Membrane Localization of Cyclic Nucleotide Phosphodiesterase 3 (PDE3)

TWO N-TERMINAL DOMAINS ARE REQUIRED FOR THE EFFICIENT TARGETING TO, AND ASSOCIATION OF, PDE3 WITH ENDOPLASMIC RETICULUM*

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Subcellular localization of cyclic nucleotide phosphodiesterases (PDEs) may be important in compartmentalization of cAMP/cGMP signaling responses. In 3T3-L1 adipocytes, mouse (M) PDE3B was associated with the endoplasmic reticulum (ER) as indicated by its immunofluorescent colocalization with the ER protein BiP and subcellular fractionation studies. In transfected NIH 3006 or COS-7 cells, recombinant wild-type PDE3A and PDE3B isoforms were both found almost exclusively in the ER. The N-terminal portion of PDE3 can be arbitrarily divided into region 1 (aa 1–300), which contains a large hydrophobic domain with six predicted transmembrane helices, followed by region 2 (aa 301–500) containing a smaller hydrophobic domain (of ~50 aa). To investigate the role of regions 1 and 2 in membrane association, we examined the subcellular localization of a series of catalytically active, Flag-tagged N-terminal-truncated human (H) PDE3A and MPDE3B recombinants, as well as a series of fragments from regions 1 and 2 of MPDE3B synthesized as enhanced green fluorescent (EGFP) fusion proteins in COS-7 cells. In COS-7 cells, the localization of a mutant HPDE3A, lacking the first 189 amino acids (aa) and therefore four of the six predicted transmembrane helices (H3A-Δ189), was virtually identical to that of the wild type. M3B-Δ202 (lacking region 1) and H3A-Δ397 (lacking region 1 as well as part of region 2) retained, to different degrees, the ability to associate with membranes, albeit less efficiently than H3A-Δ189. Proteins that lacked both regions 1 and 2, H3A-Δ510 and M3B-Δ604, did not associate with membranes. Consistent with these findings, region 1 EGFP-MPDE3B fusion proteins colocalized with the ER, whereas region 2 EGFP fusion proteins were diffusely distributed. Thus, some portion of the N-terminal hydrophobic domain in region 1 plus a second domain in region 2 are important for efficient membrane association/targeting of PDE3.

By hydrolyzing cAMP and cGMP, cyclic nucleotide PDEs1 are critical in terminating cyclic nucleotide signals and regulating biological processes mediated by these second messengers. Eleven different, but structurally related, PDE gene families (PDE1–11) have been identified (1–3). Although intracellular location of PDEs is thought to be important in compartmentalization of cyclic nucleotide-mediated processes and regulation of discrete signaling pathways (4), little is known of the mechanisms that target PDEs to their intracellular destinations.

PDE3 isoforms are characterized by their high affinity for both cAMP and cGMP and their sensitivity to inhibition by a number of positive inotropic agents (5, 6). The two PDE3 subfamilies, PDE3A and PDE3B, are products of distinct but related genes, with additional diversity generated within the PDE3A subfamily by transcription initiation from alternative sites (5, 7). PDE3 isoforms have different subcellular locations, being predominantly membrane-associated in adipocytes, cytosolic in platelets, and both cytosolic and sarcoplasmic reticulum (SR)-associated in myocardium (8–11). SR-associated and cytosolic PDE3s in myocytes may be functionally distinct (11–13). Since particulate and soluble forms of PDE3 in cultured vascular smooth muscle cells are differentially regulated by cAMP (12), subcellular location may also be important in defining mechanisms by which PDE3 isoforms are regulated. Studies using specific inhibitors suggest that PDE3 isoforms regulate cAMP and cGMP pools involved in control of lipolysis, glyco- genolysis, myocardial contractility, smooth muscle relaxation, mesangial cell proliferation, and insulin and renin secretion (14–18). Since these processes occur in cells that contain multiple PDE isoforms, compartmentalization could provide a mechanism by which PDE3 isoforms selectively alter specific cAMP pools and regulate distinct signaling pathways.

Isoforms from PDE2 and PDE4 families also differ in their subcellular locations. Three PDE2 (19) splice variants, PDE2A1, PDE2A2, and PDE2A3 (20–23), have divergent N-terminal sequences; a hydrophobic domain at the N terminus of rat PDE2A2 may be involved in its association with brain membranes (23). Divergent N-terminal regions of PDE4 iso-

† The abbreviations used are: PDE, phosphodiesterase; MPDE3B, mouse adipocyte PDE3B; HPDE3A, human cardiac PDE3A; RPDE3B, rat adipocyte PDE3B; aa, amino acid(s); nt, nucleotide(s); DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; EGFP, enhanced green fluorescent protein; PKA, protein kinase A; PKB, protein kinase B; PI3-kinase, phosphatidylinositol 3-kinase; PDGF, platelet-derived growth factor, IRS-1, insulin receptor substrate-1; AKAP, protein A kinase anchoring protein; ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; GFP, green fluorescent protein; PCR, polymerase chain reaction.

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§§ These abbreviations are used: PDE, phosphodiesterase; MPDE3B,
forms (24, 25) also may account for several differences in their catalytic properties and subcellular locations. The N-terminal 25 amino acids of rat PDE4A1A exert an inhibitory effect on catalytic activity (26) and contain information for its targeting to brain membranes (27, 28). The N terminus of human PDE4A4B may also associate with membranes, since the splice variant PDE4A4C, which lacks this region, is entirely soluble (29). The proline-rich N terminus of PDE4A5 interacts with the v-Src-SH3 domain (30).

N-terminal domains may also confer specific properties upon PDE3A and B enzymes. Analysis of the N-terminal regions of PDE3A and B predicts a hydrophobic domain of ∼200 amino acids containing six transmembrane helices (5), which seems to be important for membrane association (31, 32). Just following this region are consensus sites for phosphorylation by protein kinase A (PKA) and protein kinase B (PKB), thought to be important in phosphorylation/activation of PDE3 (33–36); subcellular location could influence the specificity of protein kinase interactions with PDE3. To analyze some of the structural determinants involved in membrane targeting/association of PDE3, a series of Flag-tagged full-length and N-terminal deletion mutants of PDE3A and PDE3B, as well as a series of N-terminal MPDE3B-EGFP fusion proteins, were constructed and expressed in mammalian cells. The intracellular location of endogenous PDE3B in murine 3T3-L1 adipocytes and of the different PDE3A and PDE3B recombinants was determined by immunofluorescence techniques.

MATERIALS AND METHODS

Construction of Flag-tagged HPDE3A, H3A-Δ189, H3A-Δ397, H3A-Δ510, MPDE3B, M3B-Δ392, and M3B-Δ604 cDNAs—All nucleotide numbers mentioned here have been assigned by virtue of their relative position (from the start codon +1). An HPDE3A cDNA fragment containing the entire open reading frame (311 was used as template for HPDE3A constructs that lacked the first 189, 397, and 510 amino acids (aa) (supplied by J. Colicelli, UCLA, Los Angeles, CA) and were named H3A-Δ189, H3A-Δ397, and H3A-Δ510, respectively, and cloned into pBluescript. MPDE3B cDNA was used as a template to generate cDNA fragments lacking the coding region for the first 302 (M3B-Δ302) and 604 (M3B-Δ604) aa, which were cloned into pcRII (Invitrogen) vectors and designated as pCRM3B-Δ302 and pCRM3B-Δ604, respectively (32).

All Flag-tagged constructs were generated by inserting an eight amino acid Flag epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) immediately upstream of the stop codon and contains a 3′ extension with the Flag epitope (de- noted by the flag symbol), a new stop codon, and an XhoI site, in that order. The resulting PCR product was ligated to XhoI/AlwI III fragment isolated from pCR3B-Δ9 from the 5′ end of EGFP cDNA (C-terminal fusion). The primer pair indicated by the arrows was used to amplify the sequences downstream of the unique DraIII sites in PDE3A and PDE3B. The sense primer corresponds to the region flanking and including the DraIII site. The antisense primer corresponds to the region immediately upstream of the stop codon and contains a DraIII site, noted by the flag symbol, a new stop codon, and an XhoI site, in that order. The resulting PCR product was ligated to XhoI/AlwI III fragment isolated from pCR3B-Δ9 to generate Flag-tagged PDE3 recombinants. For Flag-tagged M3B-Δ302, an XhoI/AlwI N-terminal fragment was ligated to an AlwI/ XhoI Flag-tagged C-terminal fragment from Flag-tagged MPDE3B.

In an analogous fashion, in a three fragment ligation, the 5′ XhoI AlwI fragment from pCRM3B-Δ302 was ligated to a 3′ AlwI/XhoI III flanking fragment isolated from pCR3B-Δ9 was ligated to a MPDE3B Flag epitope-containing fragment and XhoI cut pBluescript (SK), generating the construct pSK-M3B-Δ302Flag. In a three fragment reaction, the 5′ XhoI/DraIII fragment isolated from pCR3B-Δ604 was ligated to a MPDE3B Flag-tagged 3′ DraIII/XhoI fragment and XhoI cut pZero, to give pZ-M3B-Δ604Flag (Fig. 1).

The reconstituted DraIII sites and all sequences downstream from this site were confirmed by sequencing. For expression studies, Flag-tagged HPDE3A and MPDE3B constructs were excised from the pZ vectors with XhoI and XhoI, and then ligated to the XhoI/XhoI sites or the XhoI site of pCDNAs, respectively (Invitrogen).

Construction of cDNAs Encoding MPDE3-EGFP Fusion Proteins—A series of PCR fragments encoding MPDE3B aa 1–100, 1–200, 1–250, and 1–400, 201–250, 184–250, 301–450, and 401–500 were generated using pZ-MPDE3B (MPDE3B cDNA in pZero; ClonTech) as template. Since the pZ-MPDE3B vector contains a T7 promoter next to a unique DraIII site, which is situated adjacent to the 5′ end of the MPDE3B Kozak sequence, T7 primer was used in reverse orientation for generation of MPDE3B PCR fragments encoding aa 1–100, 1–200, 1–250, and 1–400. Antisense primers used for generation of the indicated PCR fragments are listed in Table I. To allow for 3′-in-frame fusion of PDE3B PCR fragments to the 5′ end of EGFP cDNA (C-terminal fusion), all antisense primers had a 3′ extension with a SalI site. Using 50 ng of template DNA and the appropriate primer pair (3 pmol each), PCR fragments were amplified in a GeneAmp PCR system (PerkinElmer Life Sciences model 9600) with Pfu polymerase (Stratagene). The resulting PCR products contain the unique PDE3 DraIII site at the 5′ end and a stop codon at the 3′ end; the stop codon is flanked upstream by a Flag epitope-coding sequence and downstream by an XhoI site. The PCR products were purified by PCR Purifier (Invitrogen) and isolated from this vector as DraIII/XhoI fragments.

XhoI/DraIII fragments containing sequence upstream from the unique DraIII site of HPDE3A, H3A-Δ189, H3A-Δ397, and H3A-Δ510, were isolated from pBluescript. In a three-way ligation, these 5′ XhoI DraIII fragments were ligated, via the DraIII site, to the 3′ DraIII/XhoI HPDE3A Flag epitope-containing fragments and to XhoI cut pZero vector (Invitrogen), to give pZ-HPDE3AFlag, pZ-H3A-Δ189Flag, pZ- H3A-Δ397Flag, pZ-H3A-Δ510Flag, respectively (Fig. 1).

For generation of PCR fragments encoding MPDE3B aa 201–250,
184–250, 301–450, and 401–500, the sense primers listed in Table I were used. Sense primers for amplification of MPDE3B PCR fragments 201–250 and 184–250 contained an XhoI site followed by a Kozak sequence and an ATG start site; for fragments 301–450 and 401–500, primers contained a BglII site followed by a Kozak sequence and an ATG start site. Antisense primers are also shown in Table I. All contain a SacI site at the 3′ end. The PCR products were generated using the conditions outlined above and subcloned into pCRII (Invitrogen). The fragments were cloned from this vector as XhoI/SacI or BglII/SacI fused, in-frame, to the 5′ end of EGFP cDNA contained in vector pEFP-N1. These PDE3EGFP fusion proteins were designated pEFP-3B-(201–250), pEFP-3B-(301–450), and pEFP-3B-(401–500), respectively. All sequences and points of fusion between the 3′ ends of PDE3B fragments and pEFP-N1 were confirmed by sequencing.

**Construction of R3B-Δ101–266—RPDE3B pSV/SPORT (full-length rat (R) PDE3B (Ref. 31) cloned into pSV/SPORT vector) was digested with SmaI to remove the putative membrane association domain of PDE3B. NarI, which digests one of the short deleted fragments at a unique site, was also used to reduce the numbers of short fragments that would have double-ended SacII sites and therefore be able to reassociate with the large linearized R3B-Δ101–266/pSV SPORT. To reduce further risks of intermolecular reigation, the small digested fragments were removed by gel electrophoresis. The linearized R3B-Δ101–266/pSV SPORT was then recircularized with T4 DNA ligase and the deletion verified by sequencing.**

**DNA Sequencing—** DNA sequences were determined using the ABI Prism dye terminator cycle sequencing ready reaction kit (PerkinElmer Life Sciences) with the following PCR conditions: 25 cycles of 98 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. The extension products were purified using Centri-Sep columns (Princeton Separations). Automated DNA sequencing was performed for 16 h on an ABI 373A sequencer or for 7 h on an ABI 377 sequencer.

**Cell Culture—** COS-7 cells were cultured (37 °C, 5% CO₂, 95% air) in Dulbecco’s modified Eagle’s medium (high glucose DMEM, Life Technologies, Inc.) containing penicillin/streptomycin (100 units/ml/100 μg/ml) and supplemented with 10% (v/v) fetal calf serum. NIH-3006 cells, stably transfected with HPDE3A or RPDE3B (31), were grown as described previously (27). Ho-
primary antibody, staining with pre-immune serum, and staining of transfected cells not treated with detergent. Cells were viewed using a Nikon Diaphot fluorescence microscope fitted with a 60× oil immersion objective. For analysis by confocal microscopy, the cells were examined with a Leica model TCSD/DMIRBE microscope equipped with argon and argon-krypton lasers for blue (488 nm) and green (568 nm) excitation and a 100× objective. The images shown in the different figures are representative of those obtained from at least three separate immunostaining experiments. Three to five images per experiment were taken for each combination of staining patterns.

Subcellular Fractionation of 3T3-L1 Adipocytes—Subcellular fractions of differentiated 3T3-L1 adipocytes (in 100-mm tissue culture dishes), 13–14 days after initiation of differentiation, were prepared by a modification of Clark (37). The cell monolayers (20 dishes/experiment) were rinsed twice with ice-cold buffer A (20 mM Tris-HCl, pH 7.4, 2.5 mM EDTA, 250 mM sucrose, 0.5 mM Pefabloc, 10 μg/ml pepstatin, 10 μg/ml aprotinin, and 10 μg/ml leupeptin), harvested in buffer A, and homogenized (2 ml/dish, 27 strokes with a Teflon pestle, in a 40-ml homogenization vessel). Although 20 dishes were used per experiment, cells from 5 plates were harvested and combined for homogenization. Approximately 2 ml of the whole cell homogenate was removed for later analysis. The homogenate was centrifuged (approximately 100,000 × g, 10 min) to remove large fragments and whole cells. This supernatant was then centrifuged (13,000 × g, 20 min) using an SS-34 rotor (−10 ml/tube) at −4 °C (at all other times, the samples were handled on ice or at 4 °C). The infranatant between the fat cake and the pellet (P1) was removed using a syringe and a pipette. The fat cake was discarded and the infranatant centrifuged (15,000 × g, 20 min) to produce P2. P2 was resuspended, centrifuged (20,000 × g, 20 min), and supernatant.

Membrane Localization of PDE3

**FIG. 2.** Activities of marker enzymes in subcellular fractions from 3T3-L1 adipocytes. Assays were performed as described under “Materials and Methods.” **Right,** specific activities of enzymes in the various fractions. **Left,** the ratio of the specific activities of enzymes in various fractions to their specific activities in the whole cell homogenate (WCH = 1). Results for P/H in all panels and H/L in the citrate synthase panels represent means ± 1⁄2 range of two separate experiments. All other values represent mean activities ± S.E., n = 3 separate experiments. WCH, whole cell homogenate; M/N, mitochondria/nuclei; PM, plasma membrane; P/H, plasma membrane/high density microsomes; HDM, high density microsomes; H/L, high density microsomes/low density microsomes; LDM, low density microsomes; SUP, supernatant.
PDE3B and anti-BiP antibodies (ER) or anti-p58 (G3T3-L1 adipocytes were costained with anti-
PDE3B activity. NAPDH-cytochrome c reductase activity was measured in the presence of 10 ml of scintillation mixture.

PDE activity was determined by a modification of Manganiello and Vaughan (41). The assay mixture contained (in 100 μl): 37.5 mM Hepes, pH 7.4, 15 mM Tris-HCl, pH 7.4, .075 mM EGTA, 22.5 mM MgCl2, 0.1 μM cAMP containing approximately 30,000 cpm [3H]cAMP/assay, and plus or minus 1 μM chloramide to differentiate between total activity and PDE3 activity. NAPDH-cytochrome c reductase activity was determined by the method of Dallner (42), except that the assay (1.5 ml) contained 0.3 mM KCN and was performed at 37 °C.

Galactosyltransferase activity was determined by a modification of the methods of Sichel (43) and Fleischer (44). Briefly, the assay mixture contained (in 150 μl): 35 mM Hepes, pH 7.2, 45 mM NaCl, 40 mM MnCl2, 40 mM 2-mercaptoethanol, 40 mM N-acetylglucosamine (or 0 for controls), 0.67% Triton X-100, and 400 μM UDP-[14C]galactose (Gal) containing approximately 200,000 cpm/assay. Incubation without UDP-Gal was performed at 37 °C for 15 min, at which time substrate was added and the incubation continued for an additional 30 min. The reaction was stopped by the method of Sichel (43) and then 5/6 of the reaction mix was applied to a QAE-Sephadex (Pharmacia Biotech) column (1 ml) and eluted with 3.6 ml of H2O to separate 5′-AMP from adenosine. Radioactivity was measured by the method of Sichel (43) and then 5/6 of the reaction mix was applied to a QAE-Sephadex (Pharmacia Biotech) column (1 ml) and eluted with 3.6 ml of H2O. Radioactivity was measured in the presence of 10 ml of scintillation mixture.

RESULTS

Subcellular Localization of Native MPDE3B in 3T3-L1 Adipocytes—3T3-L1 fibroblasts contain little or no MPDE3B activity. Following differentiation into adipocytes, however, PDE3B activity is markedly increased in cell particulate fractions (5, 45). As seen in Fig. 2, during subcellular fractionation of 3T3-L1 adipocytes by differential centrifugation, PDE3B activity was associated primarily with ER/heavy density microsomal fractions enriched in NADPH-cytochrome c reductase activity.
activity, and not mitochondrial, plasma membrane, Golgi/low density microsomal, or cytosolic fractions. A similar fractionation pattern was previously reported by Kono and associates in rat adipocytes (8).

To determine the localization of native MPDE3B, fully differentiated 3T3-L1 adipocytes were also costained with a rabbit polyclonal antibody against the N terminus of RPDE3B (31) and with mouse monoclonal antibodies against the ER protein BiP and the Golgi marker p58 (Fig. 3, panels 1 and 2). To visualize the plasma membrane, cells were stained with rhodamine-labeled lens culinaris agglutinin (Fig. 3, panel 3). To prevent excessive agglutinin staining of internal membranes, cells were chilled and treated with agglutinin prior to fixation and permeabilization. Although agglutinin staining clearly demarcated plasma membranes, some internal agglutinin staining was also observed, but not to the extent of that observed with PDE3B, BiP, and p58.

Subcellular Localization of RPDE3B in Stably Transfected NIH 3006 Cells—NIH 3006 cells stably expressing RPDE3B were costained with a rabbit polyclonal antibody against the N terminus of RPDE3B (31) and with mouse monoclonal antibodies against BiP, p58, or the microtubular protein, β-tubulin (Fig. 4). Examination of the costained cells using confocal microscopy indicated the presence of a reticular network in NIH 3006 cells, as in 3T3-L1 adipocytes. The pattern of staining for RPDE3B in NIH 3006 cells was virtually identical to that for BiP, the ER marker. A composite image of the PDE and ER staining (Fig. 4, image 1c) demonstrates superimposition of the two staining patterns. In contrast to 3T3-L1 adipocytes (Fig. 3), where some endogenous MPDE3B associated with Golgi, there was little evidence for co-localization of RPDE3B with the Golgi marker in stably transfected NIH-3006 cells (Fig. 4). PDE3 staining was distinct from that for β-tubulin as well as p58. The co-localization patterns for PDE/p58 and PDE/β-tubulin were identical to those obtained for BiP/p58 and BiP/β-tubulin, providing further evidence that recombinant PDE3 reacts like an ER protein, as does endogenous PDE3B in murine 3T3-L1 adipocytes.

The specificity of the anti-PDE3B antibody was established by the absence of PDE immunostaining after incubation of the antibody with its antigenic peptide, after incubation of cells with second antibody only, or after incubation of nonpermeabilized cells with anti-PDE3B antibody. Incubation of cells with preimmune serum gave weak nonspecific nuclear staining, not abolished by prior incubation of antiserum with the RPDE3B antigenic peptide. Additionally, the anti-PDE3B antibody did not immunostain HPDE3A expressed in NIH 3006 cells (31). Incubation of anti-PDE3B antibody with peptide antigen also abolished staining on Western blots of the 123-kDa band normally present in lysates from MPDE3B-transfected cells (data not shown).

Construction of Truncated HPDE3A and MPDE3B Recombinants—In order to investigate which regions of PDE3 are int-
involved in membrane association, we first constructed a series of catalytically active N-terminal deletion mutants and studied their localization in COS cells and SF9 cells (32).

From analysis of secondary structure, the N-terminal portion of PDE3A and PDE3B can be arbitrarily divided into region 1 (aa 1–300) and region 2 (aa 301–500). Hydrophobicity plots (Fig. 5) reveal the presence of a large hydrophobic domain (spanning aa 60–255 and 68–250 in HPDE3A and MPDE3B, respectively) in region 1, followed by region 2, largely hydrophilic but containing a small hydrophobic domain.

The topology for HPDE3A and MPDE3B was predicted using the TMBASE data base for membrane-spanning protein segments (46). As illustrated in Fig. 6, one algorithm predicts the existence of six transmembrane helices within the hydrophobic domain in region 1. In this model, the N terminus begins near the cytoplasmic surface; subsequent sequence traverses the membrane six times, forming three transmembrane loops of different sizes, and ends on the cytoplasmic side. This model further predicts that succeeding amino acid sequence, including the catalytic domain, is situated on the cytosolic face of the membrane. In region 2, smaller hydrophobic domains (aa 340–390 and 320–370 in HPDE3A and MPDE3B, respectively) could also potentially be involved in membrane association.

Several PDE3 mutant proteins with deletions at the N terminus were constructed from HPDE3A and MPDE3B (Fig. 7). The HPDE3A mutants H3A-Δ189, H3A-Δ397, and H3A-Δ510 lack, respectively, the first 189, 397, and 510 aa. MPDE3B mutants M3B-Δ302 and M3B-Δ604 lack the first 302 and 604 aa, respectively. Positions of deletions are indicated in Figs. 5 and 6. H3A-Δ189 lacks the first two transmembrane loops. Both M3B-Δ302 (without region 1) and H3A-Δ397 (lacking region 1 and part of region 2) lack all six transmembrane helices: M3B-Δ302, not H3A-Δ397, retains the small hydrophobic domain in region 2 downstream to the hydrophobic domain in region 1. H3A-Δ510 and M3B-Δ604 lack both region 1 and region 2, as well as much of the downstream sequence preceding the catalytic domain.

To facilitate immunofluorescence detection of PDE3, full-length HPDE3A and MPDE3B and the N-terminal mutants were tagged with the Flag epitope, which was inserted at the extreme end of the hydrophilic C terminus to maximize accessibility of the epitope for antibody binding (Fig. 7). PDE activities observed after expression of untagged and Flag-tagged PDE constructs in COS-7 cells (data not shown) were similar, indicating that the Flag tag did not disrupt enzyme activity.

We expressed wild-type HPDE3A, MPDE3B, and N-terminal truncation mutants in SF9 insect cells and determined the subcellular distribution of their activities (32). HPDE3A, MPDE3B, and H3A-Δ189 activities were predominantly recovered in particulate fractions of SF9 cells. These recombinants, which contained all or part of the hydrophobic domain in region 1, were like integral membrane proteins, in that they could be solubilized with salt plus detergent (32). The activity of M3B-Δ302, which lacked region 1, was recovered in both cytosolic (60% of total) and particulate fractions. The association of M3B-Δ302 with particulate structures reflected that of a peripheral, non-integral membrane protein, and was disrupted by salt extraction (32). The small hydrophobic domain in region 2 could be responsible for this salt-sensitive association, since the activity of H3A-Δ397 (which lacks this domain) was predominantly cytosolic and its residual particulate activity was not salt-sensitive. Activities of M3B-Δ604 and H3A-Δ511, which lacked both regions 1 and 2, were almost completely cytosolic (32).

Expression of Flag-tagged Full-length and Truncated HPDE3A and MPDE3B Isoforms in COS-7 Cells—The Flag-tagged PDE constructs were expressed transiently in COS-7 cells; Western blots (Fig. 8) indicated that the subcellular distribution of the expressed proteins in these cells was similar to that in SF9 cells (32). Flag-tagged MPDE3B (Fig. 8a) and HPDE3A (Fig. 8b) were present in particulate but not in soluble fractions; H3A-Δ189 (Fig. 8b) was also predominantly in particulate fractions. M3B-Δ302 (Fig. 8a) was present in both fractions, but predominantly in the soluble fraction, as was H3A-Δ397 (Fig. 8b), which lacked region 1 and part of region 2. M3B-Δ604 (Fig. 8a) and H3A-Δ510 (Fig. 8b), were exclusively cytosolic. The immunoreactive bands of lower Mr in the MPDE3B, HPDE3A, H3A-Δ189, and H3A-Δ397 lysates could represent proteolytic fragments of PDE3 or a soluble translation product generated by utilization of a downstream initiation site. HPDE3A contains several internal methionines flanked by potential Kozak sequences, e.g. initiation at codons for methionine 484 or 485 may explain the lower Mr band seen in the HPDE3A, H3A-Δ189, and H3A-Δ397 Western blots. Initiation at any of the several potential alternative sites in MPDE3B does not appear to account for the size of the lower Mr band in the supernatant.
To visualize the intracellular localization of full-length and truncated PDE3 constructs, transfected COS-7 cells were immunostained with anti-Flag antibody (Fig. 9). This antibody did not stain COS-7 cells transfected with empty vector or nonpermeabilized, transfected COS-7 cells (data not shown). As seen in Fig. 9, both HPDE3A and MPDE3B showed strong reticular staining, with intense staining in the perinuclear region, similar to that observed for the ER marker BiP (data not shown). Staining for H3A\textsubscript{-}D\textsubscript{189} was identical to that of HPDE3A and MPDE3B. Distribution of M3B\textsubscript{-}\text{D}302 was similar to that of full-length MPDE3B (Fig. 9). In addition to reticular staining, in some images, M3B\textsubscript{-}\text{D}302 exhibited diffuse staining at the cell periphery. Localization of M3B\textsubscript{-}\text{D}302 in the extended ER is consistent with a significant portion (~40%) of the activity of expressed M3B\textsubscript{-}\text{D}302 in particulate fractions of Sf9 cells (32), and suggests that the small hydrophobic domain or other sequences in region 2 allow for interaction of M3B\textsubscript{-}\text{D}302 with the ER in COS-7 cells (Fig. 9). On the other hand, removal of region 1 in M3B\textsubscript{-}\text{D}302 does result in cytosolic localization of M3B\textsubscript{-}\text{D}302 in homogenates of COS-7 (Fig. 8) and Sf9 cells (32), and, in Sf9 cells, particulate M3B\textsubscript{-}\text{D}302 was almost completely solubilized by salt alone without detergent (32). Taken together, these results indicate that although the hydrophobic domain in region 2 might promote targeting of M3B\textsubscript{-}\text{D}302 to the extended ER (Fig. 9), the molecules associated as non-integral membrane proteins and could be readily dissociated or released by homogenization (Fig. 8). The transmembrane helices in region 1 are most likely responsible for the strong association of MPDE3B, HPDE3A, and H3A\textsubscript{-}D\textsubscript{189} with, and/or their insertion into, the ER (Fig. 9) as integral membrane proteins (32). In a related study, immunostaining (using the anti-N-terminal PDE3B antibody; Ref. 31) of R3B\textsubscript{-}D\textsubscript{101–266}, a RPDE3B mutant in which most of the hydrophobic domain in region 1 was deleted but which retained the small hydrophobic domain in region 2, demonstrated that the mutant, like M3B\textsubscript{-}\text{D}302, colocalized with BiP (data not shown).

As also seen in Fig. 9, H3A\textsubscript{-}D\textsubscript{397} staining was mainly diffuse, except in the perinuclear region, where some semblance of the reticular staining pattern observed with the larger constructs was evident. Staining for H3A\textsubscript{-}D\textsubscript{510} with or without detergent (32). In subcellular fractionation and immunoblotting, three localization patterns were observed. MPDE3B, HPDE3A, and H3A\textsubscript{-}D\textsubscript{189} were predomi-
nantly membrane-bound; M3B-D604 and H3A-A510 were predominantly cytosolic; and M3B-Δ302 and H3A-Δ397 were present in both membrane and cytosolic fractions. To evaluate the subcellular localization in more detail, one construct from each category, i.e. MPDE3B, M3B-Δ604, and H3A-Δ397, was expressed in COS-7 cells, alone or with soluble EGFP. MPDE3B-transfected cells were costained for PDE3 and either an ER or a Golgi marker. Cells cotransfected with MPDE3B and EGFP cDNA were stained for PDE3. Since mouse monoclonal anti-Flag antibody was used to stain Flag-tagged PDE3, it was not possible to co-stain the ER or Golgi with the mouse monoclonal anti-BiP or anti-p58 antibodies used in previous experiments. Rabbit polyclonal BiP or p58 antibodies gave poor results. Therefore, rabbit polyclonal anti-calreticulin and anti-β-COP antibodies, which gave staining patterns similar to BiP and p58, respectively, were used to stain the ER and the Golgi, respectively.

As shown in Fig. 10, in COS-7 cells, as in NIH 3006 cells, the MPDE3B staining pattern was identical to that of the ER marker calreticulin (panel 1), but not that of β-COP (data not shown) or EGFP (panel 2). The staining pattern for M3B-Δ604, on the other hand, was not like that for calreticulin (panel 3) or β-COP (data not shown), but was identical to that observed for EGFP (panel 4). The staining pattern for H3A-Δ397 differed from that for β-COP (data not shown), but was in some ways similar to those for calreticulin (panel 5) and EGFP (panel 6), which reinforces data from Western blotting experiments suggesting that this protein is present in the ER as well as the cytosol. The similarity of staining patterns of MPDE3B/EGFP and M3B-Δ604/ER to each other and to that of EGFP-transfected cells stained for the ER marker (data not shown) provides further proof that MPDE3B and M3B-Δ604 behaved like reticular and soluble proteins, respectively.

Expression of MPDE3B-EGFP Fusions in COS-7 Cells—Green fluorescent proteins have been widely used as a tool for identifying molecular targeting signals. Fusions of the putative transmembrane spanning domain of type I inositol 1,4,5-triphosphate receptor to GFP identified this region as the domain involved in association with the ER and in homotetramer formation (47). The first 35 aa of endothelial nitric-oxide synthase, which directed GFP from the cytosol to the Golgi network (48), were identified as a Golgi-targeting signal.

To more closely examine the roles of regions 1 and 2 in the membrane association of MPDE3B, a series of cDNAs, encoding PDE3B N-terminal fragments fused to the N terminus of EGFP, were constructed (Fig. 11). COS-7 cells expressing EGFP or the PDE3-EGFP fusion proteins were stained for the ER marker BiP and examined using confocal microscopy.

EGFP-3B-(1–400) encompasses all six transmembrane helical segments plus the smaller hydrophobic domain between aa 328 and 370, while EGFP-3B-(1–250), EGFP-3B-(1–200), and EGFP-3B-(1–100) encompass transmembrane segments 1–6, 1–4, and 1 in region 1, respectively (Fig. 11). As seen in Fig. 12, EGFP was diffusely distributed throughout the cell, including the nucleus. EGFP-3B-(1–100), which contained the first transmembrane helical segment, and the larger fusion proteins, EGFP-3B-(1–400), -3B-(1–250) (data not shown), and -3B-(1–200) (data not shown) colocalized with the ER marker BiP and were not detected in the nucleus. However, unlike the larger fusion proteins, EGFP-3B-(1–100) (similar to H3A-Δ397; Fig. 9) appeared predominantly in the perinuclear ER and not throughout the extended ER network. Since H3A-Δ189, which lacks the first four putative transmembrane helices, efficiently associated with the ER (Fig. 9), the role of helical segments 5–6 in membrane association were further examined using the fusion proteins EGFP-3B-(184–250), which encompasses transmembrane segments 5 and 6, and EGFP-3B-(201–250), which encompasses part of segment 5 and all of segment 6 (Fig. 11). As shown in Fig. 12, both EGFP-3B-(201–250) and EGFP-3B-(184–250) also colocalized with BiP.

Interestingly, unlike M3B-Δ302, EGFP-3B-(301–450), which contains the small hydrophobic domain between aa 328 and 370 (Fig. 11), did not display reticular staining, but was observed in the cytoplasm and nucleus (Fig. 12). This result suggests that additional structural information is needed for efficient targeting to the ER. It is also consistent with our results in COS-7 cells (Figs. 8 and 9) and SF9 cells (32), and indicates that, although structural elements in the hydrophobic domain in region 2 might be important in targeting to the ER (Fig. 9), this domain supports weak membrane association of M3B-Δ302 (which is readily disrupted by homogenization (Fig. 8) and salt extraction (Ref. 32), but does not allow for movement of EGFP-3B-(301–450) to, and/or its retention by, the ER (Fig. 12). EGFP-3B-(401–500) (data not shown), which is predominantly hydrophilic (Fig. 11), did not co-localize with BiP, but exhibited diffuse cytoplasmic and nuclear staining (Fig. 12).

DISCUSSION

Earlier studies demonstrated that native PDE3 is membrane-associated in both adipocytes and liver cells (8, 49). In rat adipocytes, PDE3, which is activated by insulin and plays a key role in mediating its antilipolytic action (14), constitutes...
PDE3 associated with the ER in NIH-3006 and COS-7 cells. These results, however, do not exclude the possibility of an association of PDE3 with other cellular membranes.

Our studies demonstrate that recombinant HPDE3A and MPDE3B contain domains that enable these proteins to associate with the ER in COS-7 cells and NIH-3006 fibroblasts and, for native MPDE3B, with ER in 3T3-L1 adipocytes. Although the primary structures of the N-terminal portions of HPDE3A and MPDE3B are quite different, the secondary structures appear to be highly conserved, and the predicted topology for the two isoforms is almost identical (see Figs. 5 and 6). The absence of the first two predicted transmembrane loops in region 1 (aa 1–300) of H3A-Δ189 (Fig. 6) did not appear to have a substantial effect on membrane-targeting/association. In Sf9 cells, MPDE3B, HPDE3A, and H3A-Δ189, which contained all or some of the transmembrane helical segments in the hydrophobic domain of region 1, exhibited characteristics of integral membrane proteins and were released from particulate fractions with salt and detergent (32). Thus, either the first 189 amino acids are not essential, or any part of the hydrophobic domain in region 1 may be sufficient for strong association with, or insertion into, ER membranes.

Unlike that of the other constructs, the membrane localization of M3B-Δ302 observed by immunofluorescence was not reflected in the immunoblots of subcellular fractions from COS-7 cells in which a considerable portion of M3B-Δ302 was cytosolic. In Sf9 cells, the M3B-Δ302 that associated with particulate fractions exhibited characteristics of peripheral, non-integral membrane proteins, and was solubilized with salt alone (32). Thus, although the small hydrophobic domain lying between aa 328 and 370 in region 2 of M3B-Δ302 may be adequate for targeting to, and some interactions with, ER membranes in intact cells, the weak association that is preserved during immunostaining may not be robust enough to withstand homogenization and subcellular fractionation in COS-7 cells, and extraction of particulate fractions with salt in Sf9 cells (32).

In Sf9 cells (32) and COS-7 cells, H3A-Δ397 is predominantly cytosolic. Although most of H3A-Δ397 was located in the cytoplasm, the fraction that associated with the ER seemed to be present predominantly in the perinuclear region. M3B-Δ302 lacks region 1, but contains the small hydrophobic domain between aa 328 and 370 in region 2 of MPDE3B. H3A-Δ397 lacks the small hydrophobic domain between aa 340 and 390 in region 2 of HPD3A. The localization of M3B-Δ302 and H3A-Δ397 suggests that the small hydrophobic domain between aa 340 and 390 in region 2 of HPD3A (absent in H3A-Δ397) contains information sufficient for targeting to the extended ER membrane network (as does the domain between aa 328 and 370 in M3B-Δ302) and that PDE3 membrane interactions may not be uniform throughout the ER. Although H3A-Δ397 retains information for targeting to the perinuclear ER (which might be a site of synthesis), in the absence of region 1 and the hydrophobic domain in region 2, this mutant lacks the ability to move to and/or anchor efficiently to the extended ER, and is easily dissociated from membranes. It is also possible that PDE3 staining is more intense in the perinuclear region than in other parts of the ER network because sites of specific PDE3 interaction are more numerous in this region, or because the sequence between aa 397 and 510 targets H3A-Δ397 to this region. Whether the greater intensity of staining in the perinuclear region is an artifact of overexpression remains to be determined, although perinuclear staining for the endogenous ER markers BiP and calreticulin was also more intense.

Evidence does exist for non-uniform distribution of ER proteins. In rat vas deferens smooth muscle, the patterns of dis-
distribution of the Ca^{2+} buffering molecules calsequestrin and calreticulin are distinct (51). The ratio of calsequestrin:calreticulin in the perinuclear SR is ~1:1, whereas in the peripheral ER calsequestrin is more abundant, with a ratio of 5:1. The ER retention sequence KDEL may dictate the uniform distribution of calreticulin, while the non-uniform distribution of calsequestrin (which lacks the KDEL motif) may depend on interactions with other anchoring proteins (52). Several different domains may cooperate in targeting certain proteins to the ER (53). For example, although microsomal aldehyde dehydrogenase contains a C-terminal ER retention signal (54), the hydrophilic domains flanking the hydrophobic region are also involved in targeting (55). Hydroxysteroid dehydrogenase type II is anchored to the ER via its N terminus, but deletion of the N-terminal hydrophobic region does not alter its localization, perhaps because hydroxysteroid dehydrogenase type II contains other hydrophobic segments (56). The first N-terminal transmembrane domain is sufficient to permit retention of the SR ATPase-Ca^{2+} pump in the ER. However, as with PDE3, other structural determinants are also involved, since SR ATPase-Ca^{2+} pump lacking the first two transmembrane domains was retained in the ER, albeit not as efficiently as the wild type protein (57).

In general, our studies of the localization of MPDE3B-EGFP fusion proteins were consistent with those using truncated, catalytically active recombinants. EGFP-3B-(1–250) and EGFP-3B-(1–200), containing all six and the first four transmembrane helical segments of region 1, respectively, did localize to the ER, as did EGFP-3B-(201–250) and EGFP-3B-(184–250). Since EGFP-3B-(201–250) contains only one complete (the sixth) transmembrane helical segment, perhaps one segment contains sufficient information for efficient association with the ER. Whether the other transmembrane segments in region 1, if synthesized individually as EGFP-3B fusion proteins, could readily associate with the ER is not known. EGFP-3B-(1–100), which contains one transmembrane helical segment, also associated with the ER, but with a perinuclear distribution similar to that of H3A-Δ397. Consistent with the weaker ER-interactions and cytosolic distribution of MB-Δ302 and H3A-Δ397, when separated from downstream sequences, EGFP-3B-(301–450) and EGFP-(401–500) fusion proteins did not associate with ER.

Taken together, our results suggest that structural elements in both regions 1 and 2 within the N-terminal portion are involved in the interaction of recombinant PDE3 with ER membranes (Fig. 6). Although the transmembrane helices in the hydrophobic domain of region 1 contain information that allows for strong interactions with, or insertion into, membranes and may serve as an ER retention signal, this information may not be confined to any one portion of this domain. Efficient targeting seems to also involve additional weaker (but important) interactions driven by the shorter downstream hydrophobic areas (aa 340–390 and 320–370 in HPDE3A and MPDE3B, respectively) and the adjacent hydrophilic sequences (i.e. sequences N-terminal to aa 510 and 604 in MPDE3B and HPDE3A, respectively) in region 2. The exact mechanism by which these domains enable recombinant PDE3, which lacks a KDEL sequence, to be directed to and associate with the ER remains to be elucidated. It is clear, however, that the ability of PDE3 to associate with the ER was completely absent in PDE3A or PDE3B mutants lacking both region 1 and region 2, since H3A-Δ510 and M3B-Δ604 were located exclusively in the cytosol and, in the latter case, also in the nucleus. The reason for nuclear localization of M3B-Δ604 is unclear. Since analysis of PDE3A and PDE3B sequences using the PSORT II program (58) does predict the presence of weak nuclear localization signals (NLS) in H3A-Δ510 and M3B-Δ604, the slightly larger size of H3A-Δ510 may prevent its entry into the nucleus. Alternatively, truncated M3B-Δ604 may contain stronger NLS or an as yet unidentified NLS which promotes nuclear entry, or it may gain access to the nucleus in association with a protein that contains its own strong NLS.

The membrane association domains of PDE3 could potentially be involved in interactions either with molecular chaperones, which assist in targeting and membrane insertion (59, 60) or with anchoring proteins analogous to the cAMP protein

FIG. 11. **N-terminal fragments of PDE3B used to construct EGFP-3B fusion proteins.** A series of cDNA fragments coding for various portions of the N-terminal region of PDE3B were used in-frame to the 5’ end of EGFP cDNA in vector pEGFP-N1 as described under “Materials and Methods.” For reference, each fragment is shown in alignment with its predicted secondary structure in MPDE3B.

![Diagram showing N-terminal fragments of PDE3B](image-url)
kinase anchoring proteins (AKAPs) which target PKA to the plasma membrane, ER, Golgi, mitochondria, peroxisomes, and microtubules (61). AKAPs provide an effective mechanism for compartmentalization of PKAs, providing sites where PKAs can respond optimally to fluctuations in cAMP levels and are in proximity to specific substrates such as PDE3B (5). As with AKAPs and PKA, the subcellular distribution of PDE3 isoforms differs in different cell types. Such isoforms are found in both the SR and the cytoplasm in myocardium (10–13). Since many of the components that regulate cardiac contractility are present in the SR, including PKA-RII and an anchoring protein AKAP 100 in rat cardiac myocytes (62, 63), it is not surprising that PDE3 should also exist in this location, where it could effectively regulate cAMP involved in contractility and other responses mediated by PKA.

Recent studies strongly support the notion that components of particular signaling pathways may be organized into signaling complexes to achieve specificity of signaling in cells where there is considerable overlap in pathways activated by different receptors. In adipocytes, for example, activation of PI3-kinase by insulin and PDGF initiates signaling via distinct pathways; activation of PI3-kinase by insulin, but not by PDGF, results in increased glucose transport. The selection of these two pathways may be related to the specific subcellular pool of PI3-kinase that is activated. Insulin preferentially stimulates microsomal PI3-kinase, whereas PDGF activates PI3-kinase in plasma membranes (64). A pool of PI3-kinase activated by insulin may exist in a complex with IRS-1 that is associated with the actin cytoskeleton (65); cytochalasin D, an inhibitor of actin filament assembly, reduces glucose transport by decreasing the association of PI3-kinase and IRS-1 with vesicles containing glucose-transporters, suggesting that the actin cytoskeleton is involved in the insulin-induced relocalization of the PI3-kinase and IRS-1 (66).

Compartmentalization may also be important in the regulation of components of kinase cascades, such as PKB, which are activated by PI3-kinase-dependent mechanisms. Both PI3-kinase and PKB activities are required for induction of meiotic maturation in Xenopus oocytes (67), and injection of mRNA encoding a constitutively active form of PKB, but not a mutant PKB lacking its membrane-targeting sequence, induced meiotic maturation, demonstrating that alterations in the location of signaling components produce dramatic effects on these responses (67). In rat adipocytes, insulin induced translocation of PKB to plasma membrane fractions (68) and of one specific PKB isoform, PKBβ, to a microsomal vesicle fraction enriched in glucose transporters (69). Since insulin, IRS-1, PKB-kinase, and AKAPs all upstream mediators of PDE3B activation, it will be of interest to determine whether translocation of IRS-1, PI3-kinase, and PKB is involved in activation of PDE3.

Involvement of PDE isoforms in the compartmentalization of cAMP signaling responses has been documented in several cell types. In patch clamp studies conducted on isolated frog ventricular myocytes, Jurevicius and Fischmeister (70) found that the β-adrenergic agonist, isoprenaline, induced an increase in cAMP near the sarcolemma, 40-fold greater than that in the rest of the cell. The PDE inhibitor 3-isobutyl-1-methylxanthine dramatically reduced this localized rise in cAMP concentration, with diffusion of the cAMP signal to distal regions of the myocyte, suggesting that PDE activity is an absolute requirement for the maintenance of compartmentalized cAMP responses. Although the specific PDE activities involved were not characterized in this study, others have demonstrated that compartmentalization of cAMP signaling can apparently be regulated by specific PDE isoforms. Chini et al. (16) showed that PDE4 in mesangial cells regulated a cAMP pool that activates PKA involved in inhibition of the production of reactive oxygen metabolites, while PDE3 regulated a cAMP pool that suppresses cell proliferation. Since PDEs provide the only known mechanism for terminating cellular effects induced by cyclic nucleotides, knowledge of the processes that drive membrane-targeting/association and compartmentalization of these enzymes will be vital in understanding PDE function and regulation of cAMP signaling responses (4).

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Membrane Localization of Cyclic Nucleotide Phosphodiesterase 3 (PDE3): TWO N-TERMINAL DOMAINS ARE REQUIRED FOR THE EFFICIENT TARGETING TO, AND ASSOCIATION OF, PDE3 WITH ENDOPLASMIC RETICULUM

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