The RACK1 Signaling Scaffold Protein Selectively Interacts with the cAMP-specific Phosphodiesterase PDE4D5 Isoform*

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The WD-repeat protein receptor for activated C-kinase (RACK1) was identified by its interaction with the cyclic AMP-specific phosphodiesterase (PDE4) isomorph PDE4D5 in a yeast two-hybrid screen. The interaction was confirmed by co-immunoprecipitation of native RACK1 and PDE4D5 from COS7, HTK293, 3T3-F442A, and SK-N-SH cell lines. The interaction was unaffected by stimulation of the cells with the phorbol ester phorbol 2-myristate 3-acetate. PDE4D5 did not interact with two other WD-repeat proteins, β-cotransporter protein and Gβ, in two-hybrid tests. RACK1 did not interact with other PDE4D isoforms or with known PDE4A, PDE4B, and PDE4C isoforms. PDE4D5 and RACK1 interacted with high affinity (Kd approximately 7 pm) when they were expressed and purified from Escherichia coli, demonstrating that the interaction does not require intermediate proteins. The binding of the E. coli-expressed proteins did not alter the kinetics of cAMP hydrolysis by PDE4D5 but caused a 3–4-fold change in its sensitivity to inhibition by the PDE4 selective inhibitor rolipram. The subcellular distributions of RACK1 and PDE4D5 were extremely similar, with the major amount of both proteins (70%) in the high speed supernatant (S2) fraction. Analysis of constructs with specific deletions or single amino acid mutations in PDE4D5 demonstrated that a small cluster of amino acids in the unique amino-terminal region of PDE4D5 was necessary for its interaction with RACK1. We suggest that RACK1 may act as a scaffold protein to recruit PDE4D5 and other proteins into a signaling complex.

Modulation of the levels of the second messenger cAMP in cells is important in the regulation of numerous physiological processes, including those in the immune/inflammatory system, vascular smooth muscle, and the brain. Cyclic nucleotide phosphodiesterases (PDEs)

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†† The abbreviations used are: PDE(s), cyclic nucleotide phosphodiesterase(s); PDE4(s), cAMP-specific phosphodiesterase(s); β-COP, β-

that hydrolyze cAMP and cGMP and thus play an important role in modulating cAMP levels (1). The cAMP-specific phosphodiesterases (PDE4s) can be differentiated from other PDEs by sequence homology of the catalytic region 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 PDE4 enzymes are also characterized by the presence of unique regions of amino acid sequence outside the catalytic region of the proteins, which are called upstream conserved regions 1 and 2 (UCR1 and UCR2) and are located in the amino-terminal half of the proteins (3). The PDE4s are comprised of a large family of isoforms, encoded by four different genes (PDE4A, PDE4B, PDE4C, and PDE4D) in humans, with additional diversity being generated by alternative mRNA splicing (2).

We and other groups (3–5) have characterized five different isoforms encoded by the human PDE4D gene, all of which appear to be conserved among mammals (6, 7). The five isoforms differ by the substitution of unique blocks of amino acids at the amino-terminal regions of their respective proteins (5). The two smaller PDE4D isoforms, PDE4D1 and PDE4D2, are located exclusively in the cytosolic fraction of the cell (5, 7). The larger isoforms PDE4D3, PDE4D4, and PDE4D5 are each found both in the cytosol as well as in association with cellular particulate fractions (5, 7). The functional consequences of this diversity are poorly understood. However, the PDE4D3 isoform is a substrate for protein kinase A (PKA), which serves to activate this isoform (6, 8–10).

In this study, we identify a novel property of a different PDE4D isoform, PDE4D5. This human isoform was recently isolated by us and is found in a variety of tissues and cell types, including the brain (5). PDE4D5 can be distinguished from other PDE4D isoforms by the presence of a unique amino-terminal region of 88 amino acids (5). We now demonstrate that PDE4D5 interacts specifically and with high affinity with the RACK1 protein (receptor for activated C-kinase). RACK1 is a 36-kDa WD-repeat protein (11) that binds to certain protein kinase C (PKC) isoforms subsequent to coatomer protein; GST, glutathione S-transferase; GST-RACK1, a fusion protein of GST and RACK1; MBP, maltose-binding protein; MBP-PDE4D3 and MBP-PDE4D5, fusion proteins of MBP and PDE4D3 or PDE4D5, respectively; ORF, open reading frame; PKC, protein kinase C; PCR, polymerase chain reaction; PMA, phorbol 2-myristate 3-acetate; RACK1, receptor for activated protein C kinase; rolipram, 4-[3-(cyclopentoxyl)-4-methoxyphenyl]-2-pyrrolidinone; UCR, upstream conserved region; WD-repeat, a protein sequence motif containing repetitive sequences bounded by tryptophan and aspartic acid residues; VSV, vesicular stomatitis virus; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis.
to their activation by diacylglycerol or phospholipase C. PMA. RACK1 appears to serve as a scaffold or "anchor" protein for these PKC isoforms (12–15). Recently, RACK1 has been shown to interact with the β subunit of integrins (16) and the Src protein tyrosine kinase (17). Scaffold and anchor proteins physically connect various signal transduction components, such as receptors, kinases, and elements of the cytoskeleton, into stable complexes (18). Our data suggest that RACK1 may recruit the PDE4D5 isoform into a such a complex in a variety of cell types.

**EXPERIMENTAL PROCEDURES**

**Materials**—A HeLa cell (human HeLa S3 cells; American Type Culture Collection) two-hybrid library cloned into the EcosRI and XhoI sites of the pGADGH vector (19) 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. Antibodies to RACK1 were obtained from Transduction Laboratories. The RACK1 antibody detects a 36-kDa species in Jurkat cells (16, 17). A monoclonal antibody to human PDE4D proteins, which does not cross-react with other PDE4 species and which we have described previously (5), was obtained from Sharon Wolda, Roche Molecular Biochemicals, and detected PDE4D3 and PDE4D5 with very similar affinity (5). The PDE4D antibody also detects PDE4D species in rats and mice (5). cDNA clones for Gβ and β-COP were obtained from A. G. Gilman and K. J. Harrison-Lavoie, respectively. The L40 *S. cerevisiae* strain and the pBTM116 plasmid were obtained from A. B. Vojtek.

**Two-hybrid Screens**—These were performed using methods we have described previously (20). In brief, the open reading frame (ORF) of the pPDE79 cDNA (GenBank accession number AF012073) encoding human PDE4D5 (5) was cloned into the NotI site of pLEXAN, to generate pLEXAN79, which encodes a fusion between the glutathione S-transferase (GST) domain and PDE4D5. Similarly, the full ORF of human RACK1 ([GenBank accession number NM124194], obtained from a positive isolated in the two-hybrid screen, was cloned into the NotI site of pGADN, to produce pGADNRACK1. pGADN is a derivative of pGADGH but with a NotI site inserted into the polynkry.

**Generation of Bacterial Expression Constructs**—The full ORFs of PDE4D3, PDE4D5, and RACK1 were cloned into the NotI site of pMALN, to generate pMALPDE4D3 (also called pMALP79), and pMALPDE4D5 (also called pMALN43), respectively. pMALN is a derivative of pMAL2 (New England Biolabs (24)), with a NotI site inserted into the polynkry. The full ORF of RACK1 was cloned into the NotI site of pGEX5X-3 (Amersham Pharmacia Biotech (25)) to generate pGEXRACK1. All constructs named pMAL . . . generate fusions between the maltose-binding protein (MBP) and the amino terminus of the protein encoded by the insert. Constructs named pGEX . . . constructs generate fusions between glutathione S-transferase (GST) and the amino terminus of the protein encoded by the insert.

**Generation of COST Cell Expression Constructs**—The full ORF of PDE4D5 was cloned into the NotI site of pcDNA3 (Invitrogen), to create pcDNA-PDE4D5SVS (also called pcDNA79VS). In this construct, the insert is placed under the control of the cytomegalovirus immediate early gene promoter. The full ORF of PDE4D3 was cloned into pCIneo to produce pCNeo-PDE4D3 (5). In both cases, a sequence corresponding to the vesicular stomatitis virus (VSV) glycoprotein epitope (26) was added immediately downstream from the last codon of the PDE to encode a carboxy-terminal fusion. The native PDE4D stop codon was removed in this process, but a synthetic stop codon was placed immediately downstream from the epitope sequence. The full ORFs of PDE4D1, PDE4D2, PDE4D4, PDE4B1, PDE4B2, PDE4B3, and PDE4C2 were cloned into the NotI site of pcDNA3 (without a carboxy-terminal epitope).

**Generation of cDNAs Encoding Mutant Forms of PDE4D5**—To generate deletions in the amino-terminal region of PDE4D5, PCR was used to amplify various regions of PDE4D5. PCR product were then cloned into pLEXAN or pcDNA3. NotI sites were added to the PCR primers to aid in cloning. To generate point mutations in PDE4D5, the full-length PDE4D5 cDNA was subjected to site-directed mutagenesis with the QuikChange site-directed mutagenesis kit (Stratagene).

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

**Growth of Cell Lines**—All cell lines used in this study were obtained from the American Type Culture Collection. The lines were grown in Dulbecco's modified Eagle's medium, supplemented with fetal calf serum and antibiotics.

**Co-immunoprecipitations**—COS7, SK-N-SH, Jurkat, 3T3-F442A, or HEK293 cells were harvested in 0.5 ml of lysis buffer (55 mM Tris-HCl, pH 7.4, 132 mM NaCl, 22 mM sodium fluoride, 11 mM sodium pyrophosphate, 1.1 mM EDTA, 5.5 mM EGTA) containing complete protease inhibitor mixture (Roche Molecular Biochemicals) and lysed with 8 strokes of a 26-gauge needle attached to a disposable syringe. This method was used because we wanted to process a large number of samples quickly. It produced complete lysis of cells on the basis of vital dye staining and absence of latent dehydrogenase activity. After lysis, cell debris was removed by centrifugation at 12,000 × g for 10 min.

For immunoprecipitation of RACK1, 500 μg of cleared cytosol was mixed with 30 μl of pre-equilibrated anti-mouse IgM-agarose (Sigma) and incubated for 30 min at 4 °C. The beads were removed by centrifugation at 2,000 × g for 5 min, and the cleared lysates were incubated with 16 μl of anti-RACK1 antibody (Transduction Laboratories) in the presence of anti-mouse IgM agarose beads for 3 h at 4 °C. For immunoprecipitation of PDE4D5 from COS7 cells, 500 μg of cleared cytosol was mixed with 30 μl of pre-equilibrated protein A-agarose beads (Sigma) and incubated for 30 min at 4 °C. The beads were removed, and lysates were incubated for 3 h at 4 °C with 10 μl of monoclonal anti-PDE4D antibody (5) in the presence of protein A-agarose beads. In both cases, the beads were then collected by centrifugation (2,000 × g for 5 min) and washed three times with lysis buffer. Co-immunoprecipitation of PDE4D5 with RACK1 was analyzed by immunoblotting with the anti-PDE4D monoclonal antibody and the anti-RACK1 antibody.

**Library Expression of Glutathione S-Transferase (GST) and Maltose-binding Protein (MBP) Fusions in E. coli**—Cultures of E. coli JM109 containing pGEXRACK1, pMAL-PDE4D3, or pMAL-PDE4D5 were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (Roche Molecular Biochemicals) for 4 h at 30 °C. Bacteria were harvested by centrifugation at 2,500 × g for 10 min at 4 °C, and the bacterial pellet was frozen at –80 °C overnight. The bacterial pellets were resuspended in 10 ml of ice-cold resuspension buffer (55 mM Tris-HCl, pH 8.0, 2 mM EDTA, 10 mM β-mercaptoethanol, and complete protease inhibitor mixture) and sonicated with 4 × 30-s bursts at maximal setting. Triton X-100 was added to a final concentration of 0.02%, and cell debris was then removed by centrifugation at 15,000 × g for 10 min at 4 °C. The cleared supernatant was incubated with 1/10th volume of pre-equilibrated glutathione-Sepharose beads (for GST fusions) or amylose resin (for MBP fusions) on an orbital shaker for 30 min at 4 °C. The beads were collected by centrifugation at 2,000 × g for 1 min and washed three times with ice-cold resuspension buffer. The fusion proteins were eluted by the addition of 50 mM glutathione, 50 mM Tris-HCl, pH 8.0 (for GST fusions), or 10 mM maltose, 50 mM Tris-HCl, pH 8.0 (for MBP fusions), and dialyzed three times against 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 5% glycerol. The purified fusion proteins were stored at 80 °C until required.

**RACK1 Pull-down Assays**—COS7 cells were transfected with either 10 μg of plasmid pcDNA-PDE4D5SVS or 10 μg of plasmid pCNeo-PDE4D3. Transfections were done as described previously (5, 27). After 72 h, cells were harvested and lysed with 8 strokes of a 26-gauge needle in 0.5 ml of lysis buffer. Cell debris was removed by centrifugation (12,000 × g for 10 min at 4 °C), and 500 μg of cleared lysate was incubated with GST or GST-RACK1 and 60 μl of glutathione-Sepharose beads for 1 h at 4 °C. Beads were pelleted by centrifugation at 2,000 × g for 5 min at 4 °C and washed three times in lysis buffer. Protein complexes were eluted by the addition of 50 mM glutathione, 50 mM Tris-HCl, pH 8.0, and co-precipitation of PDE4D5 was analyzed by immunoblotting with an anti-VSV monoclonal antibody (26). In two
cases (see below) immunoblotting was performed with the PDE4D monoclonal antibody.

**ELISA Protein Interaction Assay**—Reacti-Bind glutathione-coated ELISA plates (Pierce) were treated with 1 μg of purified GST or GST-RACK1 for 16 h at 4 °C and then washed three times with 100 μl/bell wash buffer. Protein complexes were fixed with the addition of 100 μl/well 4% (v/v) paraformaldehyde in phosphate-buffered saline for 30 min at 4 °C. Paraformaldehyde fixation was used at this step because of concerns that the complexes might dissociate during the subsequent detection procedure. However, it was determined subsequently that the binding of PDE4D5 and RACK1 was so avid that the addition of paraformaldehyde made little difference. After fixation, protein complex formation was detected by the addition of anti-PDE4D monoclonal antibody (1:10,000; v/v) in dilution buffer (10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.05% Tween 20). Dilutions of the MBP fusions of PDE4D3 and PDE4D5 were incubated with immobilized GST or GST-RACK1 for 3 h at room temperature, and the wells were then washed three times with 100 μl/well ice-cold wash buffer. Protein complexes were visualized with the BCIP Microwell 2 Component Phosphatase Substrate System (Kirkegaard & Perry Laboratories) following the manufacturer’s instructions and quantified using an MRX microplate reader (Dynex Technologies) set at a test wavelength of 630 nm. RACK1 was visualized with the BCIP Microwell 2 Component Phosphatase Substrate System (Amersham Pharmacia Biotech).

**Preparation and Fractionation of Tissue Homogenates**—These were as described previously (5, 27–31). Confuent cultures of COS7 cells were scraped into 0.5 ml of lysis buffer and lysed with 20 strokes of a Dounce homogenizer equipped with a tight-fitting pestle. These homogenates were then fractionated as follows. For the low speed pellet (P1), they were centrifuged at 1000 × g, for 10 min. The supernatant from this step was then centrifuged at 100,000 × g for 1 h to yield a high speed pellet (P2, particulate) fraction and a supernatant (SN, cytosol) fraction. The pellets were then resuspended in 0.5 ml of lysis buffer.

**SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting**—These were as described previously (32). In brief, samples were resuspended in Laemmli buffer and boiled for 5 min. Membranes were blocked in 5% (w/v) low-fat milk powder in TBS (10 mM Tris-HCl, pH 7.4, 150 mM NaCl) for 1 h at room temperature, followed by alkaline phosphatase-conjugated anti-mouse IgG (Sigma; 1:2000 (v/v) in dilution buffer) for a further hour at room temperature. Immunoreactivity was visualized with the BCIP Microwell 2 Component Phosphatase Substrate System (Kirkegaard & Perry Laboratories).

**PDE Assays**—PDE activity was assayed as described previously (33). All assays were conducted at 30 °C, and in all experiments a freshly prepared slurry of Dowex:H2O:ethanol (1:1:1; v/v) was used. In all of the cases (see below) immunoblotting was performed with the PDE4D monoclonal antibody.

**RESULTS**

**Isolation of RACK1 as a Protein Interacting with PDE4D5 in a Two-hybrid Screen**—We have recently described a novel PDE4D isoform, called PDE4D5 (5). PDE4D5 differs from other PDE4D isoforms by the presence of a unique amino-terminal end, 88 amino acids in length (5), which has no detectable sequence homology to any other phosphodiesterase (Fig. 1). To determine more about the properties of PDE4D5, we wished to investigate whether specific proteins might bind to it. For this purpose, we used full-length PDE4D5 as a “bait” in a two-hybrid screen. Two independent screens were performed, with identical results. The results of one screen are shown (Table I). In both screens, a large number of cDNA clones were obtained, all of which encoded the full ORF of the RACK1 protein (12–15, 23).

**Specificity of the PDE4D5-RACK1 Interaction**—To obtain preliminary evidence that the PDE4D5-RACK1 interaction was specific, we used two-hybrid β-galactosidase assays to test the interaction of RACK1 with a variety of “baits” expressed as LexA fusions. These included lamin (22), casein kinase II, Ras, Rab, several transcription factors, and LexA itself (i.e. not as a fusion). In a similar manner, we tested PDE4D5 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 where we could demonstrate an interaction between PDE4D5 and RACK1 (data not shown). We also tested PDE4D5 for its ability to bind to two other WD-repeat proteins, the G-protein Gβ subunit and the coatomer subunit protein β-COP. Our rationale for testing β-COP was that, like RACK1, it has also been shown to bind to PKC isoforms, although with selectivity for different PKC isoforms than RACK1 (35). We were unable to detect an interaction between PDE4D5 and either of these two WD-repeat proteins, using β-galactosidase assay conditions that did detect the interaction between PDE4D5 and RACK1 (The results for β-COP are shown in Fig. 2.) These data suggest that PDE4D5 interacts specifically with RACK1 and not with WD-repeat proteins generally.

**Endogenous Expression of RACK1 and PDE4D5 in COS7 Cells**—To confirm and expand our two-hybrid data, we tested whether PDE4D5 and RACK1 could be co-immunoprecipitated from mammalian cells. We performed experiments first on COS7 cells (this and the next two sections), which were later expanded to the study of other cell types (see below). We have reported previously that PDE4D exists both in the
cytosol and in the cellular particulate fraction of various tissues and cell lines (5). To determine whether this was also true in COS7 cells, COS7 cells were disrupted and fractionated to yield a high speed (S2) supernatant fraction, reflecting cytosolic components, and also P1 and P2 particulate fractions (see “Experimental Procedures”). The fractions were subjected to SDS-PAGE and immunoblotted with antibodies specific for either PDE4D or RACK1. Both specific polyclonal and monoclonal PDE4D antibodies were used, with similar results (5). The PDE4D antibodies were all generated to a carboxyl-terminal region of the PDE4D protein and have been shown to detect all PDE4D antibodies were analyzed. The PDE4D antibodies (either polyclonal or monoclonal) did not immunoprecipitate purified GST-RACK1. The RACK1 antibody did not immunoprecipitate purified MBP-PDE4D5 (data not shown).

cytosol (S2) fraction, but some immunoreactivity was also seen in the pellet fractions.

The specific antibody to RACK1 detected in untransfected COS7 cells was a single 36 ± 1-kDa species (Fig. 3b), indicative of the presence of endogenous RACK1 (12). As also found for PDE4D5, the major fraction of RACK1 was located in the cytosolic (S2) fraction, although RACK1 immunoreactivity was also evident in the pellet fractions, to a similar extent to the level seen for endogenous PDE4D5 (Fig. 3a).

PDE4D5 and RACK1 Can Be Co-immunoprecipitated from COS7 Cells—In order to determine whether endogenous PDE4D5 and RACK1 interacted in COS7 cells, we took the cytosolic (S2) fraction from COS7 cells and subjected it to an

| Table I | Results of a two-hybrid screen with PDE4D5 as a “bait” |
|---------|-----------------------------------------------------|
| Number of transformants screened | 24,000,000 |
| Number of His+ colonies isolated | 3,000 |
| Number of His+ colonies that were also LacZ+ (of total tested) | 96/96 |
| Number of plasmids analyzed by restriction analysis and/or sequencing | 26 |
| Number of different clones present | 2 |
| Number of different mRNAs | 1 |

**Fig. 2. PDE4D5 interacts with RACK1 but not with the related WD-repeat protein β'-COP.** The PDE4D5 cDNA was cloned into pLEXAN to produce fusions with the LexA DNA binding domain. Various WD-repeat proteins were cloned into pGADN to produce fusions with the GAL4 activation domain. S. cerevisiae cells containing the appropriate plasmids were patched onto plates that selected for both plasmids and subjected to a filter β-galactosidase assay, as described (20). Positive results in the assay produce a change in the color of the patches from pink to blue. The bottom two patches serve as internal positive and negative standards, respectively (the oncoproteins RAS-Val12 and RAF (22), and the vectors without inserts). As a positive control, the GAL4 activation domain fusion of β'-COP was shown to interact with LexA-c-OP in a two-hybrid test (data not shown), consistent with results reported previously (50).

**Fig. 3. Endogenous expression and co-immunoprecipitation of RACK1 and PDE4D5 in COS7 cells.** a, extracts from untransfected COS7 cells were fractionated (see “Experimental Procedures”) and then immunoblotted with the PDE4D antibody (5). This identified in the high speed supernatant (S2) fraction two immunoreactive species of 95 ± 3 and 105 ± 2 kDa. On the basis of co-migration with recombinant species, these represented PDE4D3 and PDE4D5, respectively (5). PDE4D5 and to a lesser extent PDE4D3 were also found in the low speed (P1) and high speed (P2) pellet fractions. b, extracts from untransfected COS7 cells were fractionated and then immunoblotted with the RACK1 antibody. This identified in the high speed supernatant fraction a single immunoreactive species of 36 ± 1 kDa consistent with RACK1 (12). A similar immunoreactive species was also found in both the low speed (P1) and high speed (P2) pellet fractions. c, cytosolic (S2) fractions were prepared from untransfected COS7 cells and either immunoblotted directly (lanes Total) or subjected to immunoprecipitation with either the PDE4D antibody (lanes 4D-IP) or the RACK1 antibody (lanes RACK1-IP). The fractions/immunoprecipitates were then run on SDS-PAGE. The upper half of the gel was immunoblotted with the PDE4D-specific antibody. The arrows indicate the location of PDE4D3 and PDE4D5 as 95- and 105-kDa species, respectively. The lower half of the gel was immunoblotted with the RACK1-specific antibody, and the position of the 36-kDa RACK1 species is indicated. In each case, analyses were performed on cells treated (lanes +) or not treated (lanes -), with the PKC-activating phorbol ester PMA (10 μM), d, immunoblots of the combined pellet (lanes p) and S2 fractions (lanes s) of untransfected COS7 cells treated with 10 μM PMA. Cells were harvested at the indicated times (in minutes), fractionated as in a–c, and subjected to SDS-PAGE, followed by immunoblotting with PKCα (top panel), RACK1 (middle panel), and PDE4D (bottom panel). All data are typical of experiments done at least three times. Each lane on the gels represents 50 μg of protein, with PDE activities in the range of 25–35 pmol/min/mg protein. Equal amounts of protein from the P1, P2, and S2 fractions were analyzed. The PDE4D antibodies (either polyclonal or monoclonal) did not immunoprecipitate purified GST-RACK1. The RACK1 antibody did not immunoprecipitate purified MBP-PDE4D5 (data not shown).
immunoprecipitation protocol using either the RACK1 antibody (Fig. 3c; lanes RACK1-IP or the PDE4D antibody (Fig. 3c; lanes 4D-IP). The resulting immunoprecipitates were then subjected to SDS-PAGE and immunoblotting. As PDE4D migrates as a 105-kDa protein (5) and RACK1 as a 36-kDa protein (12), we treated the top half of the blot with the PDE4D antibody and the bottom half with the RACK1 antibody. Cytosolic extracts (i.e., not subjected to immunoprecipitation) were immunoblotted as controls (Fig. 3c; lanes Total). From this analysis, it was clear that the RACK1 antibody not only immunoprecipitated RACK1 but that it also co-immunoprecipitated PDE4D5 (Fig. 3c). Conversely, the PDE4D antibody not only immunoprecipitated PDE4D3 and PDE4D5 but it also co-immunoprecipitated RACK1. These data demonstrate that endogenously expressed PDE4D5 and RACK1 are in a complex in the cytosol of COS7 cells. We were also able to show that particulate RACK1 and PDE4D5, when solubilized by an Nonidet P-40 detergent system described by others (17), could be similarly co-immunoprecipitated (data not shown).

PMA Does Not Affect Binding of RACK1 to PDE4D5—It has been demonstrated previously that treatment of cells with phorbol esters, which activate PKC, is a necessary prerequisite for the interaction of RACK1 with both PKC (12) and the integrin β subunit (16) but not for the interaction of RACK1 with Src (17). Our co-immunoprecipitation data suggest that PDE4D5, like Src, can interact with RACK1 without PKC activation, as the treatment of COS7 with the phorbol ester PMA did not affect the ability of PDE4D5 and RACK1 to be co-immunoprecipitated, regardless of which antibody was used (Fig. 3c). In order to ascertain that PMA was able to activate PKC in COS7 cells, we treated COS7 cells with PMA, harvested them at various times, prepared cytosolic (S2) fractions, and subjected them to SDS-PAGE, followed by immunoblotting with antibodies for PKCa, RACK1, or PDE4D (Fig. 3d). These experiments showed clearly that endogenous PKCa was rapidly and completely translocated from the cytosol to the particulate fraction within 5–10 min (Fig. 3d, top panel). These data also indicate that PKCa was not constitutively activated in COS7 cells, as PKCa was clearly cytosolic prior to challenge with PMA. These observations suggest that PDE4D5, like Src, can interact with RACK1 without PKC activation. Additionally, they suggest that PMA does not trigger any translocation of either RACK1 or PDE4D5 to the particulate fraction of COS7 cells (Fig. 3d, bottom two panels).

PDE4D5 and RACK1 Can Be Co-immunoprecipitated from Various Cell Types—To determine whether the PDE4D5-RACK1 interaction occurred in cells generally, we examined a number of other cell lines, specifically HEK293 (human embryonic kidney), 3T3-F442A (mouse fibroblast/pre-adipocyte), SK-N-SH (human neuroblastoma), and Jurkat (human T-cell). Cytosolic extracts from these cell lines were subjected to SDS-PAGE with subsequent immunoblotting. The top half of the immunoblot was treated with a PDE4D antibody and the bottom half with the RACK1 antibody. With the PDE4D antibody, we detected two immunoreactive species of 105 ± 3 kDa and 95 ± 2 kDa in extracts from both HEK293 and 3T3-F442A cells (Fig. 4, lanes T). These isoforms co-migrated (data not shown) with recombinant PDE4D5 (105 kDa) and PDE4D3 (95 kDa) (5), respectively, and migrated very distinctly from the other PDE4D isoforms (5), namely PDE4D1 (68 kDa), PDE4D2 (68 kDa), and PDE4D4 (119 kDa). We did observe a faint band indicative of an ~68-kDa immunoreactive species in immunoblots of 3T3-F442A cells (Fig. 4), which may reflect either or both of the PDE4D1/2 isoforms (5). In contrast, a single ~105-kDa immunoreactive species, indicative of PDE4D5 (5), was noted in SK-N-SH cells, and no immunoreactive PDE4D species was noted in cytosolic extracts from Jurkat cells (Fig. 4). Immunoblotting of the lower half of the gel with an antibody specific for RACK1 identified a single 36 ± 2-kDa species present in all of these cell types.

To test for an interaction between PDE4D5 and RACK1 in cytosolic extracts from these cell lines, we subjected them to an immunoprecipitation protocol with either the RACK1 antibody (Fig. 4; lanes i or rIP) or a mouse nonspecific antiserum (Fig. 4, lanes n). The resulting immunoprecipitates were then subjected to immunoblotting with the PDE4D and RACK1 antibodies, as described above. These experiments showed that RACK1 could be immunoprecipitated from all cells by the RACK1 antibody but not by the nonspecific antiserum. In the HEK293, 3T3-F442A, and SK-N-SH cell lines, all of which express the PDE4D5 isoform, a single 105-kDa species in the RACK1 immunoprecipitates was detected upon immunoblotting with a PDE4D antibody (Fig. 4). No PDE4D immunoreactive species was detected in RACK1 immunoprecipitates from Jurkat T-cells, which did not express PDE4D5 (Fig. 4). These data indicate that natively expressed cytosolic RACK1 is complexed with natively expressed cytosolic PDE4D5 in both human (HEK293 and SK-N-SH) and mouse (3T3-F442A) cell lines. Our data are consistent with PDE4D5 being quantitatively immunoprecipitated with RACK1 from HEK293 and 3T3-F442A cells. Additionally, the data support the concept that PDE4D3 does not interact with RACK1, as PDE4D3 was not immunoprecipitated from HEK293 and 3T3-F442A cells using the RACK1 antibody (Fig. 4).

PDE4D5 and RACK1 Interact Directly In Vitro in a Dose-dependent Manner—We wished to determine whether RACK1 and PDE4D5 interacted directly, rather than through an intermediate protein. For this purpose, we studied the interaction between recombinant RACK1 and PDE4D5 as synthesized in E. coli. PDE4D5 was expressed as a maltose-binding protein fusion (MBP-PDE4D5) in E. coli. It was then purified (Fig. 5a) on a maltose affinity column (see "Experimental Procedures") and had an activity of 29 ± 2 pmol/min/mg protein (with 1 µM cAMP as substrate) and a Ki value of 5.1 ± 0.7 µM cAMP (mean ± S.D., n = 3 experiments). RACK1 was generated and purified as a GST fusion in E. coli GST-RACK1; see "Experimental Procedures")

To measure the interaction of PDE4D5 with RACK1, the purified protein was used in a capture plate assay. This demonstrated (Fig. 5b) that PDE4D5 bound to RACK1 in an appar-
RACK1 Interacts with cAMP-specific Phosphodiesterase

In order to evaluate whether interaction with RACK1 altered the catalytic activity of PDE4D5, we tested the effect on PDE4D5 activity of complexing E. coli-purified recombinant PDE4D5 with E. coli-purified recombinant RACK1. Under conditions (using a “pull-down” procedure; see Experimental Procedures) where all of the PDE4D5 could be shown to be complexed with RACK1, we found that RACK1 produced little or no change in either the $K_m$ or $V_{max}$ for the hydrolysis of cAMP by PDE4D5. Specifically, PDE4D5 exhibited a $K_m$ of 5.1 ± 0.7 μM when free from RACK1 and a $K_m$ of 6.9 ± 0.9 when complexed with RACK1 (mean ± S.D.; n = 3 experiments). The ratio of maximal catalytic activity for cAMP hydrolysis between RACK1-bound and uncomplexed forms of PDE4D5 was 0.95 ± 0.06 (mean ± S.D.; n = 3 experiments). We also determined the effect of RACK1 on the thermal denaturation profile of PDE4D5, which was measured upon incubation of the enzyme at 50 °C. Whether complexed or not to RACK1, the activity of PDE4D5 decayed as a single exponential (data not shown), indicative of a single homogeneous enzyme in both instances. The half-lives for decay of PDE activity were very similar for both the RACK1-complexed and uncomplexed forms of PDE4D5 (38.3 ± 2.1 and 34.3 ± 2.6 min; mean ± S.D.; n = 3 experiments). This suggests that interaction of PDE4D5 with RACK1 does not elicit a major conformational change affecting enzyme activity.

A number of investigators have noted that PDE4 isoforms can undergo conformational changes, which alter their sensitivity to inhibition by rolipram and other PDE4-selective inhibitors (31, 36, 37). Therefore, we analyzed the sensitivity of E. coli-purified MBP-PDE4D5 to rolipram when both free and complexed with E. coli-purified GST-RACK1. Dose-effect analyses (Fig. 5c) showed that rolipram inhibited the cAMP PDE purified recombinant PDE4D5 and RACK1 was tested in an ELISA as described under “Experimental Procedures.” The MBP-PDE4D5 fusion bound to GST-RACK1 in a dose-dependent manner, with an EC$_{50}$ of 7.4 ± 1.1 μM (mean ± S.D.; n = 3 separate experiments). As a control, parallel experiments were performed for MBP-PDE4D3, $c$, dose-response curves were calculated for the inhibition of PDE4D5 by rolipram, at a concentration of substrate (cAMP) of 1.0 μM. Assays were performed on MBP-PDE4D5 (“4D5-MBP”) alone, and also on MBP-PDE4D5 complexed with GST-RACK1. Assays were performed using an excess of GST-RACK1 so that all of the PDE4D5 would be complexed with RACK1 (see “Experimental Procedures”). In pull-down experiments, all of the PDE4D5 could be shown to complex with GST-RACK1 under these conditions (data not shown). As a control, assays were performed with GST alone, added at comparable levels. The IC$_{50}$ values for rolipram inhibition were 0.13 ± 0.1, 0.16 ± 0.05, and 0.52 ± 0.07 μM for MBP-PDE4D3 alone, MBP-PDE4D5 mixed with GST, and MBP-PDE4D5 mixed with GST-RACK1, respectively (mean ± S.D., n = 3). These values are significantly different (MBP-PDE4D5 alone compared with MBP-PDE4D5 complexed with GST-RACK1; p < 0.005, t test). Protein assays were performed, and molar concentrations were determined on the basis of the calculated molecular weights.

**Fig. 5.** Interaction of recombinant forms of PDE4D5 and RACK1, as purified from E. coli. a, fusions between GST and RACK1, and also between MBP and PDE4D3 or PDE4D5, were expressed and purified from E. coli (see “Experimental Procedures”). GST alone (i.e. not as a fusion) was expressed and purified in an identical manner. Cell lysates (lanes b) and purified proteins (lanes c) obtained after elution from the appropriate affinity column were run on SDS-PAGE and stained with Coomassie Blue. The species were purified to apparent homogeneity as analyzed by SDS-PAGE. The positions of the arrows marks the relative molecular weight of the purified proteins as follows: GST, 27.3 ± 1.1 kDa; GST-RACK1, 59.8 ± 1.4 kDa; MBP-4D3, 128 ± 3.8 kDa; MBP-4D5, 135.3 ± 2.6 kDa. These data are typical of experiments done at least three times. b, the interaction of E. coli-
activity of MBP-PDE4D5 in a dose-dependent manner, with an IC₅₀ value of 0.16 ± 0.07 μM. In contrast, this enzyme was complexed with GST-RACK1 a small shift in sensitivity to rolipram inhibition was seen (Fig. 5c), which was reflected in an increase in the IC₅₀ to 0.52 ± 0.07 μM. This effect was unlikely to be caused by the GST portion of GST-RACK1, as the addition of GST alone to the assays, at a level identical to that of GST-RACK1, had negligible effect on rolipram inhibition (Fig. 5c; IC₅₀ of 0.19 ± 0.02 μM).

RACK1 Interacts with PDE4D5 but Not with Other PDE4 Isoforms—Five PDE4D isoforms have been identified to date ([5] Fig. 1). We have already demonstrated that RACK1 does not interact with PDE4D3 (Figs. 3c, 4, and 5b). To determine whether any of the PDE4D1, PDE4D2, PDE4D3, or PDE4D4 isoforms interact with RACK1, cDNAs encoding these isoforms were expressed in S. cerevisiae as LexA fusions, and two-hybrid assays were used to test for their ability to interact with a GAL4-RACK1 fusion. No interactions were detected (data not shown), indicating that RACK1 interacts specifically with the PDE4D5 isoform.

To confirm this observation in mammalian cells, we also tested the ability of RACK1 to associate with recombinant PDE4 isoforms expressed in COS7 cells. For these experiments, we utilized a GST “pull-down” method, as modified by us previously (38). In brief, transiently transfected COS7 cells with cDNAs encoding various PDE4 isoforms. Cytosolic fractions were prepared from the transfected cells and incubated with GST-RACK1, and the resulting complex was absorbed onto glutathione-agarose beads (see “Experimental Procedures”). The beads were then harvested by centrifugation, washed, and immunoblotted with the PDE4D antibody (Fig. 6).

As shown before by us (5, 27, 29–31, 39), in homogenates of the transfected COS7 cells the activity of the transfected PDE4D species accounted for over 98% of the total cellular cAMP PDE activity (Fig. 6a, lanes ly). When cytosolic extracts from PDE4D5-transfected cells were subjected to “pull-downs” with GST-RACK1, a 105-kDa PDE4D immunoreactive species consistent with PDE4D5 was identified on the immunoblots (Fig. 6a, lanes rg). In contrast, when pull-downs were performed with GST alone, no PDE4D species was seen on the immunoblots (Fig. 6a, lanes g). In parallel experiments, we performed pull-downs from COS7 cells transfected with a cDNA encoding PDE4D3. We were unable to pull down any immunoreactive PDE4D3 with GST-RACK1 (Fig. 6a). However, endogenous PDE4D5 was pulled down in these experiments to a level similar to that seen using an equivalent amount of cytosol from mock-transfected cells.

In similar experiments, we determined that PDE4D1, PDE4D2, and PDE4D4, when expressed in COS cells (5), could not be pulled down by GST-RACK1 (data not shown). In still additional experiments, we determined that the human PDE4A4/5 isoform (3, 31), the human PDE4B isoforms PDE4B1, PDE4B2, and PDE4B3 (39), and also the human PDE4C2 isoform (41), when expressed in COS7 cells, could not be pulled down with GST-RACK1. These data imply that, of the known PDE4 isoforms, RACK1 binds selectively to PDE4D5.

The Interaction of PDE4D5 with RACK1 Is Mediated by Specific Amino Acids in the PDE4D5 Amino-terminal Region—As RACK1 interacts specifically with PDE4D5, and not with other PDE4 isoforms, it is likely that RACK1 interacts with regions of sequence that are unique to PDE4D5. PDE4D5 differs from all known PDE4 isoforms (2, 5) in the presence of a unique region of 88 amino acids at its amino terminus (Fig. 1). We wished to determine which specific amino acids within this region are essential for the binding of PDE4D5 to RACK1.

As a first step, we created a two-hybrid construct containing just the unique 88-amino acid amino-terminal region of PDE4D5, and we demonstrated that it could interact with RACK1 (Fig. 7a, patche NT). We then created constructs encoding deletion mutations in the amino-terminal region of PDE4D5, and we tested them for their ability to interact with RACK1. The interaction was tested by both a two-hybrid assay (Fig. 7a) and by pull-down experiments (Fig. 6b). For the two-hybrid experiments, constructs encoding fusions between LexA and various amino-terminal deletions of PDE4D5 were tested for interaction with GAL4-RACK1. For the pull-down experi-
Experiments (Fig. 6 tested the ability of the various PDE4D5 amino-terminal deletions of the unique 88-amino acid amino-terminal region of PDE4D5 as a fusion with the GAL4 activation domain (5, 10). The interactions were tested with the filter β-galactosidase assay used in Fig. 2. 

b, individual amino acids in the amino-terminal region of PDE4D5 were mutated to alanine, and the resulting constructs were expressed as LexA fusions and tested for their ability to interact with pGADN-RACK1. Also included as controls are LexA fusions of unmutated PDE4D5 (wt) and the signal produced with pGADN-RACK1 and LexA alone (nb).


discernments, we tested the ability of mutant PDE4D5 forms expressed in COS7 cells to interact with GST-RACK1. For the COS7 cell experiments, it was essential that we could distinguish between the transfected mutant protein and endogenous PDE4D5 proteins in COS7 cells. For this purpose, we utilized PDE4D5 constructs that encoded an epitope of the vesicular stomatitis virus envelope (26), attached to the carboxy-terminal end of the protein (5, 10). Expression of these VSV-tagged PDE4D5 species in COS7 cells could be detected by immunoblotting with a monoclonal antibody specific for the VSV epitope (26). Preliminary experiments demonstrated that COS7 cell-expressed, VSV-tagged unmutated PDE4D5 could be pulled down with GST-RACK1 (Fig. 6b, lane wt). In contrast, COS7 cells transfected with the vector alone demonstrated no detectable VSV immunoreactivity (Fig. 6b, lane m). We then tested the ability of the various PDE4D5 amino-terminal deletion mutants to interact with GST-RACK1. The pull-down experiments (Fig. 6b) demonstrate that a single region of the PDE4D5 amino terminus, comprising amino acids 12–29, is necessary for interaction with RACK1 but that other regions appear to be dispensable. Identical conclusions were obtained using two-hybrid methods (Fig. 7a).

To obtain additional data localizing the region of interaction, we created two mutants with deletions in the middle of the PDE4D5 amino-terminal region. One deletion (D2, Fig. 1) removed amino acids 22–27 and the other (D1) amino acids 19–51. Neither of these two mutations interacted with GST-RACK1 in a pull-down assay (Fig. 6b). These data narrowed down a region essential for interaction with RACK1 to a small region within the PDE4D5 amino terminus. Secondary structure analyses performed with the Wisconsin package of DNA analysis programs (data not shown) demonstrated that this region of PDE4D5 (approximately amino acids 19–50) may form an α-helix but otherwise did not have any distinguishing sequence motifs. It has no obvious primary sequence homology to PKC, Src, or integrins.

We then analyzed the effects of mutations in individual amino acids in the region defined by the deletion mutation analysis. Site-directed mutagenesis was used to mutate individual codons in the region to alanine. The effect of each of these mutations on the interaction with RACK1 was tested with the two-hybrid assay. Ten codons were individually mutated, and mutations of four of these (Asn-22, Pro-23, Trp-24, or Asn-26) were each shown to block completely the interaction (Fig. 7b). Two of these mutations (Asn-22 and Trp-24) were then expressed in COS7 cells and tested for their ability to co-immunoprecipitate with RACK1. Both the mutations blocked the interaction in this assay as well (Fig. 6c). These mutations were clustered in a very small region of the protein, which presumably serves as the major, or possibly only, region of interaction between PDE4D5 and RACK1.

**DISCUSSION**

Regulation of the levels of the second messenger cAMP has the potential to influence multiple processes in cells and tissues. PDE4 enzymes have been implicated in numerous cellular functions in the brain, airway smooth muscle, endocrine tissues, and in the immune/inflammatory systems (for review see Ref. 2). However, the regulation of many PDE4 isoforms is poorly understood. We have recently isolated a novel PDE4 isoform, PDE4D5, which is expressed in numerous cell lines and also in the brain (5). In the present study, we have demonstrated that PDE4D5 interacts specifically and at high affinity with the RACK1 WD-repeat scaffold protein. The interaction between these two proteins was demonstrated by multiple independent methods, including two-hybrid screening, pull-down assays with recombinant RACK1, and binding studies with purified recombinant proteins. We have also demonstrated by co-immunoprecipitation studies that native, endogenously expressed RACK1 and PDE4D5 interact in cells.

The term RACK1 stands for receptor for activated protein C kinase, and the first identified functional role for RACK1 was as a protein capable of binding to various PKC isoforms after they had been activated through treatment of cells with either diacylglycerol or phorbol esters (12–15). It is a member of the large family of WD-repeat proteins (11), which have numerous functions in cells. It is most homologous to the Gβ subunit, which participates in many isoforms of adenylyl cyclase and various ion channels (11, 42) and which also serves as an anchor for the β-adrenergic receptor kinase (43). X-ray diffraction studies of the Gβ subunit have demonstrated that it has a “propeller” structure (44, 45), and the “blades” of the propeller are believed to be sites of interaction between Gβ and other proteins. It is likely that RACK1 has a similar structure (46). The coatomer protein β′-COP is also a WD-repeat protein that also interacts with PKC isoforms (35). However, we have shown that PDE4D5 does not bind to either Gβ or β′-COP, demonstrating that PDE4D5 interacts specifically with RACK1 and not with WD-repeat proteins in general.

More recently, RACK1 has been shown to interact with the integrin β subunit (16) and the protein tyrosyl kinase Src (17). RACK1 may serve as a scaffold or “adapter” protein for either Src or the integrin β subunit. Overexpression of RACK1 inhibits the tyrosine kinase activity of Src and inhibits the growth of NIH-3T3 cells (17). The physiologic role of RACK1 in integrin function remains to be determined (see below).

Although RACK1 has been shown to interact with at least four different proteins, the mechanisms for these interactions appear to be quite different. PKC isoforms need to be activated by PKC, Src, or integrins.

**FIG. 7.** Yeast two-hybrid analysis of PDE4D5 deletions and point mutations. a, plasmids encoding fusions between the DNA binding domain of LexA and various amino-terminal deletions of PDE4D5 were tested for their ability to interact with RACK1, expressed as a fusion with the GAL4 activation domain (left column, pGADN-RACK1). The identical LexA fusions were tested for their ability to interact with the GAL4 activation domain alone (right column, pGADN). The regions of PDE4D5 included in the various constructs are shown in Fig. 1. Also shown is the interaction generated by a LexA fusion containing the unique 88-amino acid amino-terminal region of PDE4D5 (NT). The interactions were tested with the filter β-galactosidase assay used in Fig. 2. b, individual amino acids in the amino-terminal region of PDE4D5 were mutated to alanine, and the resulting constructs were expressed as LexA fusions and tested for their ability to interact with pGADN-RACK1. Also included as controls are LexA fusions of unmutated PDE4D5 (wt) and the signal produced with pGADN-RACK1 and LexA alone (nb).
by treatment with phorbol esters and Ca\(^{2+}\) before they can interact with RACK1 (47). This treatment is believed to produce a conformational change in the PKC enzyme, which exposes its C2 region, allowing it to interact with RACK1 (13–15). A number of domains within the C2 region of PKC are involved in its binding to RACK1 (13, 14). These domains show no obvious sequence homology to the region in the amino terminus of PDE4D5 that we have shown to be essential for its interaction with RACK1 (Figs. 6 and 7). The interaction of RACK1 with the β subunit of integrins also requires the stimulation of cells with PMA (16). This suggests that PKC activation is necessary for the interaction of the integrin β subunit with RACK1 or that PMA can directly promote the interaction. This interaction may involve a conformational change in RACK1, as the interaction between RACK1 and the integrin β subunit can only be demonstrated in vitro if a truncated form (WD-repeats 5–7, inclusive) of RACK1 is used.

In some ways, the interaction that we observe between PDE4D5 and RACK1 appears to resemble that between RACK1 and the SH2 region of Src (17). Src interacts with full-length GST-RACK1 in pull-down assays, which is also true for PDE4D5 (Figs. 3, 4, and 7). Co-immunoprecipitation of Src and RACK1 from cell lysates did not require PMA, which is also true for the interaction between RACK1 and PDE4D5. Therefore, RACK1 may interact with different proteins through different mechanisms, some of which involve phorbol ester-induced conformational changes and some of which apparently do not.

Several other PDE4 isoforms have also been demonstrated to bind to other signaling proteins, and it is of interest that these interactions also appear to be mediated by sequences in the unique amino-terminal ends of the PDE4 proteins. The PDE4A5 isoform has been demonstrated to bind to proteins containing SH3 domains (38). The PDE4A1 isoform is targeted to membranes, and the membrane targeting of this isoform is mediated by specific amino acids in its amino-terminal region (27–29, 48).

The physiologic implications of the RACK1-PDE4D5 interaction may be related to the ability of RACK1 to serve as a scaffold or adaptor protein that mediates the recruitment of PDE4D5 into a protein complex. A single scaffold or adaptor protein that mediates the recruitment of a number of signaling proteins, protein kinase A-anchoring proteins can interact with proteins containing SH3 domains (38). The PDE4A1 isoform is targeted to membranes, and the membrane targeting of this isoform is mediated by specific amino acids in its amino-terminal region (27–29, 48).

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