Activation of the cAMP-specific Phosphodiesterase PDE4D3 by Phosphorylation

IDENTIFICATION AND FUNCTION OF AN INHIBITORY DOMAIN*

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Splicing variants of type 4 phosphodiesterases (PDE4) are regulated by phosphorylation. In these proteins, a conserved region is located between the amino-terminal domain, which is the target for phosphorylation, and the catalytic domain. Previous studies have indicated that nested deletions encompassing this region cause an increase in catalytic activity, suggesting this domain exerts an inhibitory constraint on catalysis. Here, we have further investigated the presence and function of this domain. A time-dependent increase in hydrolytic activity was observed when PDE4D3 from FRTL-5 cells was incubated with the endoproteinase Lys-C. The activation was abolished by protease inhibitors and was absent when a phosphorylated enzyme was used. Western blot analysis with PDE4D-specific antibodies indicated the Lys-C treatment separates the catalytic domain of PDE4D3 from the inhibitory domain. Incubation with antibodies recognizing an epitope within this domain caused a 3- to 4-fold increase in activity of native or recombinant PDE4D3. Again, PDE activation by these antibodies had properties similar to, and not additive with, the activation by protein kinase A phosphorylation. An interaction between the inhibitory domain and both regulatory and catalytic domains of PDE4D3 was detected by the yeast two-hybrid system. Mutations of Ser54 to Ala in the regulatory domain decreased or abolished this interaction, whereas mutations of Ser54 to the negatively charged Asp strengthened it. These data strongly support the hypothesis that an inhibitory domain is present in PDE4D and that phosphorylation of the regulatory domain causes activation of the enzyme by modulating the interaction between inhibitory and catalytic domains.

Hormones, neurotransmitters, and cytokines control the functions of a target cell by regulating both synthesis and degradation of the second messenger cAMP. Diffusing from the site of synthesis where adenylyl cyclases are located, the cyclic nucleotide binds to the regulatory subunits of the oligomeric PKA (1) or to binding sites on cyclic nucleotide-gated channels (2). The cyclic nucleotide binding and subsequent changes in conformation of these intracellular effectors are thought to be at the basis of most, if not all, cAMP effects on cell function. Alternatively, cAMP is degraded by phosphodiesterases, enzymes that inactivate and terminate the cAMP signaling (3, 4).

Cyclic nucleotide phosphodiesterases (PDEs) belong to a large superfamily of enzymes regulated by a number of signal transduction pathways including the cAMP-signaling pathway (5). Elegant work with nonhydrolyzable cAMP analogs (6, 7) has demonstrated that an increase in intracellular cAMP and activation of PKA cause a rapid increase in PDE activity and cAMP degradation in hepatocytes. Despite the several hypotheses proposed to explain this phenomenon, the exact function of this feedback mechanism in the cell remains uncertain. It may represent a dampening mechanism to prevent overstimulation, a mechanism for termination of the stimulus or a means by which the cells increase the sensitivity of responses.

Although it was originally thought that a cGMP-inhibited phosphodiesterase (PDE3) is the enzyme involved in this feedback control of cAMP levels (8–10), more recent data have indicated that a type 4 cAMP-specific phosphodiesterase (PDE4) may also fulfill this regulatory function in the cell. A short term activation of a PDE4 has been observed in thyroid cells where TSH-dependent activation of PKA leads to phosphorylation and activation of PDE4D3, a splicing variant derived from the pde4d gene (11, 12). Similar regulations of PDE4s have been described in myoblasts (13), aortic smooth muscle cells (14), AT20 pituitary cells (15), osteoclasts (16), and a glioma cell line (17), underscoring the ubiquitous nature of this regulation.

The exact mechanism by which phosphorylation causes the activation of PDE4D3 is unknown. Upon inspection of the sequence of the PDE4D3 splicing variant, it is evident that the catalytic domain, which is highly conserved in all PDEs, is flanked by two additional domains. In vitro phosphorylation and site-directed mutagenesis have identified a regulatory domain target for phosphorylation at the amino terminus of the rat PDE4D3 (12). This domain coincides with a region named upstream conserved region 1 (UCR1) (18) conserved in different PDE4s. The regulatory domain lies upstream of an additional domain conserved in all PDE4s (18, 19) named UCR2 (18). Of the splicing variants derived from the pde4d gene, PDE4D1 contains the entire UCR2 but lacks the phosphorylation domain, whereas the PDE4D2 splicing variant contains only a portion of the UCR2 domain. The so-called long variants, PDE4D3, PDE4D4, and PDE4D5, contain both domains as well as unique amino terminus regions (5, 21).

PCR, polymerase chain reaction; ONPG, o-nitrophenyl-β-D-galactopyranoside; TSH, thyroid-stimulating hormone; PAGE, polyacrylamide gel electrophoresis.
During studies to map the boundaries of the catalytic domain, it was observed that nested deletions removing the conserved region corresponding to UCR2 of PDE4D1 yield an enzyme with increased catalytic activity (22). Further deletion mutagenesis studies in PDE4D1 (23) and a PDE4A (24) have strengthened this hypothesis that a domain constraining the catalytic capacity of PDE4 is present upstream of the catalytic domain. These findings on PDE4s are in line with earlier observations on the calmodulin-regulated PDE (PDE1) and the cGMP-stimulated PDE (PDE2). Controlled proteolytic cleavage of these enzymes yields a constitutively activated catalytic fragment, indicating that calmodulin or cGMP binding alters the interaction of an inhibitory domain with the catalytic domain (25–28).

From the data accumulated thus far, it is unclear whether the inhibitory domain coincides with the regulatory domain or whether a separate inhibitory domain does indeed exist in the PDE4D variants. Previous attempts to measure the catalytic activity of purified full-length enzymes or enzymes with truncations in the putative inhibitory domain have yielded inconsistent results because PDE4D3 is often inactivated during purification. Here we have used several alternative strategies to investigate the presence of an inhibitory domain in a PDE4D3. Our findings lend further support to the hypothesis that an inhibitory domain is present within the conserved region of PDE4 (UCR2) and that this domain mediates the phosphorylation-dependent activation of the enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials—** Yeast strains of *Saccharomyces cerevisiae* and cloning vectors for the yeast two-hybrid system (29) were obtained from CLONTECH, (Palo Alto, CA) (Match Maker I and II). Yeast media was from Difco, Inc. (Angers, Germany), and amino acids and 3-amino-1,2,4-triazole were from Sigma. Restriction enzymes were purchased from Roche Molecular Biochemicals, and DNA-modifying enzymes and PCR reagents were from Life Technologies, Inc. or Stratagene (La Jolla, CA). All the other reagents were of the highest analytical grade available and were purchased from Sigma.

**Strains and Growth Conditions—** The yeast strains Y190, HPTc, and Y187 were obtained from CLONTECH. Yeast transformations were performed using the lithium acetate method. Single transformants were spread on selection medium (SD-medium) lacking either Leu or Trp. Cotransformants were propagated on SD-medium containing adenine (10 mg/ml) and 50 μg/ml 3-amino-1,2,4-triazole only, thus increasing the selection pressure for protein-protein interaction and suppressing His leakiness of Y190. In the mating experiment Y187 was used to mate with Y190.

**Domain Interaction Using the Yeast Two-hybrid System—** Rat PDE4D3 amino terminus (amino acids 1–113) encoding the regulatory domain was generated by PCR using a partial cDNA clone of rPDE4D3 as template. The clone contained several changes in the 5′ end of nucleotide sequence of rPDE4D3, which gave a single amino acid change. Forward and reverse oligonucleotides with added restriction sites and a stop codon were used (Fig. 7 and 8) to amplify the full-length cDNA using the following forward and reverse primers with incorporated restriction sites and a stop codon (EcoRI: 5′-CGG AAT TCG AGG CCT ACC AGA AAC-3′; GUPA4 and SalI/Tag: 5′-TGA GTC GAC TAC TGT ACA GAA CAA TGG TC-3′; GUPA3). The PCR products were cloned into EcoRI/SalI site of pGAD424 (CLONTECH) downstream of the Gal4 activation domain. The clone was named pGAD1.6 (see Figs. 7 and 8). A switch in yeast colonies during vector restrictions was also carried out by subcloning procedure. The PCR was performed in the presence of recombinant Pfu polymerase (Stratagene) at a low cycle number (10 cycles) to ensure high fidelity reading. The insertions were entirely sequenced to confirm the correct reading frame and sequence. Sequencing was performed by the Molecular Biology facility at Stanford University using the ABI PRISM dye terminator cycle sequencing ready reaction kit with AmpliTaq DNA polymerase, FS (Perkin-Elmer).

The fusion proteins were expressed in the yeast strain Y190 (CLONTECH) of *S. cerevisiae*: Ura 3-52, his 3-200, lys 2-801, ade 2-101, trp 1-901, leu 2-3, gal4Δ, gal80Δ, cyh2, LYS2-GALI1/¿AII1/¿XIS1/¿XIS1, HIS3:GALI1/¿AII1/¿XIS1/¿XIS1, URA3:GALI1/¿AII1/¿XIS1/¿XIS1/¿XIS1. Intrinsic transcriptional autoactivation of pGBT9/0.4 was determined by transformation alone or cotransformation with control plasmids containing the Gal4 transcriptional activation domain alone (pGAD424) or with other in-frame cDNAs such as the SV40 large T-antigen (pTD1, CLONTECH) or the isoform of 14–3-3 (pGADG1/H/4–3). The pGAD1.6 construct was tested with the Gal4 binding domain alone (pGBT9) and murine p53 (pVAc-1, CLONTECH) or an apoptotic protein (pGBP7/BAD) expressed with the Gal4 binding site. No background for β-galactosidase activity detection was observed after overnight incubation at 30 °C. No background for β-galactosidase expression was observed for the constructs after 5 days of incubation at 30 °C on selection medium (SD-medium, yeast nitrogen base without amino acids 6.7 g/liter, pH 5.8, agar 18 g/liter, adenine 10 mg/liter, 2% dextrose, and 50 mM 3-amino-1,2,4-triazol).

**Constructions Deleting the Subdomains Present in UCR2—** Subdomains present in the putative autoinhibitory domain of rPDE4D3 (Fig. 1) were progressively deleted to map the site of interaction between inhibitory and regulatory domain. Three constructs were generated by PCR amplification or restriction digestion of the full-length cDNA. Construct PDEtruA (see Fig. 8) containing amino acids 164-end and missing the first α-helical region of UCR2 was generated by PCR using the full-length cDNA of rPDE4D3 as a template and forward and reverse primers with incorporated restriction sites (EcoRI: 5′-CGG AAT TCG TCT CCA TCG AGG AGG CCT ACC AGA AGG AGA-3′; GUPA3) to clone PDEtruB an additional amphipathic helix is deleted (amino acids 179-end; see Fig. 8). A forward primer (EcoRI: 5′-CGG AAT TCC TCA CCC ACC TCT TGT-3′; GP2) and reverse primer (SalI/Stop; GUPA3) was used. In construct PDEtruC (Fig. 8) containing amino acids 214-end, the epitope recognized by K116 was removed. In this latter case the forward primer was GP2 (EcoRI: 5′-CGG AAT TCC TCA CCC ACC TCT TGT AGG AGG AGG CCT ACC AGA AGA-3′; GUPA3) whereas the reverse primer again was GUPA3. The PCR products were purified by agarose gel electrophoresis and extracted using a gel extraction kit (Qiagen, Chatsworth, CA). The final constructs were obtained by following a three-point ligation strategy using two fragments for subcloning. The fragments containing the truncations in UCR2 were digested with EcoRI and XhoI and again purified by gel electrophoresis. The second fragment was cloned into the restriction digestion of the full-length cDNA of rPDE4D3 with XhoI and BglII. Both fragments were cloned into pGAD424 cut by EcoRI and BglII. Positive clones were sequenced up to the XhoI cloning site to confirm the correct reading frame and sequence of the PCR fragment. The complete inserts were also subcloned into the EcoRI site of pGBP7.

**β-Galactosidase Activity Assays—** For qualitative detection of β-galactosidase activity in the transformants, the colony-lift-filter assay was carried out according to the manufacturer’s protocol (CLONTECH). Blue colonies were developed after incubation at 30 °C.

When the measurement of the interaction required quantification, liquid culture β-galactosidase assay with o-nitrophenyl-β-d-galactopyranoside (ONPG) as substrate was used. Yeast colonies expressing the different constructs were inoculated into 5 ml of SD-medium lacking Leu, Trp, and His and were grown overnight at 30 °C with shaking. The overnight culture (2 ml) was inoculated into 8 ml of yeast-peptide (YPD) liquid medium and grown at 30 °C until the A600 of 1 ml reached 0.5–0.8. The A600 of 1 ml of the culture was measured and recorded. An aliquot (1.5 ml) of culture was taken and centrifuged at 14,000 rpm for 1 min. The supernatant was discarded, and the pellet was washed and resuspended in 1.5 ml of Z buffer containing 60 mM NaHPO4, 40 mM Na2HPO4, 10 mM KCl, 10 mM MgSO4. The solution was centrifuged again at 14,000 rpm for 1 min, and processed as described above.

**References**

1. S.-L. C. Jin, W.-P. Kuo, and M. Conti, submitted for publication.
and the pellet was resuspended in 0.3 ml of Z buffer. The cell suspension (0.1 ml) was placed into a fresh microcentrifuge tube and frozen in liquid nitrogen. The frozen cell was thawed in 37 °C water bath for 1 min. A 0.7 ml aliquot of Z buffer containing 0.2% β-mercaptoethanol was added to the reaction tubes, and then 0.16 ml of ONPG in Z buffer was added. ONPG was dissolved in Z buffer at 4 mg/ml for 1–2 h. After adding ONPG, the incubation time was measured to calculate β-galactosidase activity units. The reaction mixture was incubated at 30 °C until a yellow color developed. To stop the reaction, 0.4 ml of 1 M Na2CO3 was added. The reaction tube was centrifuged for 10 min at 14,000 rpm to pellet cell debris. The supernatant was removed with care, and absorbance was measured at 420 nm. A unit of β-galactosidase was defined as the amount that hydrolyzes 1 μmol of ONPG to p-nitrophenol and p-galactose/min (30). β-Galactosidase units were calculated as follows: β-galactosidase units = 1,000 × A540/(t × V × A0.05). where t = elapsed time (in min) of incubation, V = volume of the culture used for the assay, and A0.05 = A540 of 1 ml of culture.

 Yeast Mating—Yeast strain Y187: MATα, ura3–52, his3–200, ade2–101, trp1–901, leu2–3, 112, gal4Δ, metα, gal80Δ, URA3::GAL1-IAAS, GAL1-IAAT, lacZ was single-transformed with pGBT9, pGBT/0.4, pGAD/T1.6, pGAD/2kb, and p Va3 as the positive control and selected for Trp+ transformants. The yeast strain, HP76c, was single-transformed with the AD-vector pGAD424 alone, with pGAD/0.4, pGAD/1.6, pGAD/2kb, and the control plasmid pTD1. Transformants were propagated on SD-Leu. Single positive clones for Trp and Leu were separately streaked in strips on the corresponding SD-medium and incubated for 3–4 days at 30 °C. The different transformants of Trp+ and Leu+ were replica-plated in a grid pattern on YPD medium and incubated for 1 day at 30 °C. Colonies of diploid cells were replica-plated on SD-medium lacking Leu, Trp, and His. His+ colonies were observed 6 days after incubation at 30 °C. LacZ expression was detected using the β-galactosidase colony-lift-filter assay.

 FRTL-5 Cell Cultures—Cells were grown in Coon’s F-12 medium supplemented with 5% calf serum, TSH (1 millunit/ml), insulin (10 μg/ml), and transferrin (5 μg/ml) as previously reported (growth medium) (11). At 70–80% confluence, growth medium was removed, cells were rinsed twice with Hank’s buffered saline solution, and quiescent medium (Coon’s F12 medium with 0.1% bovine serum albumin without insulin or TSH) was added to the cells. After 3 days in quiescent medium, cells were rinsed, and fresh medium was added to the cultures. After a 1-h equilibration, TSH (30 nm) or forskolin (100 μM) was added to some plates, whereas vehicle was added to the control plates. In those experiments where rolipram was used, the inhibitor was added at a final concentration of 10 μM for 15 min before the TSH addition. At the end of the treatment, cells were rinsed several times with PBS and harvested in homogenization buffer containing 20 mM Tris, pH 8.0, 10 mM NaF, 1 mM EDTA, 0.2 mM EGTA, and 5.4 mM β-mercaptoethanol with protease inhibitors including 0.01 mg/ml aprotinin, 0.7 μg/ml pepstatin, 0.2 μg/ml leupeptin, 0.01 μg/ml trypsin inhibitor, and 50 μM benzamidine. Supernatants were prepared by microcentrifugation at maximum speed for 30 min at 4 °C. We have previously demonstrated that most of the PDE activity recovered in the supernatant of FRTL-5 cells corresponds to PDE4D3.

 Controlled Proteolysis of PDE4D3—Lys-C was used at a concentration of 0.1 μg/μl in buffer containing 50 mM Tricine, pH 8.0, 10 mM EDTA. Crude extracts of PDE4D3 from FRTL-5 cells were prepared in homogenization buffer without protease inhibitors. The crude enzyme preparation was incubated with Lys-C (Sigma) in a ratio of 1:150 (w/w) at room temperature for 10 min. At the end of the incubation, the reaction was stopped by adding excess bovine serum albumin (1 mg/ml), and aliquots of the reaction products were immediately used for the PDE assay. For the SDS-PAGE analysis of the Lys-C cleavage products, purified recombinant PDE4D3 was partially digested with Lys-C in a ratio of 1:100 (w/w) at room temperature for 5 min. The reaction was stopped by adding SDS sample buffer and boiling for 5 min. SDS-PAGE (10% gels) was used to separate the fragments was generated by Lys-C digestion.

 Purification of the Recombinant PDE4D3—The recombinant PDE4D3 was expressed in SF-9 cells using baculovirus as previously reported (12). The monoclonal antibody (M3S1) was used to purify the recombinant PDE4D3. After incubation for 3 days at 27 °C, SF-9 cells were centrifuged at 100 × g for 10 min, and supernatant was discarded. The pellet was homogenized in homogenization buffer with protease inhibitors and centrifuged at 188,000 × g for 40 min at 4 °C in an Ultracentrifuge (SW41Ti rotor, Beckman Coulter, Inc., Fullerton, CA). The supernatant was diluted 1:1 with sample binding/washing buffer (10 mM Tris-Cl, pH 7.6) and then applied to a M3S1 affinity column. The column was rinsed with washing buffer until base-line absorbance at 280 nm was reached. The enzyme was eluted with 0.1 M glycine, pH 2.8, and the 500-μl fractions were neutralized with 1 M Tris-Cl, pH 10. The purified PDE4D3 was stored in the presence of 30% ethylene glycol at −20 °C for further analysis. The details of the preparation of the column and purification procedure were previously reported in Salanova et al. (31).

 The purified PDE4D3 from SF-9 cells had a specific activity of approximately 3–5 μmol/min/mg and showed a single 93-kDa band on SDS-PAGE. The properties of the purified recombinant PDE4D3 are similar to those of native PDE4D3 from FRTL-5 cells. The Kα for cAMP is ~1.5 μM for both enzymes. They both are inhibited by rolipram with IC50 values of ~10–30 nM.

 Incubation of PDE4D3 with Antibodies—Crude PDE4D3 extracts from FRTL-5 cell or purified recombinant PDE4D3 were diluted in 50 mM Tris HCl, pH 7.4, 0.1% bovine serum albumin. The antibodies used included the polyclonal antipeptide antibodies (K111, K115, and K116) and the monoclonal antibody (M3S1) against PDE4D and the polyclonal antibody against the carboxyl terminus domain of PDE4A (AC55). The properties of these antibodies have been previously described (32).

 Antibodies were added at final concentrations ranging between 0.05 to 25 μM incubation mixture. The samples were incubated for 1 h on ice with occasional mixing. At the end of the incubation, aliquots of the incubation were used for the PDE assay. The PDE activity was measured following the method of Thompson and Appleman (33) with minor
Although activation was less pronounced than that with native
experiments were repeated with a purified recombinant PDE4D3. 
phosphorylation and activation by proteolysis are not additive. 
treated cells were comparable, indicating that activation by 
recovered after proteolysis from quiescent and forskolin-
were subjected to incubation with Lys-C, only a marginal in-
specific activity 3-fold higher than those of preparations de-
with Lys-C, at room temperature for 10 min in the absence or presence of protease inhibitors including 0.01 mg/ml 
activity recovered after proteolysis was completely inhibited by 
the result of the proteolysis because inclusion of protease in-
modifications (34). In those experiments where antibody binding was 
blocked with the peptide used as an immunogen, the peptide was added 
at a final concentration of 5 μg/ml.

RESULTS

Limited Proteolysis of Native and Recombinant PDE4D3—In 
the initial experiments, crude soluble preparations for FRTL-5 
cells were the source of PDE4D3, because this is the enzyme most readily activated by phosphorylation. This PDE4D3 is the predominant form recovered in the soluble fraction of FRTL-5 cells as judged by DEAE-ion exchange chromatography and Western blot analysis (data not shown) (11, 35). The crude PDE4D3 preparation from quiescent FRTL-5 cells was incubated with the endoproteinase Lys-C for times up to 60 min at room temperature, and PDE activity was measured. This treatment caused a time-dependent increase in PDE activity (Fig. 2) with a greater than 4-fold increase observed after a 30-min incubation. A longer incubation time with Lys-C caused an overall reduction in activity, indicating a proteolytic inactivation of the catalytic domain. This increase in PDE activity is the result of the proteolysis because inclusion of protease inhibitors completely blocked the PDE activation (Fig. 3). The activity recovered after proteolysis was completely inhibited by 10 μM rolipram (data not shown), confirming that a PDE4 is indeed affected by the digestion and that the treatment does not uncover unrelated cryptic PDE activity.

Together with PDE4D3 preparations from quiescent cells, crude preparations from cells incubated with forskolin for 30 min were used. Forskolin produces phosphorylation of the PDE4D3, thus yielding an activated enzyme. The enzyme preparations derived from forskolin-treated FRTL-5 cells had a specific activity 3-fold higher than those of preparations derived from quiescent cells (Fig. 2). When these preparations were subjected to incubation with Lys-C, only a marginal increase in PDE activity was observed (Fig. 2). The activities recovered after proteolysis from quiescent and forskolin-
treated cells were comparable, indicating that activation by phosphorylation and activation by proteolysis are not additive.

To confirm the observation with crude FRTL-5 extracts, experiments were repeated with a purified recombinant PDE4D3. Although activation was less pronounced than that with native enzyme, the recombinant PDE4D3 expressed in insect cells was also activated by partial proteolysis (PDE activity, μmol/min/mg of protein: control = 3.1 ± 0.4; Lys C-treated: 6.2 ± 0.2). The activation by partial proteolysis was because of an increase in Vmax without significant changes in Km for cAMP (Km without proteolysis = 1.15 μM, Km after proteolysis = 1.14 μM). To determine the site of cleavage, the recombinant enzyme was incubated with Lys-C, and the proteolytic products were separated by SDS-PAGE followed by Western blot analysis with antibodies that recognize two epitopes in the PDE4D3 (Fig. 4). Lys-C partial digestion produced a major fragment of 59 kDa recognized by an antibody (M351) specific for the carboxyl terminus of PDE4D3 and a 34-kDa fragment that was recognized by the K116 antibody (Fig. 4). An additional minor product of 69 kDa was often observed (Fig. 4). These data demonstrate that partial proteolysis yields a major fragment containing the catalytic domain but lacking the autoinhibitory domain.

Activation of PDE4D3 by Antibodies against the Putative Inhibitory Domain—Several antisera (K111, K115, K116) were generated against the peptide sequence VSEYSNTFLD-KQHEVEIPSPT in the putative inhibitory domain of PDE4D3 (36) (Fig. 1). During immunoprecipitation experiments using these antibodies, it was observed that the PDE activity recovered often exceeded the input activity (36); therefore, the possibility that this increase in activity is because of binding and activation by the antibody was reevaluated.

Peptide antisera from three different rabbits were incubated at 4 °C for 1 h with crude PDE4D3 preparations from quiescent FRTL-5 cells. At the end of the preincubation, the PDE activity was measured. All the antisera recognizing the putative auto-

inhibitory domain (K111, K115, K116) produced a 3- to 4-fold increase in PDE activity, whereas normal rabbit serum or a monoclonal antibody against the carboxyl terminus of PDE4D3 (M351) were without effect (Table I). An antiserum against the carboxyl terminus of the unrelated PDE4A (AC55) was equally ineffective (Table I). Although a trace of PDE activity was detectable in the antiserum used, this was less than 1% of the total activity measured (data not shown). Furthermore, inclusion of the peptide used as an immunogen in the incubation completely prevented the antibody activation (Fig. 5), indicating that the interaction of the antibody with PDE4D3 is required for the activation. The activation was proportional to the

![Fig. 2. Activation of PDE4D3 by partial digestion with endo-
protease, Lys-C. PDE4D3 extracts were prepared from FRTL-5 cells 
incubated in the absence or presence of forskolin (100 μM). At the end of 
the 30-min treatment, cells were harvested, and crude soluble extracts 
of PDE were prepared as detailed under “Experimental Procedures.” 
Extracts were incubated with Lys-C (0.5 μg/ml) at room temperature 
for the times reported in the abscissa. The proteolytic digestion was 
terminated by adding 0.1% bovine serum albumin, and the PDE activity 
was assayed at 34 °C for 5 min. A representative experiment of the 
three performed is reported.

![Fig. 3. PDE4D3 activation by Lys-C treatment is blocked by 
protease inhibitors. Crude extracts of PDE4D3 were prepared from 
FRTL-5 cells as detailed under “Experimental Procedures.” Extracts 
were incubated with Lys-C at room temperature for 10 min in the 
absence or the presence of protease inhibitors including 0.01 mg/ml 
aprotinin, 0.7 μg/ml pepstatin, 0.2 μg/ml leupeptin, 0.01 μg/ml trypsin 
inhibitor, and 50 μM benzamidine. The proteolytic digestion was 
terminated by adding 0.1% bovine serum albumin, and the PDE activity 
was assayed at 34 °C for 5 min. A representative experiment of the 
three performed is reported.](http://www.jbc.org/Downloaded from http://www.jbc.org/ on July 25, 2018)
amount of antibody used (Fig. 6). The possibility that the activation is because of stabilization of the enzyme was excluded by a time course study. In the absence of the antibody, the activity was not significantly reduced after a 60-min incubation (data not shown). That the effect of the antisera is not because of partial proteolysis of the PDE was further indicated by immunoprecipitation and Western blot analysis. The 93-kDa intact enzyme was the form recovered (data not shown). In addition, increased activity was recovered in the pellet of the immunoprecipitation (data not shown), again indicating that the autoinhibitory domain has not been cleaved. Similar results were obtained with purified IgG from the antisera used (data not shown), thus decreasing the possibility of proteolytic activation by serum proteases. The activation by the antibody causes an increase in \(V_{\text{max}}\) of the enzyme without affecting the \(K_{m}\) for cAMP (\(K_{m}\) without K116 = 1.4 \(\mu\)M, \(K_{m}\) with K116 = 1.5 \(\mu\)M). In addition, the antibody binding caused a decrease in Mg\(^{2+}\) requirements for catalysis (without K116 = 22.9 \(\pm\) 1.5 mM; with K116 = 2.4 \(\pm\) 1.6 mM; mean \(\pm\) S.E., \(n = 3\)). Finally, the activity of the enzyme bound to the antibody was completely inhibited.

**TABLE 1**

| Antiserum | PDE activity (pmol/min/mg) |
|-----------|--------------------------|
| NRS       | 89 \(\pm\) 5             |
| K111      | 93 \(\pm\) 10            |
| K115      | 383 \(\pm\) 20           |
| K116      | 344 \(\pm\) 31           |
| K116      | 340 \(\pm\) 22           |
| AC55      | 93 \(\pm\) 4             |
| M3S1      | 90 \(\pm\) 5             |

The activation of PDE4D3 by the antibody against the autoinhibitory domain requires binding of the antibody with the PDE. Crude preparations of PDE4D3 were incubated with K116 antibody (10 \(\mu\)g/ml incubation mixture) in the presence or absence of 5 \(\mu\)g/ml peptide 2224. At the end of the preincubation, the PDE activity was assayed at 34 °C for 5 min.

**FIG. 4.** Partial digestion of the recombinant PDE4D3 with Lys-C. A, schematic representation of the PDE4D3 domain with a potential site for Lys-C cleavage. Domains are represented with boxes connected by lines. R is the regulatory domain, and C is the catalytic domain. The putative autoinhibitory domain is a filled box (I). The position of the epitopes recognized by the two antibodies, K116 and M3S1, are highlighted by bars. B, SDS-PAGE analysis of partial proteolytic products of PDE4D3. The fragments of the partially digested enzyme were separated by 10% SDS-PAGE, and then the gel was transferred onto the polyvinylidene difluoride membrane. Two different antibodies, K116 and M3S1, were used for the Western blot analysis. The calculated mass of the two major proteolytic fragments were \(\approx\)34 kDa and 59 kDa.

**FIG. 5.** The activation of PDE4D3 by the antibody against the autoinhibitory domain requires binding of the antibody with the PDE. Crude preparations of PDE4D3 were incubated with K116 antibody (10 \(\mu\)g/ml incubation mixture) in the presence or absence of 5 \(\mu\)g/ml peptide 2224. At the end of the preincubation, the PDE activity was assayed at 34 °C for 5 min.

**FIG. 6.** Activation of phosphorylated and dephosphorylated PDE4D3 by the K116 antibody. Crude extracts of PDE4D3 were prepared from FRTL-5 cells treated with vehicle only (■, ●, ●), with rolipram (10 \(\mu\)M) and TSH (10 \(\mu\)M) (△), or with forskolin (10 \(\mu\)M) (▲). At the end of the incubation, the extracts were harvested, and crude PDE4D3 extracts were prepared as described under "Experimental Procedures." Aliquots of the extracts were incubated for 1 h on ice with increasing concentrations of K116 (■, △, ●), preimmune serum (○), or M3S1 (●). At the end of this incubation, the PDE activity was measured as described under "Experimental Procedures." A representative experiment of the three performed is reported. AB, antibody.
by rolipram (data not shown). However, the antibody binding caused a decrease in the IC50 for rolipram (PDE4D3 without K116 = 25 ± 5 nM; PDE4D3 with K116 = 320 ± 76 nM; mean ± S.E., n = 3).

To determine whether activation of PDE4D3 by antibody binding to the putative autoinhibitory domain is additive to activation by phosphorylation, the antibody incubation was repeated with extracts from cells treated with either TSH plus rolipram or forskolin (Fig. 6). The activation of PDE4D3 by antibody binding was not additive to the activation by phosphorylation. In three experiments performed, activation of the enzyme from quiescent cells was 2.78 ± 0.78-fold. The activation was reduced to 1.59 ± 0.06-fold and 1.12 ± 0.05-fold for the enzymes prepared from TSH + rolipram and forskolin-treated cells, respectively. That the antibody binds equally well to the phosphorylated enzyme was confirmed by immunoprecipitation and Western blot analysis. In both cases K116 recognized phosphorylated PDE4D3 as well or better than the nonphosphorylated enzyme (data not shown). In addition, cell-free experiments where the enzyme was phosphorylated with PKA and then incubated with K116 confirmed that the activation by antibody and by phosphorylation is not additive (data not shown).

Interaction between the Phosphorylation Domain and the Autoinhibitory Domain—The above data support the hypothesis that an inhibitory domain is present upstream from the catalytic domain of PDE4D. Removal or disruption of the interaction of this domain with the contiguous catalytic or regulatory domains causes activation of catalysis. Because activation by proteolysis or by interaction with the antibody is not additive to activation by phosphorylation, we hypothesized that phosphorylation at the amino terminus of PDE4D regulates the catalytic domain indirectly by affecting the autoinhibitory domain. To test this hypothesis, the yeast two-hybrid system was used to study the interaction between the regulatory, the autoinhibitory, and catalytic domains.

Constructs containing the regulatory domain and the autoinhibitory domain as well as the catalytic domain were subcloned as fusion proteins with the DNA binding and activation domain of Gal-4 and tested in the yeast two-hybrid interaction assay. Strong interaction was detected between the regulatory, and catalytic domain was first observed with the alignment of the regulatory domain and the carboxyl terminus domain (Fig. 7). This interaction was independent of the vector used and was evident when yeast growth or the β-galactosidase activity was used for the assay (Fig. 7). Conversely, constructs containing only the catalytic domain or the catalytic domain and the carboxyl terminus domain did not interact with the regulatory domain (Fig. 7). This indicated that the regulatory domain interacts directly with the autoinhibitory domain. A further deletion study where the putative autoinhibitory domain was progressively truncated indicated that the amino terminus portion of the inhibitory domain is responsible for the interaction with the regulatory domain (Fig. 8). Finally, an additional strong interaction between the autoinhibitory domain and the catalytic domain was detected with this yeast two-hybrid assay (see Fig. 9).

Because the regulatory domain is the target for phosphorylation by PKA, a change in charge in this domain may affect the interaction with the autoinhibitory domain. This possibility was tested by substituting the Ser24 in the regulatory domain to Asp or to Ala. Ser24 mutation to Asp increased the interaction between the regulatory domain and the autoinhibitory domain (Fig. 9) when compared with the wild type regulatory subunit (Fig. 9), suggesting a tighter interaction between the two domains may occur. Conversely, when Ser24 was mutated to Ala, a significant decrease of the interaction was observed (Fig. 9).

DISCUSSION

Using several independent strategies, we demonstrated the presence of an inhibitory domain in the PDE4D protein, which maintains the catalytic domain in a less active state. Originally proposed on the basis of deletion mutagenesis (22), this hypothesis is now substantiated by the activation of a PDE4D3 variant by partial proteolysis and by the interaction with an antibody that binds to an epitope in this domain. That the function of this inhibitory domain is physiologically relevant is supported by the finding that activation by phosphorylation and removal of the inhibitory constraint are not additive. These findings, as well as the interaction between the inhibitory and the regulatory domains detected by the yeast two-hybrid system, provide the basis for a model of PDE4D3 activation where phosphorylation of the regulatory domain relieves an inhibitory constraint on catalysis (Fig. 10).

A region of homology in PDE4 sequences upstream of the catalytic domain was first observed with the alignment of PDE4D1 and PDE4B2 sequences (ratPDE3 and ratPDE4 in the original publication) (19). Subsequent studies have shown that this conserved region is also present in PDE4A and PDE4C and have defined this region as the upstream conserved region 2 (UCR2) (18). Nested deletion mutagenesis of 97 amino acid stretch between Ala48 and Lys145 in PDE4D1 yielded an enzyme with a 3- to 4-fold increase in Vmax without any significant change in the Km for cAMP (22). Similarly, a truncation up to Pro120 in PDE4D1 expressed in bacteria as a fusion protein showed a 6-fold increase in PDE activity (23). Furthermore, the naturally truncated form PDE4D2 with the putative initiation methio-
Partial proteolysis of the recombinant PDE4D3 generates two fragments that migrate on SDS-PAGE with an apparent mobility of 59 and 34 kDa, respectively. Because the 34-kDa fragment is recognized by the antibody against the inhibitory domain but not by two antibodies against the carboxyl terminus domain, we can conclude that it cannot encompass the entire catalytic domain, and therefore, it is not catalytically active. Conversely, the 59-kDa fragment cross-reacts with the antibodies against the carboxyl terminus of PDE4D but not with the antibody corresponding to the inhibitory domain. Thus, the Lys-C cleavage separates the catalytic domain from the putative inhibitory domain, the most likely cleavage site being the stretch of Lys present between residue 217 and 222 in PDE4D3. The 59-kDa proteolytic fragment is 3–4 times more active than the full-length enzyme because the inhibitory domain has been removed.

Our data on partial proteolysis are in agreement and extend an earlier observation by Sanwal and co-workers (13, 38). In L6 myoblasts, dibutyryl cAMP increases the PDE activity expressed in these cells. One of the three forms of PDE separated by gel filtration chromatography is activated by incubation with snake venom proteases or with chymotrypsin. In addition, stimulation with dibutyryl AMP rendered the enzyme less sensitive to activation with snake venom. Although the nature of this enzyme was not described at that time, we believe that this form corresponds to PDE4D3, which is activated by phosphorylation through the cAMP pathway. As we have shown for the form expressed in thyroid cells, the form described in myoblasts is activated by snake venom proteolysis, and the activation is not additive to the activation by phosphorylation.

The activation of PDE4D3 by antibody binding or proteolysis has the same characteristics as the activation induced by phosphorylation of the enzyme. An increase in $V_{\text{max}}$ without concomitant changes in $K_m$ occurs when the enzyme is phosphorylated (12, 39), when it interacts with K116, or when it is activated by proteolysis. In addition, the changes in $Mg^{2+}$ requirements for catalysis are similar in the three conditions. These findings support the idea that similar changes in conformation of the catalytic domain take place in the enzyme after phosphorylation, proteolysis, or antibody binding, suggesting that modification of the interaction between I and C mediate the activation by phosphorylation. Instead, the findings with rolipram are difficult to accommodate in our model. Proteolysis or binding to K116 cause a decreased affinity for rolipram, a finding predictive from the deletion mutagenesis data. It is established that PDE4s exist in two conformers that recognize the inhibitor rolipram with different affinities (40–42). Removal of a region in PDE4A corresponding to the inhibitory domain causes a loss of high affinity rolipram binding (24). On the contrary, phosphorylation of PDE4D causes an increase in affinity for rolipram and related compounds or has no effect on the affinity state, a view that we favor. In a recent report, we have summarized the arguments in favor of the idea that phosphorylation increases the activity of an enzyme already in a high affinity conformation rather than causing a transition between the low and high affinity (12). We should point out that enzyme activation and increase in rolipram affinity do not always go together. In addition to the observation reported herein, Hoffmann et al. (43) report data that mutagenesis of Ser$^{54}$ causes an increased affinity for rolipram without increasing catalytic activity. Thus, it is possible that changes in rolipram affinity and activation of catalysis are two distinct phenomena.

By using the yeast two-hybrid system, we have detected an interaction between the regulatory domain and the amino terminus of the conserved domain UCR2. The interaction is increased when the Ser$^{54}$ is mutated to Asp, which indicates that a negative charge in that region is compatible with, or reinforces, the interaction between the regulatory and inhibitory

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3 M. Conti, unpublished observation.
The decrease or loss of the interaction after Ser 54 mutation to Ala can be explained as follows. It is possible that the removal of the hydroxyl group of Ser54 disrupts a hydrogen bond, weakening the interactions between the two domains, which would be in agreement with a model proposed by Houslay and co-workers (43) to explain the site-directed mutagenesis of Ser54 to different residues (43). Alternatively, it is possible that Ser 54 is phosphorylated when the amino terminus domain is expressed as a fusion protein in yeast. The mutation of Ser54 to Ala then prevents phosphorylation, thereby weakening or obliterating the interaction with the inhibitory domain. Although at the present time we cannot distinguish between the two possibilities, our data reinforce the conclusion that Ser54 plays a crucial role in the interaction between the two domains and the introduction of a negative charge that mimics phosphorylation does not alter, and possibly enhances, this interaction.

Collectively, the above findings can be accommodated in the following model for PDE4D3 activation by phosphorylation. In the dephosphorylated enzyme, a subdomain within UCR2 exerts an inhibitory constraint on the catalytic domain. Upon phosphorylation of the regulatory domain, interactions between the regulatory domain and the amino terminus of UCR2 are modified, perhaps strengthened, thus altering the interaction between the inhibitory and catalytic domains. These changes in conformation release the constraint exerted on the catalysis, thus increasing the rate of cAMP hydrolysis (Fig. 10).

It should be emphasized that our findings do not exclude the possibility that the inhibitory domain coincides with the binding site for an allosteric regulator of the PDE. Proteolysis of the PDE may cause both the removal of the putative regulator binding site and activation of catalysis. Similarly, the antibody against the autoinhibitory domain may simply displace an allosteric regulator from its binding site. Although there is no evidence for a factor that binds and inhibits PDE4D3, there is a precedent for an allosteric regulator of other PDEs forms. For instance, the activity of the α and β subunits of the retina PDE6 is suppressed by the inhibitory γ subunit (20, 44). Furthermore, we cannot exclude that deletions of UCR2 in the PDE4D3 alter the quaternary structure of the enzyme. Both PDE4D1 and PDE4D3 behave as dimers or tetramers (23).4 In conflict with this hypothesis, however, is the finding that regions important for dimerization or oligomerization have been tentatively mapped at the carboxyl terminus of the protein and not at the amino terminus, where the inhibitory domain is located (23).

In summary, the data presented above indicate that a region between Met167 and Pro212 in PDE4D3 is responsible for holding the catalytic domain in a less active conformation. On the basis of our data we propose the model that the phosphorylation causes activation by relieving an inhibitory constraint on the catalytic domain. As suggested by others for the calmodulin regulation of PDE1 (27), the putative inhibitory domain may act as a pseudosubstrate, inactivating the enzyme in a compet-

4 G. Pahlke, unpublished observation.
itive manner. The elucidation of the three-dimensional structure of PDE4D3 will certainly answer these questions.

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