UCR1 and UCR2 Domains Unique to the cAMP-specific Phosphodiesterase Family Form a Discrete Module via Electrostatic Interactions*

(Received for publication, June 15, 1999, and in revised form, December 21, 1999)

Matthew B. Beard‡, Aileen E. Olsen§, Randy E. Jones§, Suat Erdogan‡,†, Miles D. Houslay§,¶, and Graeme B. Bolger§,**

From the ²Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, Institute of Biology and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland, United Kingdom and ²Veterans Affairs Medical Center, Huntsman Cancer Institute, Departments of Medicine (Division of Oncology) and Oncological Science, University of Utah Health Science Center, Salt Lake City, Utah 84132

The cAMP-specific phosphodiesterases (PDE4) enzymes contain unique “signature” regions of amino acid sequence, called upstream conserved regions 1 and 2 (UCR1 and UCR2). UCR1 and UCR2 are located between the extreme amino-terminal region and the catalytic region of the PDE4 enzymes. The UCR1 of the PDE4D3 isoform was used as a “bait” in a two-hybrid screen, which identified a PDE4D cDNA clone containing UCR2 and the catalytic region but not UCR1. Two-hybrid and “pull down” analysis of constructs incorporating various regions of the PDE4D3 cDNA demonstrated that the carboxyl-terminal region of UCR1 interacted specifically with the amino-terminal region of UCR2. The interaction was blocked by mutations of two positively charged amino acids (Arg-98 and Arg-101 to alanine) located within an otherwise largely hydrophobic region of UCR1. Mutation of three negatively charged amino acids in UCR2 (Glu-146, Glu-147, and Asp-149, all to alanine) also blocked the interaction. The phosphorylation of UCR1 by cAMP-dependent protein kinase (PKA) in vitro attenuated the ability of UCR1 to interact with UCR2. Mutation of the PKA substrate site in UCR1 (Ser-54) to aspartic acid, which mimics the activation of PDE4D3 by PKA, profoundly reduced the interaction between UCR1 and UCR2. Our data are consistent with a model in which UCR1 and UCR2 act as independent domains whose interaction is determined by electrostatic interactions and which may be disrupted by PKA phosphorylation. We suggest that the UCR1 and UCR2 domains may form a module that interacts with and regulates the PDE4 catalytic region.

Modulation of the levels of the second messengers cAMP and cGMP by cyclic nucleotide PDEs plays an important role in the regulation of numerous physiological processes, including those in the immune/inflammatory systems, vascular smooth muscle, and the brain. The cAMP-specific phosphodiesterases (PDE4s) are members of a large family of cyclic nucleotide phosphodiesterases (1). PDE4 enzymes can be differentiated from other PDEs by sequence homology in the catalytic regions of the enzymes (2) and by their ability to be specifically inhibited by the drug rolipram. Rolipram and other specific PDE4 inhibitors have been shown to have anti-depressant, anti-inflammatory, and smooth muscle relaxant activity in humans (2). The mammalian PDE4s show strong evolutionary conservation to the dunce gene of Drosophila melanogaster, which was first isolated as a mutation affecting learning and memory in that organism (3, 4). In mammals, the PDE4s are comprised of a large family of isoforms, encoded by four different genes (PDE4A, PDE4B, PDE4C, and PDE4D), with additional diversity being generated by alternative mRNA splicing (2).

We have demonstrated previously (2, 5) that the PDE4 enzymes are uniquely characterized by two regions of amino acid sequence, called upstream conserved regions 1 and 2 (UCR1 and UCR2, respectively). UCR1 and UCR2 are located between the extreme amino-terminal regions of the proteins and their catalytic regions (Fig. 1). UCR1 and UCR2 appear to be distinct, in that they lack homology to each other and are separated by a region of relatively low homology (2, 5). Significantly, UCR1 and UCR2 show strong evolutionary conservation throughout mammalian PDE4s (2) and are also conserved in PDE4 homologs in organisms as distantly related as D. melanogaster (4) and Caenorhabditis elegans (6). This strong evolutionary sequence conservation suggests that UCR1 and UCR2 are functionally important, as sequence motifs that are strongly conserved in evolution are often of functional significance. The function(s) of UCR1 and UCR2 are not known. However, a phosphorylation site for the cAMP-dependent protein kinase (PKA) is located at the beginning of UCR1 (Ser-54 in PDE4D3, Fig. 1) and phosphorylation of the PDE4D3 isoform activates the enzyme and changes its ability to be inhibited by rolipram (7–10).

* This work was supported in part by a Merit Review award from the Office of Research and Development, Medical Research Service, Department of Veterans Affairs, National Institutes of Health Grant 84132 (to G. B. B.), and by a grant from the Medical Research Council (UK) (to M. D. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1744 solely to indicate this fact.

‡ Present address: Dept. of Biochemistry, Faculty of Veterinary Medicine, University of Mustafa Kemal, Antakya, Turkey.

§ Supported by a collaborative travel grant from the Wellcome Trust, UK.

** To whom correspondence should be addressed: Dept. of Veterans Affairs Medical Center, 500 Foothill Blvd., Salt Lake City, UT 84148. Tel.: 801-582-1565 (ext. 2155); Fax: 801-583-9624; E-mail: Graeme.Bolger@m.cc.utah.edu.

* The abbreviations used are: PDE(s), cyclic nucleotide phosphodiesterase(s); PDE4(s), cAMP-specific phosphodiesterase(s); Cat, catalytic; DTT, dithiothreitol; GST, glutathione S-transferase; LR, linker region; MBF, malse-binding protein; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PKA, cAMP-dependent protein kinase; RACK1, receptor for activated c-kines; rolipram, 4-[3-(cyclopentoxyl)-4-methoxyphenyl]-2-pyrrolidinone; UCR, upstream conserved region; VSV, vesicular stomatitis virus.
In this study, we demonstrate, by two independent approaches, that UCR1 and UCR2 interact directly with each other. We also show that the interaction involves discrete regions of UCR1 and of UCR2 and that it is dependent on specific charged amino acids within these regions. We also show that this interaction is disrupted by the phosphorylation of UCR1 by PKA and of mutation of Ser-54 in UCR1 to aspartic acid, which mimics the stoichiometrically PKA-phosphorylated form of PDE4D3 (9, 10). We propose a model in which UCR1 and UCR2 form an interacting module that serves to regulate the catalytic region of the enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**—A HeLa cell (HeLa S3 cells; American Type Culture Collection) two-hybrid library cloned into the EcoRI and XhoI sites of the pGADGH vector (11) was obtained from David Beach (Cold Spring Harbor Laboratory). This vector expresses proteins as fusions with the activation domain of the Saccharomyces cerevisiae GAL4 protein. A monoclonal antibody to human PDE4D proteins, which does not cross-react with other PDE4 species and which we have described previously (12), was a gift from Sharon Wolda, ICOS Corp. This antibody was generated against the extreme carboxy-terminal region of the PDE4D3 protein. The PDE4D3 protein can be detected at the amino terminus, such as those lacking UCR1 and UCR2 (12).

**Two-hybrid Screens**—These were performed using methods we have described previously (13, 14). In brief, various regions of the pPDE43 cDNA encoding human PDE4D3 (Fig. 1; GenBank™ accession number L20970 (5)) were cloned into the NotI site of pLEXAN, to produce constructs encoding fusions between the PDE and the DNA-binding domain of the Escherichia coli LexA protein. These constructs were prepared by the addition of NotI sites to the cDNA regions by the use of PCR, as described previously (5). Screens were performed with the HeLa two-hybrid cDNA library in the S. cerevisiae strain L40 (15). To screen the library, positive clones were initially selected for growth in the absence of histidine (in the presence of 1 mM 3-aminotriazole) and then patched to plates selecting only for the two plasmids and assayed for β-galactosidase activity using a filter β-galactosidase assay, as described (15). Library plasmid DNA was then isolated from the positives and re-assayed for interaction with the LexA fusion, using methods described previously (13). For additional two-hybrid experiments, various pieces of the PDE4D3 cDNA were cloned into the NotI site of pGADN, using PCR as described for the LexA fusions.

**QuikChange Site-directed Mutagenesis**—QuikChange site-directed mutagenesis kit (Stratagene). Specifically, pMALU1DTR encodes the fusion protein MBP-UCR1-C, consisting of amino acids 17–136 of PDE4D3. pMAL43U1 encodes the fusion protein MBP-UCR1, consisting of amino acids 1–134 of PDE4D3. pMALC2 (de-scibed above) was grown overnight in LB medium (20) supplemented with 100 μg/ml ampicillin and 2 mg/ml glucose. These cultures were then patched to plates selecting only for the two plasmids and assayed for interaction with the LexA fusion, using methods described above. The activity units were defined as follows: (OD420(reaction) – OD660(reaction)) / (time in min)OD660(culture)), where OD420(reaction) and OD660(culture) are the optical densities (at the indicated wavelengths, in nm) of the final β-galactosidase reaction and OD660(culture), is the optical density at 660 nm of the cultures at the time of harvest.

**Generation of COS7 Cell Expression Constructs**—Various pieces of PDE4D3 cDNA were cloned into the NotI site of pMALN, using PCR as described above for the LexA fusions. pMALN is a derivative of pMALC2 (New England Biolabs (17)), with a NotI site inserted into the polylinker. These clones generate fusions between maltose-binding protein (MBP) and the amino terminus of the protein encoded by the insert. Specifically, pMALU1DTR encodes the fusion protein MBP-UCR1-C, consisting of amino acids 80–116 of PDE4D3. pMALA3U1 encodes MBP-UCR1, consisting of amino acids 17–136 of PDE4D3. pMALC2 (described above) was grown overnight in LB medium (20) supplemented with 100 μg/ml ampicillin and 2 mg/ml glucose. These cultures were then patched to plates selecting only for the two plasmids and assayed for interaction with the LexA fusion, using methods described above. The activity units were defined as follows: (OD420(reaction) – OD660(reaction)) / (time in min)OD660(culture)), where OD420(reaction) and OD660(culture) are the optical densities (at the indicated wavelengths, in nm) of the final β-galactosidase reaction and OD660(culture), is the optical density at 660 nm of the cultures at the time of harvest.

**Generation of Expression Constructs**—Various pieces of the PDE4D3 cDNA were cloned into the NotI site of the vector pcDNA3 (Invitrogen). In these constructs, the insert is placed under the control of the cytomegalovirus intermediate early gene promoter. pcDNA3V5SVF encodes the full open reading frame of PDE4D3. pcDNAFN43N2SVSF encodes UCR2 and the catalytic regions of PDE4D3 (i.e. UCR2+Cat). pcDNAFN43N3SVSF encodes only the catalytic region of PDE4D3 (i.e. Cat). In all cases, a sequence corresponding to the vesicular stomatitis virus (VSV) glycoprotein epitope (18) was added immediately downstream from the last native codon of the PDE to encode a carboxy-terminal fusion. The native PDE4D3 stop codon was removed in this process, but a synthetic stop codon was placed immediately downstream from the epitope sequence, as described (10, 14). A portion of the PDE4D3 cDNA corresponding to UCR2 (amino acids 134–212) was cloned into pEBG, to produce pEBGUCR2. pEBG is a derivative of pEBG2 (19), but with a NotI site in the polylinker. It encodes a fusion between glutathione S-transferase (GST) and the amino terminus of the protein encoded by the insert. The expression of the fusion protein in mammalian cells is driven by the polypeptide chain elongation factor 1α (EF-1α) promoter.

**Generation of cDNAs Encoding Mutant Forms of PDE4D3**—Portions of the PDE4D3 cDNA insert cloned into the various vectors described above were subjected to site-directed mutagenesis with the QuikChange site-directed mutagenesis kit (Stratagene).

**Verification of Two-hybrid, Expression, and Mutagenesis Constructs**—All mutant or PCR-generated constructs were verified by sequencing prior to use.

**Expression and Purification of MBP Fusions**—In E. coli—E. coli JM109 transformed with pMALU1DTR, pMALA3U1, or pMALC2 (described above) were grown overnight in LB medium (20) supplemented with 100 μg/ml ampicillin and 2 mg/ml glucose. These cultures were...
Interaction between PDE4 UCR1 and UCR2

then used to inoculate 400-ml cultures of the same medium, which were grown at 37 °C with agitation until the OD600 was 0.6 to 1.0. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.3 mM, and the cultures were grown for an additional 4–6 h at 30 °C. The bacteria were collected by centrifugation at 2500 × g for 5 min at 4 °C and then resuspended in 20 ml of KHEM buffer (50 mM HEPES-KOH, pH 7.2, 10 mM EDTA, 1.92 mM MgCl2) containing 1 mM dithiothreitol (DTT) and complete protease inhibitor mixture (Roche Molecular Biochemicals). The resuspended bacteria were stored in 10-ml aliquots at −20 °C until needed.

The aliquots were thawed at room temperature and held on ice. They were sonicated in 20-s pulses, separated by 30-s intervals, until cell lysis was complete. The sonicate was centrifuged at 9,000 × g for 30 min at 4 °C. The supernatant was then incubated end-over-end for 2–4 h at 4 °C with 100 μl (bed volume) amylose resin (New England Biolabs) equilibrated in KHEM buffer/DTT/protease inhibitor mixture. The beads were collected by centrifugation at 2500 × g for 5 s at room temperature, held on ice, and then washed three times, each time with 1 ml of KHEM/DTT/protease inhibitor mixture. The purified protein was then eluted from the beads by three incubations, end-over-end, for 10 min at 4 °C with 250 μl of KHEM buffer plus 10 mM maltose. The eluted fractions were pooled and then dialyzed for 6 h at 4 °C against three changes of dialysis buffer (20 mM Tris–Cl, pH 7.6, 50 mM NaCl; 2 liters total volume). The fusion protein was assayed for protein concentration and stored at −80 °C.

**FIG. 2.** The carboxyl-terminal half of UCR1 interacts with the amino-terminal region of UCR2. a, structure of PDE4D deletion mutants. The regions of PDE4D3 incorporated into various two-hybrid or COS7 cell expression constructs are shown schematically, along with the amino acid coordinates of the regions encoded by each construct. Cat. indicates the catalytic region only. The open boxes refer to UCR1, UCR2, or the catalytic regions of the protein, as indicated. The wavy lines refer to regions of amino acids that separate UCR1, UCR2, and the catalytic region. The cross-hatched box (Tag) represents the GAL4 activation domain or, in the case of U1D and U1E, the LexA DNA-binding domain in the two-hybrid constructs pLEXAU1D and pLEXAU1E, respectively. b, various regions of PDE4D3, as described in a and listed to the right of the figure, were cloned into pGADN to encode fusion proteins with the trans-activation domain of the GAL4 protein. They were then tested for interaction with amino acids 80–109 of UCR1, expressed as the LexA DNA-binding domain fusion pLEXAU1E (indicated as UCR1-C for this figure only), pLEXAN or pGADN without inserts (vector) were used as controls. F.I. indicates full-length PDE4D3. A filter β-galactosidase two-hybrid assay was used as described (13). The two patches at the right serve as internal positive and negative controls, respectively (the oncogenes RasV12 and RafI (15) and the vectors without inserts). The β-galactosidase reactions were deliberately overdeveloped to demonstrate the lack of interaction of isolated UCR1 with full-length PDE4D3. This produced a few weak false-positive signals (i.e. the negative controls are weakly blue).
FIG. 3. UCR1 interacts with UCR2 in vitro, as demonstrated by pull down assays. A, pull down assays were performed with various MBP fusion proteins on extracts of COS7 cells transfected with constructs expressing various truncations of PDE4D3 (see “Experimental Procedures”). Equal amounts of the bound and unbound fractions (16% of the total amount of material in the fraction) were then immunoblotted with a monoclonal antibody specific for the PDE4D carboxyl-terminal region. Arrows to the left of each panel indicate the migration of each PDE4D3 truncation. Arrows at the top of each panel indicate the MBP fusion used in the pull downs. In the left panel are analyses of COS7 cells expressing UCR2+Cat (odd-numbered lanes) or the isolated catalytic region (Cat, even-numbered lanes). Lanes 1 and 2, standards for UCR2+Cat and Cat.
Interaction between PDE4 UCR1 and UCR2

Lanes 3–8 analyze unbound fractions, and lanes 9–14 analyze bound fractions from pull downs. In the right panel are analyses of COS7 cells expressing full-length PDE4D3 (even-numbered lanes) and UCR2; Cat (odd-numbered lanes). Lanes 1–4 analyze unbound fractions, and lanes 5–8 analyze bound fractions. Full-length PDE4D3 had an apparent molecular mass under denaturing conditions of 105.5 ± 2.8 kDa, UCR2-Cat of 94.3 ± 1.4 kDa, and Cat of 76.4 ± 1.5 kDa, as determined by comparison to pre-stained molecular weight markers. These data are typical of experiments performed on three separate occasions. b, histogram of interactions between MBP-UCR1 and the PDE4D3 truncations. The data show the proportion of the specific PDE4D3 truncation (full-length PDE4D3, UCR2+Cat, or Cat, respectively) captured in the bound fraction as a percentage of the amount of the truncation present in the pull down assay. Detection was performed by immunoblotting with the PDE4D4 antibody, as described under “Experimental Procedures.” The figures represent mean ± S.E. for n = 3 separate experiments. c, histogram of interactions between the MBP-UCR1 and the PDE4D3 truncations. The proportion of the specific PDE4D3 truncation in the bound fraction was determined by PDE assay. These data are typical of experiments performed on three separate occasions. d, interaction of GST-UCR2 and MBP-UCR1. Bound and unbound fractions were immunoblotted with an antibody to GST. GST-UCR2 had an apparent molecular mass under denaturing conditions of 61 ± 0.3 kDa (arrow at right of panel). Lane 2 (”blank”) contained no sample. The right panel shows pull downs with immobilized GST-UCR2 or GST alone, as derived from transfected COS7 cells, on MBP or MBP-UCR1. Immunoblotting was performed with an antibody to MBP. Only the fraction bound to the MBP fusion is shown. The data show that MBP-UCR1 binds only to GST-UCR2 and not to GST alone and that MBP alone does not bind to GST or GST-UCR2. MBP had an apparent molecular mass under denaturing conditions of 67 ± 1.0 kDa (arrow at left of panel) and MBP-UCR1 of 77 ± 1.6 kDa (arrow at right of panel). These data are typical of experiments performed on three separate occasions.
the two-hybrid screen was specific, we used yeast two-hybrid β-galactosidase assays to test the interaction of the two-hybrid positive with a variety of baits expressed as LexA fusions. These included lamin (15), casein kinase II, Ras, Raf, several transcription factors, and the DNA-binding region of LexA itself (i.e., not as a fusion). In similar fashion, we tested pLEXAU1D for its ability to bind to these proteins expressed as GAL4 fusions and also to the GAL4 activation domain itself (i.e., not as a fusion). No interaction was detected under conditions that demonstrated an interaction between pLEXAU1D and the positive from the screen. Therefore, we felt it was unlikely that our results could be explained by nonspecific interactions between UCR1 and other proteins.

The Carboxyl-terminal Half of UCR1 Interacts with the Amino-terminal Third of UCR2—We wished to confirm the results of the two-hybrid screen and to determine precisely which region(s) of PDE4D3 interacted with the carboxyl-terminal half of UCR1. Therefore, we created constructs encoding various portions of the PDE4D3 cDNA as GAL4 activation domain fusions (Fig. 2a) and used yeast two-hybrid β-galactosidase assays to test them for interaction with the carboxyl-terminal half of UCR1. These data are representative of experiments performed on three separate occasions. B, pull downs with mutations in MBP-UCR1. Pull down assays were performed as in the legend to Fig. 3a, with various MBP-UCR1 mutant fusion proteins used to probe extracts from COS7 cells transfected to express UCR2+Cat. Comparable amounts, as measured by immunoreactivity, of UCR2+Cat were incubated with constant amounts of MBP-UCR1. Immunoblots were then performed on the bound fraction only, using the PDE4D antibody. These data are representative of experiments performed on three separate occasions. C, histogram of the interactions between MBP-UCR1 mutants and UCR2+Cat. The figure showing data from both quantitative immunoblotting (solid bars) and determination of PDE activity (shaded bars). The figures represent mean plus S.E. for n = 3 separate experiments. Quantitation was performed as described in the legend to Fig. 3c.
Interaction between PDE4 UCR1 and UCR2

Site-directed mutagenesis was used to mutate codons in pGADNU2J (UCR2-N (W.T.)) to alanine. The mutagenized constructs (listed in the left-hand column) were then tested by two-hybrid quantitative β-galactosidase assay for their ability to interact with pLEXA1D (UCR1-C (W.T.)) or pLEXAU1D containing the mutations R98A or R101A. Vector, pLEXAN or pGADN. Values are the mean ± S.D. for assays performed in triplicate.

| Vector                 | R101A | R98A | UCR1-C (W.T.) |
|------------------------|-------|------|---------------|
| Vector                 | 4.3 ± 0.1 | 2.9 ± 1.3 | 3.5 ± 0.1 | 3.4 ± 0.8 |
| UCR2-N (W.T.)          | 1.7 ± 0.2 | 14.4 ± 1.3 | 111.2 ± 23.7 | 77.7 ± 17.7 |
| 143.146                | 3.1 ± 0.1 | 38.0 ± 1.7 | 154.8 ± 18.3 | 122.7 ± 11.8 |
| 153.156                | 4.7 ± 0.7 | 7.1 ± 0.9 | 96.7 ± 7.5 | 22.6 ± 5.3 |
| 146.147,149            | 4.1 ± 0.6 | 3.6 ± 0.3 | 4.0 ± 0.4 | 9.0 ± 1.1 
| 147                    | 3.8 ± 0.2 | 33.2 ± 7.1 | 92.4 ± 8.5 | 33.1 ± 8.0 |
| 146,147                | 3.4 ± 0.2 | 38.3 ± 10.2 | 11.2 ± 2.3 | 54.6 ± 3.7 |
| 149                    | 4.3 ± 0.5 | 4.2 ± 0.2 | 8.1 ± 2.8 | 28.2 ± 10 |
| 144,147                | 4.3 ± 0.8 | 4.5 ± 0.8 
| 147,149                | 5.5 ± 0.5 | 4.8 ± 0.1 

*p < 0.003 by t test, compared with UCR2-N/W.T./UCR1-C(W.T.). p < 0.003 by t test, compared with UCR2-N/R98A.

Specifically Charged Amino Acids in UCR2 Are Required for Its Interaction with UCR1—We then attempted to determine...
which amino acids in UCR2 are necessary for it to interact with UCR1. Site-directed mutagenesis was used to mutate various combinations of glutamic acids or aspartic acids within the amino terminus of UCR2 to alanine (Fig. 1). The resulting mutants were then tested for their ability to interact with MBP fusions on protein staining. These data are representative of experiments performed on three separate occasions. Interaction between PKA-phosphorylated MBP-UCR1 and UCR2+Cat. Pull down assays using MBP, MBP-UCR1, or MBP-UCR1R51A/R52A were performed on extracts from COS7 cells expressing UCR2+Cat, as described in the legend to Fig. 3a. The lower panel shows the results when the constituents were phosphorylated by PKA (as in a), and the upper panel shows identical experiments performed without PKA. The data are representative of experiments performed on three separate occasions. a, interaction between PKA-phosphorylated MBP-UCR1 and UCR2+Cat. Quantitative immunoblotting was performed as described in the legend to Fig. 3a. The figures represent mean plus S.E. for n = 3 separate experiments. b, interaction between MBP-UCR1 with mutations in the PKA consensus site and UCR2+Cat. Pull down assays were performed as in the legend to Fig. 3a. Various mutant MBP-UCR1 constructs were used in pull down assays on COS7 cell extracts expressing UCR2+Cat. Immunoblots were performed on the bound fraction, as described in the legend to Fig. 3a. These data are representative of experiments performed on three separate occasions. c, histogram of the interactions between MBP-UCR1 with mutations in the PKA consensus site and UCR2+Cat. Data are shown from pull down assays analyzed by both quantitative immunoblotting (solid bars) and determination of the PDE activity of UCR2+Cat (shaded bars). PDE activity was determined as in Fig. 4c. The figures represent mean plus S.E. for n = 3 separate experiments.

which amino acids in UCR2 are necessary for it to interact with UCR1. Site-directed mutagenesis was used to mutate various combinations of glutamic acids or aspartic acids within the amino terminus of UCR2 to alanine (Fig. 1B). The resulting mutants were then tested for their ability to interact with pLEXAU1D, using two-hybrid quantitative β-galactosidase assays (Table 1). The results demonstrate that the simultaneous mutation of three amino acids (Glu-146, Glu-147, and Asp-149) in UCR2 significantly attenuated its interaction with UCR1. Mutation of the pair Asp-153/Glu-153 to alanine also appeared to attenuate the interaction. In contrast, mutation of the pair Glu-143/Glu-146 had no detectable effect on the interaction.

We then wished to determine if the positively charged aspartic acids (Arg-98 and Arg-101) in UCR1-C interacted with specific negatively charged glutamic/aspartic acid residues (Glu-146, Glu-147, and Asp-149) in UCR2. Therefore, we tested the ability of the single R98A and R101A mutants to block the interaction, when paired with various combinations of E146A, E147A, and D149A mutants. The results (Table 1) show that the combination of R98A and D149A mutants significantly attenuated the interaction, compared with the corresponding single mutants. The combination of the R101A mutant and the
that the S54A mutation in PDE4D3 had no effect on enzyme activity (9, 10). However, it caused a conformational change in the PDE4D3 catalytic region, as determined by increased sensitivity of the enzyme to inhibition by rolipram (10). The conservative S54T mutation did not change the sensitivity of the enzyme to rolipram (10). Conversely, mutation of Glu-53 to alanine mimicked the activation of PDE4D3 by PKA phosphorylation but did not alter sensitivity to inhibition by rolipram (10). We created these three mutations in MBP-UCR1 and showed by pull down assay that the S54A mutation prevented the interaction of UCR1 with UCR2, whereas the S54T and E53A mutations did not affect the interaction (Fig. 5, d and e). These data suggest that the UCR1-UCR2 interaction could produce conformational changes in the catalytic region that would change the sensitivity of the enzyme to rolipram (see “Discussion”).

Disruption of the PKA consensus phosphorylation site by mutation of RRES to AAES (MBP-UCR1R51A/R52A) had no effect on the UCR1-UCR2 interaction, as measured by pull down assays (Fig. 5, d and e). For this reason, we used the MBP-UCR1R51A/R52A construct as a control for examining the effect of direct PKA phosphorylation of UCR1 (see above; Fig. 5, a–c).

**DISCUSSION**

UCR1 and UCR2 are regions of sequence that are unique “signatures” of the PDE4 cAMP-specific phosphodiesterase family. They are located in the amino-terminal regions of these enzymes and are clearly separate from their catalytic regions. They are highly conserved in evolution, as they are present in isoforms encoded by all four human and rat PDE4 genes and also in PDE genes in organisms as distantly related as D. melanogaster (4) and C. elegans (6). Studies of PDE4 deletion constructs have demonstrated that UCR1 and UCR2 have no direct role in catalysis (reviewed in Ref. 2). However, regions of amino acid sequence that are highly conserved in evolution are usually of functional significance. To determine more about the properties of UCR1, it was used in a two-hybrid screen. Intriguingly, the screen demonstrated that UCR1 interacted with UCR2. Additional two-hybrid tests demonstrated that the carboxyl-terminal region of UCR1 interacted with the amino-terminal end of UCR2.

**Phosphorylation of UCR1 by PKA Attenuates Its Ability to Interact with UCR2—PDE4D3 is activated upon phosphorylation by PKA (7–10).** Phosphorylation of PDE4D3 occurs at two serines, both within the consensus RRXX: Ser-13, located in its unique amino-terminal region, and Ser-54, which is located at the beginning of UCR1 (Fig. 1B). The phosphorylation at Ser-54 leads to both enzyme activation and a change in sensitivity to inhibition by rolipram (9, 10). In this study, we show that phosphorylation of UCR1 by PKA attenuates its ability to interact with UCR2. First, we demonstrated that MBP-UCR1 was a PKA substrate (see above; Fig. 5, a–c). This phosphorylation was ablated in the PKA consensus site (RRES<sup>d</sup>, Fig. 1B) was disrupted by mutation to AAES (i.e. MBP-UCR1R51A/R52A). This mutation had been shown by us previously (10) to prevent PKA from phosphorylating and activating PDE4D3 and not to change either PDE activity or sensitivity to inhibition by rolipram. We also demonstrated that PKA was unable to phosphorylate UCR1 with a S54A mutation (i.e. MBP-UCR1S54A; data not shown). We then tested the ability of PKA-phosphorylated MBP-UCR1 to interact with UCR2-Cat. Treatment of MBP-UCR1 with PKA produced a marked attenuation in its ability to interact with UCR2-Cat in pull down assays (Fig. 5, b and c). This confirms that phosphorylation of UCR1 by PKA could attenuate its ability to interact with UCR1.

We also tested the effects of several other mutations in the PKA consensus site (Fig. 5, c–e). We have shown previously
that Ser-54 and Glu-53 were involved in forming bonds that influenced the structure of the catalytic unit of the enzyme (10). In this model, Glu-53 was involved in forming an ion pair which kept the enzyme in a low activity state and whose disruption (e.g., the E53A mutant) changed the activity of the enzyme but did not alter the sensitivity of the enzyme to inhibition by the PDE4-selective inhibitor rolipram. The model also suggested that the side chain hydroxyl group of Ser-54 was involved in forming a hydrogen bond that did not alter enzyme activity but influenced the conformation of the enzyme catalytic region, as measured by an increase in susceptibility of the enzyme to inhibition by rolipram. Phosphorylation of Ser-54 (or the S54D mutation) disrupted both the Glu-53 and Ser-54 bonds, leading to enzyme activation and enhanced sensitivity to inhibition by rolipram. However, the S54A mutation blocked only the Ser-54 bond, producing a change only in rolipram inhibition. In the present paper, we demonstrate that the S54D and S54A mutants, but not the E53A or S54T mutants, block the UCR1-UCR2 interaction. Therefore, it is likely that the Ser-54 hydroxyl bond, but not the Glu-53 ion pair, is essential for the UCR1-UCR2 interaction. In turn, this suggests that disruption of the UCR1-UCR2 interaction leads to an alteration in the conformation of the catalytic unit that is detected by altered rolipram inhibition but that is insufficient for enzyme activation.

We present a model for the structure of the PDE4 enzyme, based on our data (Fig. 6). The model proposes that UCR1 and UCR2 each contain one or more self-folding domains that interact with other regions of the PDE4 enzymes. One important interaction, as demonstrated by our data, is between the carboxyl-terminal region of UCR1 and the amino-terminal region of UCR2. This interaction is mediated by electrostatic interactions between positively charged amino acids in UCR1 and negatively charged amino acids in UCR2, as demonstrated by our mutational analysis. The interaction may be modulated by PKA phosphorylation of a serine in the extreme amino-terminal region of UCR1 (i.e., a region not interacting directly with UCR2). This is compatible with the UCR1 amino-terminal domain affecting the UCR1 carboxyl-terminal region and preventing it from interacting with UCR2. UCR1 and UCR2 thus appear to form a regulatory module that in turn regulates the PDE4 catalytic unit. Structural analysis of the PDE4 enzyme will be needed for further insight into the molecular mechanisms of this regulation.

Acknowledgment—DNA sequencing and oligonucleotide synthesis were supported by NCI Grant 5-FO-CA42014 from the National Institutes of Health.

REFERENCES

1. Beavo, J. A. (1995) Physiol. Rev. 75, 725–748
2. Houssay, M. D., Sullivan, M., and Bolger, G. B. (1998) Adv. Pharmacol. 44, 225–242
3. Dudai, Y., Jan, Y. N., Byers, D., Quinn, W. G., and Benzer, S. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 1684–1688
4. Quí, Y. H., Chen, C. N., Malone, T., Richter, L., Beckendorf, S. K., and Davis, R. L. (1991) J. Mol. Biol. 222, 553–565
5. Bolger, G., Michaeli, T., Martins, T., St. John, T., Steiner, B., Rodgers, L., Rigs, M., Wigler, M., and Ferguson, K. (1993) Mol. Cell. Biol. 13, 6558–6571
6. The Caenorhabditis elegans Sequencing Consortium (1998) Science 282, 1022–1026
7. Sette, C., Iona, S., and Coniti, M. (1994) J. Biol. Chem. 269, 9245–9252
8. Sette, C., Vicini, E., and Coniti, M. (1994) J. Biol. Chem. 269, 18271–18274
9. Sette, C., and Coniti, M. (1996) J. Biol. Chem. 271, 16526–16534
10. Hoffmann, B., Wilkinson, I. R., McCallum, J. F., Engels, P., and Houssay, M. D. (1998) Biochem. J. 333, 139–149
11. Hannon, G. J., Demetrick, D., and Beach, D. (1993) Genes Dev. 7, 2378–2391
12. Bolger, G. B., Erdogun, S., Jones, R. E., Loughney, K., Wilkinson, I., Scotland, G., Hoffman, R., Farrell, C., and Houssay, M. D. (1997) Biochem. J. 328, 539–548
13. Bolger, G. B. (1998) in Protein Targeting Protocols (Clegg, R. A., ed) pp. 101–131, Humana Press, Inc., Totowa, NJ
14. Yarwood, S. J., Steele, M. R., Scotland, G., Houssay, M. D., and Bolger, G. B. (1999) J. Biol. Chem. 274, 14909–14917
15. Vogt, A. B., Hollenberg, S. M., and Cooper, J. A. (1993) Cell 74, 205–214
16. Guarente, L. (1983) Methods Enzymol. 101, 181–191
17. Guan, C., Li, P., Riggs, P. D., and Inouye, H. (1988) Gene (Amst.) 7, 21–30
18. Kreis, T. E. (1986) EMBO J. 5, 931–941
19. Sanchez, I., Hughes, R. T., Mayer, B. J., Yee, K., Woodgett, J. R., Avruch, J., Kyriakis, J. M., and Zon, L. I. (1994) Nature 372, 749–750
20. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
21. McPhee, I., Pooley, L., Lobban, M., Bolger, G., and Houssay, M. D. (1995) Biochem. J. 310, 965–974
22. Harlow, E., and Lane, D. P. (1999) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
23. Huston, E., Pooley, L., Julien, P., Scotland, G., McPhee, I., Sullivan, M., Bolger, G., and Houssay, M. (1996) J. Biol. Chem. 271, 31334–31344
24. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
25. Marchmont, R. J., and Houssay, M. D. (1980) Biochem. J. 187, 381–392
26. Fields, S., and Song, O. (1989) Nature 340, 245–246
27. McPhee, I., Yarwood, S. J., Scotland, G., Huston, E., Beard, M. B., Ross, A. H., and Houslay, M. D. (1995) J. Mol. Biol. 253, 1684–1688
28. England, P., Sullivan, M., Muller, T., and Lubbert, H. (1995) FEBS Lett. 358, 305–310
UCR1 and UCR2 Domains Unique to the cAMP-specific Phosphodiesterase Family Form a Discrete Module via Electrostatic Interactions
Matthew B. Beard, Aileen E. Olsen, Randy E. Jones, Suat Erdogan, Miles D. Houslay and Graeme B. Bolger

J. Biol. Chem. 2000, 275:10349-10358.
doi: 10.1074/jbc.275.14.10349

Access the most updated version of this article at http://www.jbc.org/content/275/14/10349

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 27 references, 15 of which can be accessed free at http://www.jbc.org/content/275/14/10349.full.html#ref-list-1