ERK2 Mitogen-activated Protein Kinase Binding, Phosphorylation, and Regulation of the PDE4D cAMP-specific Phosphodiesterases

THE INVOLVEMENT OF COOH-TERMINAL DOCKING SITES AND NH₂-TERMINAL UCR REGIONS

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The cAMP-specific phosphodiesterase family 4, subfamily D, isoform 3 (PDE4D3) is shown to have FQF and KIM docking sites for extracellular signal-regulated kinase 2 (ERK2) (p42MAPK). These straddle the target residue, Ser579, for ERK2 phosphorylation of PDE4D3. Mutation of either or both of these docking sites prevented ERK2 from being co-immunoprecipitated with PDE4D3, ablated the ability of epidermal growth factor to inhibit PDE4D3 through ERK2 action in transfected COS cells, and attenuated the ability of ERK2 to phosphorylate PDE4D3 in vitro. The two conserved NH₂-terminal blocks of sequence, called upstream conserved regions 1 and 2 (UCR1 and UCR2), that characterize PDE4 long isoforms, are proposed to amplify the small, inherent inhibitory effect that ERK2 phosphorylation exerts on the PDE4D catalytic unit. In contrast to this, the lone intact UCR2 region found in PDE4D1 directs COOH-terminal ERK2 phosphorylation to cause the activation of this short isoform. From the analysis of PDE4D3 truncates, it is suggested that UCR1 and UCR2 provide a regulatory signal integration module that serves to orchestrate the functional consequences of ERK2 phosphorylation. The PDE4D gene thus encodes a series of isoenzymes that are either inhibited or activated by ERK2 phosphorylation and thereby offers the potential for ERK2 activation either to increase or decrease cAMP levels in cellular compartments.

CAMP provides a key second messenger system of major regulatory importance in all cell types. Its hydrolysis provides an important means whereby cAMP levels are regulated (1). This is achieved by members of the large, multigene family of cyclic nucleotide phosphodiesterases (PDEs) (2–6). Members of the PDE4 enzyme family specifically hydrolyze cAMP and are selectively inhibited by the compound, rolipram (6). These enzymes appear to be of particular regulatory significance in immune/inflammatory cells, and in the central nervous system, as PDE4-selective inhibitors, they serve as potent anti-inflammatory agents and exhibit anti-depressant properties (6–9). A “signature” feature of these enzymes is the presence of two highly conserved blocks of sequence, called UCR1 and UCR2 (3, 6, 10). The UCR regions are located in an analogous position, NH₂-terminal to the catalytic unit, to the pairs of regulatory domains found in other PDE families such as the calmodulin-binding domains found in PDE1 enzymes and the cGMP-binding domains found in PDE2 and PDE5 enzymes (2, 4, 11, 12). Each of the four PDE4 genes encodes multiple isoforms that are generated by alternate mRNA splicing or the use of alternative promoters. Each of the various isoforms encoded by a single PDE4 gene falls into two categories (6), namely “long” PDE4 isoenzymes that exhibit both UCR1 and UCR2 and “short” isoenzymes that lack UCR1 and have either an intact or NH₂-terminally truncated form of UCR2.

The functional role of UCR1 and UCR2 and the functional consequences of generating short and long isoenzymes remain to be defined. Nevertheless, it has been shown (13, 14) that the long PDE4D3 isoenzyme can be activated through the PKA-mediated phosphorylation of a single serine residue (Ser54) at the start of UCR1. PDE4D3 can also be regulated through inhibitory phosphorylation by the p42MAPK, ERK2 (15), that occurs at a single residue (Ser79) located within the PDE4D3 catalytic unit. These regulatory phosphorylation events appear to be intertwined as ERK2 inhibition of PDE4D3 was ablated by subsequent PKA-mediated phosphorylation (15). This provides a potential feedback regulatory system whereby the ERK2-mediated inhibition of PDE4D3 allows cAMP levels to rise, triggering the subsequent PKA-mediated phosphorylation of PDE4D3 (15). In this regard, PDE4D3 provides a point of cross-talk that links ERK2 activation to the regulation of cAMP signaling. The corollary of this is the ability of PKA to attenuate ERK2 activation through the inhibitory phosphorylation of c-Raf (16–20).

In this study, we demonstrate that PDE4D3 possesses FQF and KIM docking motifs for ERK2 (21–27). These straddle...
Fig. 1. Schematic representation of PDE4 isoforms, constructs, and action of ERK2. Upper panel, shown are UCR1 and UCR2, which are about 58 and 78 amino acids in length, respectively; the site for COOH-terminal ERK2 regulatory phosphorylation; the locations of the proposed KIM and FQF docking sites for ERK2; and the PKA phosphorylation-tag site in UCR1. Lower panel, a schematic for the PDE4 constructs used in this study. These are the long PDE4D3 isoform (28) with UCR1 located at residues 51–109 and UCR2 at residues 134–212; (UCR1+UCR2+CAT)4D, which reflects the common region found in all long PDE4D isoforms; the PDE4D1 short isoform that lacks UCR1; (UCR2+CAT)4D, which lacks both UCR1 and any unique NH2-terminal region; the PDE4D2 short isoform whose UCR2 is truncated of its first 32 residues; and (CAT)4D, which represents the PDE4D catalytic region and extreme COOH terminus only. 

In all cases, a sequence corresponding to the SVS glycoprotein epitope was added immediately downstream from the last native codon of the PDE to encode a carboxyl-terminal fusion (15). The native PDE4D stop codon was removed in this process, but a synthetic stop codon was placed immediately downstream from the epitope sequence (Fig. 1).

Western Blot Analysis—Immunoblotting of the PDE4D species and ERK2 phosphorylation was performed as described before by us using ~20 μg of protein samples with detection using an anti-SVS mAb, either mAbs or polyclonal antibodies specific for PDE4D enzymes, and an ERK2-specific mAb (15, 28). The quantification of immunoreactive protein was verified by sequencing before use. Sequencing was performed on an automated sequencing machine (ABI model 373). The monoclonal antibody specific for PDE4D enzymes, and an ERK2-specific mAb (15, 28) was generated against the extreme COOH-terminal 65 amino acids of the PDE4D3 protein that are found in common in all known PDE4D isoforms. This antibody can detect PDE4D3 isoforms truncated at the amino terminus, such as those lacking UCR1 and UCR2. It will detect all known PDE4D isoforms, since their COOH-terminal termini are identical (28). We also employed polyclonal antibodies specific for PDE4D (28). A monoclonal antibody to the vesicular stomatitis virus (VSV) epitope was obtained from Sigma. A monoclonal antibody specific for ERK2 and described previously by us (15) was a kind gift from Dr. M. Harnett (University of Glasgow, United Kingdom).

Generation of COS1 Cell Expression Constructs and Site-directed Mutagenesis of PDE4—The construction of the VSV epistope-tagged PDE4D3 and the various S579A and S579D mutants of PDE4D3 has been described in detail previously by us (15). The various other mutations described in this study were done on this construct using the QuickChange site-directed mutagenesis kit from Stratagene (La Jolla, CA) according to the manufacturer’s instructions. Various pieces of the PDE4D3 cDNA were cloned into the NotI site of the vector pcDNA3 (InVitrogen). In these constructs, the insert was placed under the control of the cytomegalovirus intermediate early gene promoter, pcDNAF4N3VSVF encodes amino acids 16–673 of PDE4D3, which encompasses UCR1, UCR2, and the catalytic region, yielding the construct (UCR1+UCR2+CAT)4D. pcDNAF4N3VSVF encodes amino acids 134–673 of PDE4D3, which encompasses both UCR2 and the catalytic region, yielding the construct (UCR2+CAT)4D. pcDNAF4N3VSVF encodes amino acids 225–673 of PDE4D3, which encompasses the catalytic region only, yielding the construct (CAT)4D.

Ser579, the residue that provides the target for ERK2 phosphorylation in PDE4D3. These docking sites allow PDE4D3 to be co-immunoprecipitated with ERK2 and are essential for ERK2 to regulate the activity of PDE4D3 in intact cells. In addition, we show here, through analysis of PDE4D long and short isoforms as well as truncated species, that UCR1 and UCR2 play a key role in defining the functional response of PDE4D isoforms to ERK2 phosphorylation.

**EXPERIMENTAL PROCEDURES**

Antibodies—A monoclonal antibody to human PDE4D proteins, which does not cross-react with other PDE4 species and which we have described previously (28), was a gift from Sharon Wolda (ICOS Corp., Seattle, WA). This antibody was generated against the extreme COOH-terminal 65 amino acids of the PDE4D3 protein that are found in common in all known PDE4D isoforms. This antibody can detect PDE4D3 isoforms truncated at the amino terminus, such as those lacking UCR1 and UCR2. It will detect all known PDE4D isoforms, since their COOH-terminal termini are identical (28). We also employed polyclonal antibodies specific for PDE4D (28). A monoclonal antibody to the vesicular stomatitis virus (VSV) epitope was obtained from Sigma. A monoclonal antibody specific for ERK2 and described previously by us (15) was a kind gift from Dr. M. Harnett (University of Glasgow, United Kingdom).

**Cell Culture and Transfection—**Cell culture and transfection were performed using the COS1 SV40-transformed monkey kidney cell line maintained at 37 °C in an atmosphere of 5% CO2, 95% air in complete growth medium containing Dulbecco’s modified Eagle’s medium supplemented with 0.1% penicillin/streptomycin (10,000 units/ml), glutamine (2 mM), and 10% fetal calf serum. Details have been described previously by us (15). Briefly, however, COS1 cells were transfected using DEAE-dextran. The DNA to be transfected (5 μg) was mixed and incubated for 15 min with 250 μl of 10 mg/ml DEAE-dextran (Sigma) in PBS to give a DNA-dextran mix. When cells reached 70% confluency in 100-mm dishes, medium was removed, and the cells were given 10 ml of fresh Dulbecco’s modified Eagle’s medium containing 0.1 mM chloroquine and the DNA-dextran mix (250 μl). The cells were then incubated for 4 h at 37 °C. After this period, the medium was removed, and the cells were shocked with 10% Me2SO in PBS. After PBS washing, the cells were returned to normal growth medium and left for a further 2 days before use. For determination of PDE activity, the cells were homogenized in PDE assay buffer, and only when performing immunoprecipitation analyses were the cells scraped and lysed in buffer.

**EGF Stimulation of COS-1 Cells, Harvesting, and Preparation of Cell Extracts for Analysis—**This was performed as described in detail previously by us (15).
fied by three different procedures as described before by us (29). For two of these, SDS-polyacrylamide gels were run with a range of different protein amounts/lane and then subjected to immunoblotting. For routine analysis, immunodetection of the protein was performed by the ECL method (Amersham Pharmacia Biotech). The films were scanned, and optical densities were plotted against sample protein content. Reference lanes were run with various amounts of PDE4D species to calibrate the linear portion of the range. As a confirmation, immunodetection was also performed using a 125I-labeled secondary antibody.

**Immunoprecipitation**—Cell lysates from COS1 cells were prepared as described previously (30). For harvesting, the cells were first washed in PBS before being scraped into lysis buffer (25 mM HEPES, 2.5 mM EDTA, 50 mM NaCl, 50 mM NaF, 30 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, pH 7.5, with added protease inhibitors) and mixed at 4 °C for 20 min. This was cleared by centrifugation before further procedures were carried out. For immunoprecipitation, the lysates (150 μg of protein) were precleared by incubation with 20 μl of protein G-Sepharose 4B fast flow (Amersham Pharmacia Biotech) for 30 min at 4 °C. This was removed by centrifugation and the PDE immunoprecipitated by incubation with the indicated specific antibody for 2 h at 4 °C. The immune complexes were then coupled to 50 μl of protein G-Sepharose with incubation for 1 h at 4 °C, followed by centrifugation, and the pellets were washed with lysis buffer and, finally, PDE assay buffer (20 mM Tris, pH 7.6, with protease inhibitors). Lysates of U937 human monocytic cells were similarly subjected to immunoprecipitation.

**Assay of cAMP PDE Activity**—PDE activity was determined by a modification of the two-step procedure of Thompson and Appelman (31) as described previously by us (32). All assays were conducted at 30 °C with initial rates taken from linear time courses. Activity was linear with added protein concentration. Transfection of COS1 cells led to the novel PDE4 activity comprising >97% of the total cell activity. Mock transfections (vector only) did not alter the endogenous COS cell PDE activity. As a routine, we subtracted the residual endogenous COS1 cell PDE activities found in parallel experiments from those activities found in the PDE4D transfected cells. Untransfected and mock (vector only) transfected COS1 cells exhibited a PDE activity of 10 ± 5 pmol/min/mg protein.

**Phosphorylation in Vitro of PDE4D Isoenzymes**—This was done as described previously (14, 15). PDE4 from 5 × 10⁶ transfected COS1 cells was immunoprecipitated as a complex with protein G-Sepharose. This was incubated for 30 min at 4 °C with 1 volume of phosphorylation buffer (100 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 30 mM β-mercaptoethanol, 10% glycerol) containing 0.1 μCi [γ-32P]ATP (100 MBq/nmol). The reaction was started by the introduction of either 0.5 or 1 unit of activated recombinant ERK2 (Upstate Biotechnology) and allowed to continue for a period of either 5 or 30 min at 30 °C as stated above. The Sepharose was washed four times with 1 ml of PDE buffer and resuspended in PDE buffer for analysis.

**Table I**

| Isoenzyme | KIM Sequence |
|-----------|--------------|
| PDE4D     | VET KKVTSGGVLL | L|
| PDE4C     | VET KKVTSGLVV FQF |
| PDE4B     | VET KKVTSGVV L|
| PDE4A     | VET KKVTSGVV L|
| hElk1     | QKG RKF RD     |
| PDE4D     | LA K RAKDRANL HS|
| PDE4C     | LAQ RVRK L PS  |
| PDE4B     | LTE RGNSV L S  |
| PDE4A     | LQE RGRSSV A L M L|
| hElk1     | L/V RKR/K L     |

We note here that the PDE4D3 catalytic unit contains (Table 1) a putative ERK2 docking site in the form of an FQF motif that is located some 15 amino acids COOH-terminal to the ERK2 phosphorylation site. The other is the KIM motif that has been found approximately 120–150 residues NH₂-terminal to the ERK target Ser/Thr residue. This contains a cluster of positively charged amino acids together with hydrophilic residues and the sequence 120–150 residues NH₂-terminal to the ERK target Ser/Thr residue. This contains a cluster of positively charged amino acids.

**RESULTS**

The Catalytic Unit of PDE4 Isoenzymes Contains Docking Sites for ERK MAP Kinase—We have demonstrated previously (15) that ERK2 phosphorylated the long PDE4D3 isoenzyme at a single site, Ser⁵⁷⁹, that is located within the PDE4D catalytic unit. This caused the inhibition of PDE4D3. Such phosphorylation was demonstrated (15) both in vitro, using recombinant activated ERK2, as well as in intact COS1 cells that had been challenged with EGF in order to activate endogenous ERK2. Based upon studies done on various ERK2-phosphorylated transcription factors, it has recently been suggested that authentic ERK2 substrates contain docking sites for this kinase (21–27). Such sites have been envisaged as providing a modular system that allows for enhanced ERK2 binding and thus fidelity of ERK2 phosphorylation and action in vivo (24, 27). Two such sites have been recognized to date. One of these is the FQF motif (27) that is located some 5–30 residues carboxy-terminal to the ERK2 phosphorylation site. The other is the KIM motif that has been found approximately 120–150 residues NH₂-terminal to the ERK2 phosphorylation site of various transcription factors (21–27, 36). Examples of these docking site recognition motifs are given in Table I. Indeed, a number of transcription factors, such as Elk-1 and members of its subfamily, that provide well recognized ERK substrates exhibit both of these docking sites (27).

We note here that the PDE4D3 catalytic unit contains (Table 1) a putative ERK2 docking site in the form of an FQF motif that is located some 15 amino acids COOH-terminal to Ser⁵⁷⁹, the target for ERK2 phosphorylation (15). In addition to this,
**FIG. 2.** ERK docking motifs in PDE4 isoenzymes. 

**a**, the upper panel shows a Western blot for ERK2 done on anti-VSV mAb immunoprecipitates (IP) of VSV epitope-tagged (14, 15) forms of either wild-type PDE4D3 (Wt-4D3) or a PDE4D3 species (Dbl. mutant) with mutations in both putative ERK docking sites (F597A/Q598A/F599A and K455A/K456A). The control (c) is given for protein G-agarose beads treated with lysate but without the addition of VSV-specific mAb. Equal amounts of PDE4D3 species were loaded in each instance, as determined by immunoblotting. The lower panel shows an ERK2 immunoblot (top section) for VSV-mAb-immunoprecipitated wild-type PDE4D3 (Wt-S579) and both the S579D (Mut D579) and S579A (Mut A579) mutant PDE4D3 forms. Also given is a VSV-mAb immunoblot for these PDE4D3 forms (bottom section) in order to gauge the amount of each of these proteins that was present in the probed lysates. These data are typical of experiments performed at least three times.

**b**, the percentage binding of ERK2 to various docking site mutants of PDE4D3 compared with wild-type PDE4D3 (100%). In this experiment, PDE4D3 (wild type), the F597A/Q598A/F599A PDE4D3 mutant (FXF mutant), the K455A/K456A mutant (KIM mutant), and the double mutant were expressed in COS cells. Co-immunoprecipitation experiments were then done from lysates of these cells using equal immunoreactive amounts of each of these enzymes as indicated in the inset for wild-type PDE4D3 (Wt) together with its KIM mutant (KIM), FXF mutant (FXF), and double KIM plus FXF (double) mutant forms as described above. These data are typical of experiments performed at least three times. c, PDE4D3 (wild type), the F597A/Q598A/F599A PDE4D3 mutant, the K455A/K456A mutant, and the double mutant were expressed in COS1 cells. The cells were then challenged with EGF (50 ng/ml) and harvested at the indicated time points for the determination of PDE activity. In all of these studies, >98% of the lysate PDE activity was due to the transfected PDE4D recombinants. Data are means ± S.D. for three separate experiments. d, data are shown for the in vitro ERK2 phosphorylation of PDE4D3 (D3) and the mutant F597A/Q598A/F599A (mut-FQF) and K455A/K456A (mut-KIM) forms. Equal amounts of these species were used as assessed by immunoblotting (lower panel) using a VSV.
the sequence VetKKvtssgLlL, found some 122 residues amino-terminal to Ser$_{579}$ in PDE4D3, provides a potential ERK2 docking site of the KIM form (Capital letters indicate consensus residues, Table I). The work of various investigators indicates that the core of the KIM form of ERK docking site contains a leucine residue located some 3–6 residues to the COOH-terminal side of a cluster of basic residues (23, 27). In addition to this, it has been suggested that a further leucine (or valine) residue is important, and this may be found some 2 residues to the NH$_2$-terminal side of the positively charged core residues of the KIM region (24, 26). Thus PDE4D3 appears to satisfy all of the requirements for having both the KIM and FQF forms of ERK docking sites (Table I).

We thus set out to establish whether these two motifs in PDE4D3 did indeed form functional docking sites for ERK2. First, we were able to show that endogenous ERK2 could be co-immunoprecipitated with VSV epitope-tagged PDE4D3 from transfected COS1 cells (Fig. 2, a and b). In various transfection experiments, between 52 and 86% (range; $n = 9$ separate determinations) of the total amount of ERK2 could be immunoprecipitated with the entire pool of PDE4D3. This association was also evident when COS1 cells had been treated for 10 min with EGF in order to activate ERK2 (data not shown, but see below regarding Fig. 2e). ERK2 could also be co-immunoprecipitated (Fig. 2e) equally as well as using either the S579D PDE4D3 mutant, which mimics the ERK2-phosphorylated form of PDE4D3, or the S579A mutant, which provides a form of PDE4D3 that is insensitive to ERK2 phosphorylation. Thus, the interaction between ERK2 and PDE4D3 is apparently unaffected by the status of both ERK2 phosphorylation and PDE4D3 phosphorylation at Ser$_{579}$.

We then tested for the association of ERK2 with forms of PDE4D3 that contained mutations in these putative ERK2 docking sites. The F597A/Q598A/F599A mutant was generated in order to disrupt the FQF motif. The K455A/K456A mutant was generated to disrupt the KIM region, by ablat- ing the positively charged residues that all investigators have identified as being at the core of this docking site. We also generated a PDE4D3 construct that had both of these mutations. We then showed (Fig. 2, a and b) that, unlike wild type PDE4D3, none of these three “docking site” mutants were able to co-immunoprecipitate ERK2, i.e. to our knowledge, the first demonstration that ERK2 can be co-immunoprecipitated with a substrate protein and that such an interaction is mediated through dual docking sites. Indeed, PDE4D3 and a protein-tyrosine phosphatase (25) are the only two enzymes that have, to date, been shown to have docking sites allowing interaction with ERK2.

Challenge of COS1 cells with EGF has been shown to cause the activation of ERK2 and the inhibition of transiently expressed PDE4D3 activity (15). We see here (Fig. 2c), however, that EGF failed to elicit the inhibition of both the F597A/Q598A/F599A and K455A/K456A mutant PDE4D3 forms when expressed in COS1 cells. In addition, the ability of recombinant ERK2 to phosphorylate, in vitro, these docking site mutant forms of PDE4D3 was severely attenuated compared with its action on wild type PDE4D3 (Fig. 2d, Phosphorylation (1)). However, by increasing the amount of ERK2 in the assay and extending the phosphorylation period, these mutant forms could be phosphorylated to similar levels as the wild-type form of PDE4D3 (Fig. 2d, Phosphorylation (2)). This is consistent with the notion that these docking sites serve to enhance the efficiency of phosphorylation of substrates by ERK2, presumably by recruiting ERK2 into a tight complex with them.

The interaction between PDE4D3 and ERK2 could also be demonstrated for endogenously expressed components. We have previously shown that activation of endogenous ERK2 in U937 monocytic cells led to inhibition of PDE4D3 activity (15). Here we demonstrate that PDE4D3 could be co-immunoprecipitated with ERK2 from these cells (Fig. 2e). This was true for both the unphosphorylated, faster mobility form of ERK2 found in resting cells, and the phosphorylated, slower mobility form found in EGF-treated U937 cells (Fig. 2e). We confirmed, by immunoblotting (data not shown), that the amount of antibody added was sufficient to immunoprecipitate all of the PDE4D3 and PDE4D5 isoenzymes found in the samples analyzed here. This was done from both untreated cells (−EGF) and from cells that had been treated for 10 min with 50 ng/ml EGF (+EGF).

In the untreated cells, all of the immunoprecipitated ERK2 was in the high mobility, basal state, whereas in the EGF-treated cells all of the ERK2 was in the low mobility, activated state. Thus, ERK2 could be co-immunoprecipitated with PDE4D3 in both mobility/activity states. It then seemed crucial to obtain some estimate as to what fraction of the pool of ERK2 in these cells was complexed with natively expressed PDE4D3. Clearly, if this was a significant fraction then this could have particular consequences for ERK2 signaling through restricting the availability of other ERK2 substrates. We were able to gauge that endogenous PDE4D3 was associated with 1.3 ± 0.5% of the total ERK2 in resting U937 cells and that this proportion did not change (1.4 ± 0.5%; mean ± S.D.; $n = 3$) in EGF-treated U937 cells where ERK2 was converted to its activated form.

Thus, this level of recruitment is unlikely to interfere with ERK2 signaling in these cells by complexing a large pool of ERK2 with PDE4D3 forms.

| Transfected PDE4D form | Activity in EGF-treated cells | Activity in EGF- and PD98059-treated cells |
|------------------------|-----------------------------|------------------------------------------|
| PDE4D5                 | 49 ± 5                      | 100 ± 6                                  |
| PDE4D3                 | 51 ± 7                      | 98 ± 5                                   |
| (UCR1 + UCR2 + CAT4D)  | 51 ± 6                      | 101 ± 6                                  |
| (CAT4D)                | 82 ± 5                      | 97 ± 3                                   |
| PDE4D1                 | 136 ± 4                     | 99 ± 5                                   |
| (UCR2 + CAT4D)         | 135 ± 5                     | 101 ± 4                                  |
| PDE4D2                 | 84 ± 5                      | 104 ± 7                                  |

Long PDE4D Forms Are Inhibited by ERK2 Phosphorylation—PDE4D3 is one of the three long isoenzymes (i.e. UCR1-containing) that are encoded by the PDE4D gene (28). Indeed,
FIG. 3. Action of EGF on PDE4D forms expressed in transfected COS cells. COS1 cells were transfected to express the indicated PDE4D form, challenged with EGF (50 ng/ml), and then harvested at the indicated times for determination of PDE activity. In all of these studies, >98%
both it and the long PDE4D5 form can be inhibited similarly by ERK2 action (15). These enzymes differ only by virtue of their alternatively spliced extreme NH₂-terminal region (28), and thus it would seem unlikely that these regions are of functional significance in influencing the inhibitory effect of ERK2 phosphorylation. To address this directly, we set out to evaluate whether inhibition is an inherent consequence of ERK2-mediated phosphorylation of what can be considered the “core” PDE4D long form. To do this we generated a truncated species, (UCR1+UCR2+CAT4D), that was engineered by removal of the unique 15 amino acid NH₂-terminal region of PDE4D3. This construct includes only those regions found in common to all three PDE4D long forms, namely the combined UCR1 and UCR2 regions attached to the catalytic unit (Fig. 1). When expressed in COS1 cells, the activity of (UCR1+UCR2+CAT4D) was reduced by challenge of cells with EGF to a level similar to that achieved when analyzing wild-type PDE4D3 (Table I). No inhibition of these PDE4D forms ensued when transfected cells were challenged with EGF in the presence of the MEK inhibitor, PD98059 (Table I). Thus, the extreme NH₂-terminal region is not needed in order to observe ERK2 inhibition of PDE4D long forms.

We then set out to investigate the functional outcome of ERK2 phosphorylation on the activity of the core PDE4D catalytic unit. To do this, we engineered the construct (CAT)4D, which comprises just the PDE4D catalytic region (Fig. 1). We noted that the activity of this truncated species was inhibited upon EGF challenge of transfected COS1 cells (Table II). However, the magnitude of this inhibition was considerably lower than that seen for the long PDE4D3 and PDE4D5 isoenzymes (Table II). Thus, the combined UCR1 and UCR2 regions appear to magnify the inherent inhibitory effect of ERK2 phosphorylation of the PDE4D catalytic unit.

The Short PDE4D1 Isozyme Is Activated by ERK2 Phosphorylation—The PDE4D gene encodes two short forms, called PDE4D1 and PDE4D2 (6, 28, 37). While both of these lack UCR1, they differ in two key aspects. First, PDE4D1 has a unique NH₂-terminal region, whereas PDE4D2 lacks any such region. Second, PDE4D1 has an intact UCR2, whereas the UCR2 of PDE4D2 is NH₂-terminally truncated by 32 residues (6, 37).

To our surprise, we discovered that EGF treatment led to the activation, rather than the inhibition, of PDE4D1 expressed in COS1 cells (Fig. 3a). The MEK inhibitor, PD98059 (Fig. 3a), ablated this action. We also demonstrated that recombinant ERK2 could elicit the phosphorylation of PDE4D1 (Fig. 4a). However, ERK2 failed to phosphorylate the S491A mutant form of PDE4D1 (Fig. 4a). This signifies that ERK2 phosphorylated PDE4D1 at a single site, Ser⁴⁹¹, that is cognate to the single ERK2 phosphorylation site (Ser⁵⁷⁵) found in PDE4D3 (15). Phosphorylation of PDE4D1 in vitro, with ERK2, caused an increase in its activity to 135 ± 7% of that of PDE4D1 that had not been phosphorylated by ERK2 (untreated = 100%; n = 3; mean ± S.D.; incubation without ERK2, under identical conditions, caused no change in activity, <5%). This level of activation was similar to that achieved by EGF treatment of COS1 cells that had been transfected to express PDE4D1 (Fig. 3a; Table II).

We have shown previously (15) that mutation of Ser⁵⁷⁵, in PDE4D3, to the negatively charged amino acid aspartate generated a mutant enzyme whose reduced activity mimicked that caused by ERK2 phosphorylation of wild-type PDE4D3. Applying a similar strategy here, we generated the S491D mutant form of PDE4D1. The activity of this mutant was increased to 131 ± 6% of that of wild type PDE4D1 (n = 3; mean ± S.D.; wild type = 100%). In contrast, the relative activity of the S491A mutant form of PDE4D1 was identical (100 ± 6%; n = 3; mean ± S.D.) to that of the wild-type enzyme. These data are consistent with ERK2 phosphorylation causing activation of the PDE4D1 short form.

PDE4D1 also differs from the long PDE4D isoforms not only in lacking UCR1 but also in having a distinct NH₂-terminal region. We thus set out to evaluate whether the unique NH₂-terminal region of PDE4D1 could influence the ability of ERK to stimulate the activity of this PDE4 short form. To do this we generated a species called (UCR2+CAT4D) that removed the unique 45-amino acid NH₂-terminal region from PDE4D1 (Fig. 1). When (UCR2+CAT4D) was expressed in COS1 cells, we found that, as with the short PDE4D1 isozyme, its activity was similarly stimulated by EGF and ablated by PD98059 (Fig. 3b; Table II). We also generated the S446D mutant of (UCR2+CAT4D) in order to mimic the ERK2-phosphorylated form. This mutant had an activity which was some 147 ± 12% of the lysate PDE activity was due to the transfected species. In some instances, cells were additionally challenged with the MEK inhibitor, PD98059 (20 μM). Shown here are data for PDE4D1 (positions 480–520) (a), (UCR2+CAT4D) (positions 255–262) (b), PDE4D2 (positions 256–275) (c), and (CAT4D) (positions 287–295) (d). The specific activities (pmol/min/mg of protein) of the lysates for COS cells expressing these species are given in parentheses (range). The figure shows EGF-induced changes in PDE4 activity relative to that of the untreated controls (100%) with means ± S.D. (n = 3 separate experiments shown).
**DISCUSSION**

The ERK MAP kinase system provides a pivotal route whereby a variety of growth factors and hormones exert actions on key transcriptional and other cellular processes (39). It has already been demonstrated that this pathway can be negatively regulated by cAMP in cells that express the PKA-inhibited c-Raf isoenzyme (16–19). We suggest in this study that a key role of PDE4 enzymes is to provide an additional mechanism for integrating the ERK and cAMP signaling pathways.

Here we demonstrate that PDE4D enzymes have both the KIM and FQF forms of ERK2 docking sites (Fig. 1; Table I). This can be taken as a strong indication that PDE4D enzymes do indeed provide authentic ERK substrates in vivo (21–27, 36). These sites straddle the target serine residue for ERK2 phosphorylation and appear to be integral to the action of ERK2 in regulating PDE4D enzymes. Thus, mutation of these sites in PDE4D3 ablated the ability of ERK2 to be co-immunoprecipitated with PDE4D3 and ablated the ERK2-mediated inhibition of PDE4D3 in intact COS cells challenged with EGF. In addition, both docking sites appear to be needed for ERK2 to phosphorylate PDE4D3 optimally in vitro. Intriguingly, isoenzymes encoded by the other three *PDE4* genes also exhibit both the FQF and KIM docking sites (Table I), and thus all PDE4 isoenzymes may be able to interact with ERK2. Indeed, it has been shown that PDE4B2 can be phosphorylated by an ERK
MAP kinase preparation, although the functional significance of this has not been explored in detail (40).

UCR1 and UCR2 provide unique signatures of PDE4 enzymes and are formed from two blocks of highly conserved sequence, some 55 and 76 amino acids in length, respectively. The UCR1 and UCR2 pair, found immediately NH2-terminal to the catalytic region, are similarly placed to paired regions found in members of other PDE families that allow regulation of the enzyme catalytic unit. Thus, paired domains in PDE2 and PDE5 enzymes confer cGMP binding and regulation, whereas in PDE1 enzymes they confer Ca2+/calmodulin binding and regulation (2, 4, 11, 12). It has been postulated that UCR1 and UCR2 may serve to regulate PDE4 enzymes (6). This is supported by the observation that PKA activates PDE4D3 through the phosphorylation of Ser54, located within UCR1 (13, 14, 41), and that this can be simply mimicked by mutation of this target serine to a negatively charged amino acid (14). We propose here that other key functions of UCR1 and UCR2 are both to define and mediate changes in PDE4D isoenzyme activity as a consequence of COOH-terminal phosphorylation by ERK2.

From analyses of PDE4D isoforms, and various truncated PDE4 constructs, we show that the core PDE4D catalytic unit, lacking both UCR1 and UCR2, is weakly inhibited by ERK2 phosphorylation. In marked contrast to this, the intact lone UCR2, as found in PDE4D1, redirects the effect of ERK2 phosphorylation so as to cause activation of this PDE4D short isoform. We propose that this action is mediated by the extreme NH2-terminal portion of UCR2, since PDE4D2, a short isoform whose UCR2 lacks the NH2-terminal first 32 amino acids, was weakly inhibited rather than activated as a consequence of ERK2 phosphorylation. We further propose that the combined UCR1 and UCR2 regions, found in the long PDE4D forms, form a regulatory module that serves to amplify the inherent inhibitory effect of ERK2 phosphorylation on the PDE4D catalytic unit.

The various PDE4D isoforms thus provide a panel of enzymes that can either be activated or, alternatively, inhibited to different extents by ERK2 phosphorylation. Indeed, that PDE4D1 can be activated by ERK2 phosphorylation may provide insight into recent reports that phorbol 12-myristate 13-acetate treatment of FDCP myeloid cells (42) and vascular smooth muscle cells (43) appeared to cause a small overall increase in total cellular PDE4 activity that was ablated by PD98059. Thus, the ability to control, in a selective fashion, the expression of long and short isoenzymes from the PDE4D gene offers the potential of providing a tailored signaling system that could allow ERK2 activation either to increase or decrease cAMP levels in specific cell types. Such a means of regulation may thus offer an insight into why cells might choose to express particular long and short PDE4 isoenzymes. In addition, as a number of PDE4D isoenzymes appear to be targeted to specific intracellular sites (6), differential regulation by ERK may offer the potential either to raise or lower cAMP levels in discrete intracellular domains. This would add PDE4 enzymes to the growing list of machinery responsible for compartmentalized cAMP signaling (1, 44).

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