR1/UCR2 interaction as it could be ablated by the mutagenesis of these residues. The UCR1-UCR2N interaction was thought to be intramolecular, as it could only be detected when UCR1C and UCR2N were expressed as separate constructs. Although enzyme dimerization is mediated by the same domains, we found that mutation of these charged residues had no effect on the state of oligomerization of PDE4D3. Furthermore, PKA phosphorylation of the N terminus of PDE4D3 (Ser-54), previously shown to abrogate the intramolecular UCR1-UCR2-interaction, does not affect enzyme dimerization. There are at least two possible explanations for this discrepancy that cannot be distinguished at the present time. The first possibility is that enzyme dimerization is the only interaction mediated by the UCR domains, but it involves more residues than the previously identified charged amino acids. Therefore, dimerization of the full-length enzyme cannot be ablated by site-directed mutagenesis of only these residues. Conversely, in experiments where UCR1C and UCR2N are encoded by two separate truncated constructs, this interaction is weakened and site-directed mutagenesis of only these charged residues is sufficient to abrogate the interaction. On the other hand, the UCR domains may be required for both intermolecular dimer formation and for an intramolecular interaction. In the latter case, PDE4 dimerization represents a new, additional feature of the UCR domains and may explain some of the differences between the regulatory properties of long and short PDE4 splice variants. In preliminary studies, we show that the activation of PDE4D3 upon phosphorylation by PKA is ablated in constructs lacking either UCR1C or UCR2N (Fig. 5B), thus indicating that these domains are necessary for translating the conformational changes at the PDE4 N terminus into altered catalytic functions. These issues, relating enzyme dimerization and functions (i.e. dependence of kinetic properties, inhibitor sensitivity, and metal ion dependence on the oligomerization state), will be addressed in future studies.

In summary, using several independent approaches, the present study demonstrates that PDE4D2, representative of the short splice variants, behaves as a monomer, whereas PDE4D3, a prototype of the long splice variants with complete UCR domains, is a dimer. The long PDE4 splice variants, therefore, might have a molecular organization similar to that of PDE5 and PDE6. This raises the possibility that, although different in their primary structure, the property of enzyme dimerization at the N terminus may be evolutionarily conserved among the PDE families. Thus, the possibility also should be entertained that the molecular mechanisms that regulate enzyme activity upon modulation of the N-terminal domains, via post-translational modifications or the binding of signaling molecules, may be conserved and similar in all PDEs.

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