Distinct roles of endoplasmic reticulum cytochrome b5 and fused cytochrome b5-like domain for rat Δ6-desaturase activity[S]

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Abstract The Δ6-desaturase catalyzes key steps in long-chain polyunsaturated fatty acid biosynthesis. Although the gene coding for this enzyme has been isolated in diverse animal species, the protein structure remains poorly characterized. In this work, rat Δ6-desaturase expressed in COS-7 cells was shown to localize in the endoplasmic reticulum. As the enzyme contains an N-terminal cytochrome b5-like domain, we investigated by site-directed mutagenesis the role of this domain in the enzyme activity. The typical HPGG motif of the cytochrome b5-like domain, and particularly histidine in this motif, is required for the activity of the enzyme, whatever the substrate. Neither endogenous COS-7 cytochrome b5 nor coexpressed rat endoplasmic reticulum cytochrome b5 could rescue the activity of mutated forms of Δ6-desaturase. Moreover, when rat endoplasmic reticulum cytochrome b5 was coexpressed with wild-type desaturase, both proteins interacted and Δ6-desaturase activity was significantly increased. The identified interaction between these proteins is not dependent on the desaturase HPGG motif. These data suggest distinct and essential roles for both the desaturase cytochrome b5-like domain and free endoplasmic reticulum cytochrome b5 for Δ6-desaturase activity.—Guillou, H., S. D'Andrea, V. Rioux, R. Barnouin, S. Dalaine, F. Pedrono, S. Jan, and P. Legrand. **Distinct roles of endoplasmic reticulum cytochrome b5 and fused cytochrome b5-like domain for rat Δ6-desaturase activity. *J. Lipid Res.* 2004.** 45: **32–40.**

Supplementary key words FADS2 • polyunsaturated fatty acid biosynthesis • hexadecenoic acid

Long-chain polyunsaturated fatty acids (PUFAs) such as arachidonic acid (C20:4n-6) and docosahexaenoic acid (C22:6n-3) play pivotal roles in a variety of biological functions (1). In animals, some of the daily needs in long-chain PUFAs are fulfilled from the diet. However, most of the long-chain PUFAs found in animal tissues are derived from the biosynthetic pathway involving elongations, Δ6-desaturation, and Δ5-desaturation for conversion of essential fatty acid precursors (C18:2n-6 and C18:3n-3) to their respective 20- and 22-carbon polyenoic products.

None of the desaturases involved in this biosynthetic pathway have been reproducibly purified, and their structure remains to be characterized. The only animal desaturase whose structure is known is the Δ9-desaturase (2). This enzyme is part of a multienzyme system present in the endoplasmic reticulum and is composed of Δ9-desaturase, NADH cytochrome b5 reductase, and cytochrome b5. In the process of double bond formation, the membrane-bound cytochrome b5 transfers electrons by lateral diffusion from NADH cytochrome b5 reductase to the Δ9 fatty acid desaturase (2).

Although the first mammalian Δ9-desaturase was cloned almost 20 years ago (3), mammalian desaturases involved in PUFA biosynthetic pathways, i.e., Δ6- and Δ5-desaturases, have been cloned more recently (4–8). Comparison of their respective amino acid sequences shows one major difference between Δ9-desaturase and Δ6- and Δ5-desaturases: an N-terminal cytochrome b5-like domain is present in Δ6- and Δ5-desaturases but not in Δ9-desaturase.

Numerous cytochrome b5-like domains have been identified in various desaturases from yeast, plants, and animals (9). This remarkable characteristic raises the possibility that NADH cytochrome b5 reductase transfers electrons to the catalytic site of these cytochrome b5 fusion desaturases directly via the cytochrome b5-like domain and does not require an independent cytochrome b5. The presence of such cytochrome b5-like domains in desaturase proteins is likely to have originated from a fusion with an ancestral cytochrome b5 gene that may have conferred some evolutionarily selectable advantage. Although these cytochrome

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Abbreviations: FCS, fetal calf serum; GC, gas chromatography.

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[S] The online version of this article (available at http://www.jlr.org) contains an additional three figures.

b5 fusion domains have diverged significantly, a typical HPGG motif has been conserved. This particular sequence forms an accessible heme binding core of the cytochrome b5-like domain (10). Among desaturases fused to a cytochrome b5-like domain, the cytochrome b5 domain has been demonstrated to be essential for borage Δ6-desaturase (11) and a yeast Δ9-acyl-CoA-desaturase (12).

The mammalian Δ6-desaturase, also named FADS2, has been cloned (4, 5), and its activity has been described (4, 5, 13–16). It was shown that expression of rat Δ6-desaturase (17) in a yeast strain deficient for cytochrome b5 was sufficient to confer to host cells the capacity to convert C18:2n-6 to C18:3n-6. However, coexpression of cytochrome b5 increased the level of Δ6-desaturated fatty acid (17) accumulation, suggesting that "free" cytochrome b5 is not strictly required but may play a role in Δ6-desaturation in this yeast model.

In this study, we compared, in COS-7 cells, the activity of recombinant wild-type rat Δ6-desaturase with the activity of mutated recombinant enzymes in which the typical cytochrome b5 ^{53}HPGG^{56} motif has been mutated or deleted. We also investigated in COS-7 cells the role of coexpressed microsomal cytochrome b5 in Δ6-desaturase activity and its putative capacity to compensate for the essentialness of the ^{53}HPGG^{56} motif in the Δ6-desaturase function reported here.

MATERIALS AND METHODS

Chemicals

cis-7,10,13,16,19-Docosapentaenoic acid (C22:5n-3) was purchased from Matreya (Pleasant Gap, PA). The characterized fatty acid methyl ester of C24:6n-3 (18) was a generous gift from Dr. K. Ishihara (National Research Institute of Fisheries Science, Yokohama, Japan). Other unlabeled fatty acids were from Sigma (St. Quentin Fallavier, France). Radiolabeled [1-^{14}C]18:3n-3 and [1-^{14}C]18:0 (52 mCi/mmol) were purchased from Perkin Elmer Life Science (Paris, France). Fetal calf serum (FCS) was purchased from Perbio (Bezons, France). Solvents (HPLC grade) were purchased from Fisher Scientific (Elancourt, France). Other reagents were from Sigma. The anti-cytochrome b5 (rabbit) polyclonal antibody and the anti-myc (mouse) monoclonal antibody used in this study were generous gifts from Dr. N. Borgese (University of Milan, Italy) and Dr. S. Suire (The Babraham Institute, Cambridge, UK), respectively.

Plasmid construction and site-directed mutagenesis

The pCMV-HAHA expression vector (19) was a generous gift from Dr. A. Atfi (Institut National de la Santé et de la Recherche Médicale U482, Hôpital Saint-Antoine, Paris, France). The pCMV/β-Gal expression vector was a generous gift from Dr. C. Diot (Unité Mixte de Recherche Institut National de la Recherche Agronomique-Ecole Nationale Supérieure Agronomique de Génétique Animale, Rennes, France). The plasmids constructed for the expression of rat Δ6-desaturase (referred to as pCMV/Δ6) have been previously described (13).

A plasmid coding for rat cytochrome b5 was constructed for expression in mammalian cells and is referred to as pcDNA3/cytb5. From the published (20) rat cytochrome b5 sequence (GenBank accession number D13205), oligonucleotide primers were designed to PCR amplify the entire coding sequence, with its stop codon using the high-fidelity *Pfu* polymerase from Promega (Lyon, France). The forward primer (5′-CAATGGATC-C**ATG**CCGGCCCACATGC-3′) included the translation start codon (boldface) and the *Bam*HI restriction site (underlined). The reverse primer (5′-CGTGCTCGAGC**TCA**GCTACTCTTGT-GGCT-3′) contained the translation stop codon (boldface) and the *Xho*I restriction site (underlined). The PCR product amplified from rat liver cDNA was treated with *Bam*HI and *Xho*I before cloning into pcDNA3 (Invitrogen, San Diego, CA). The *Hind*III-*Sal*I fragment containing the full-length rat cytochrome b5 cDNA was subcloned in frame from pcDNA3 into pCMV-HAHA. This construction is referred to as pCMV-HAHA/cytb5 and allows the expression of a cytochrome b5 fused N-terminally to a double hemagglutinin (HA) epitope.

A plasmid coding for a C-terminally myc-tagged Δ6-desaturase was constructed using pCMV for expression in mammalian cells and is referred to as pCMV/Δ6myc. From the published rat Δ6-desaturase sequence (5) (GenBank accession number AB021980), oligonucleotide primers were designed to amplify, by PCR, the entire coding sequence with a deleted stop codon. The forward primer (5′-CAGTGGATCC*ATG*GGGAAGGGAGGTA-3′) included the translation start codon (italics) and the *Nco*I restriction site (underlined). The reverse primer (5′-TGTGCGGCCGC*TTTGT-GGAGGTAGGCATCC-3′*) corresponded to the C-terminal sequence of the protein (italics) without its stop codon and a *Not*I site (underlined). The PCR product amplified from rat liver cDNA was treated with *Nco*I and *Not*I before cloning into pCMV/myc/cyto (Invitrogen).

Mutagenesis of the ^{53}HPGG^{56} motif in the N-terminal cytochrome b5 domain of rat Δ6-desaturase was performed using a site-directed mutagenesis kit (QuickChange; Stratagene, Amsterdam, The Netherlands) according to the manufacturer's protocol. Three sets of two mutagenic primers were designed (**Table 1**). In each set, both primers are complementary to the opposite strands of pCMV/Δ6 and insert the desired mutation or deletion. These primers were used to delete the ^{53}HPGG^{56} domain, to delete H^{53}, or to replace H^{53} with an alanine, providing new expression vectors named pCMV/Δ6−^{53}HPGG^{56}, pCMV/Δ6−H^{53}, or pCMV/Δ6−H^{53}A, respectively.

Sequences coding for Δ6-desaturase and Δ6-desaturase with deletion of the sequence corresponding to the ^{53}HPGG^{56} motif were

TABLE 1. Primers used for site-directed mutagenesis

Mutagenic Primers (5′–3′)	Mutation or Deletion	Name of the Plasmid
CCAAATGGTCCCAGCGG**GCC**CCAGGGGGGCACCGTG and CACGGTGCCCCCCTGG**GGC**CCGCTGGGACCATTTGG	Alanine substituted for H^{53}	pCMV/Δ6−H^{53}A
CCAAATGGTCCCAGCGGXCCAGGGGGGCACCGTG and CACGGTGCCCCCCTGGCCXGCTGGGACCATTTGG	H^{53} deleted	pCMV/Δ6−H^{53}
CCAAATGGTCCCAGCGGXXXXCACCGTGTCATCGGACAC and GTGTCCGATGACACGGTGXXXXCCGCTGGGACCATTTGG	^{53}HPGG^{56} deleted	pCMV/Δ6−^{53}HPGG^{56}

The mutagenic codon is indicated in boldface. The codon deletions are indicated (X).

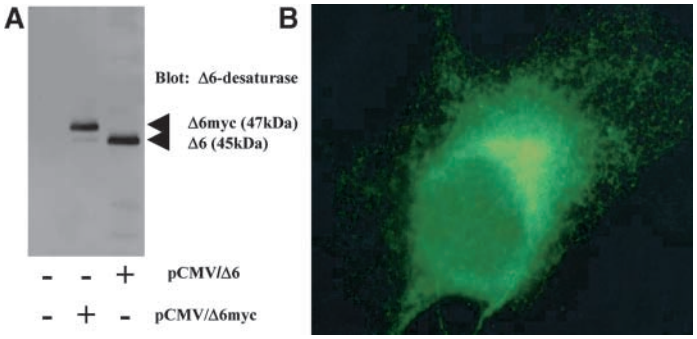

Fig. 1. A: Expression of myc-tagged rat Δ6-desaturase was assessed by Western blotting of transiently transfected COS-7 cell lysates using anti-rat Δ6-desaturase sera. B: Localization of myc-tagged Δ6-desaturase expressed in COS-7 cells. COS-7 cells were transiently transfected with pCMV/Δ6myc, fixed, permeabilized, and stained for myc epitope using FITC-labeled secondary antibodies. No signal was detected in nontransfected cells incubated under the same conditions (data not shown).

used for PCR amplification with oligonucleotide primers before subcloning into p3×Flag (Sigma). The forward primer (5′-GACTGAAGCTT*ATG*GGGAAGGGAGGTA-3′) included the translation start codon (italics) and a *Hind*III restriction site (underlined). The reverse primer (5′-CATGCGGATCC*TCA*TTTGTGGAGGTAGGCATCC-3′) contained the translation stop codon (italics) and a *Bam*HI site (underlined). The PCR products were treated with *Hind*III and *Bam*HI before cloning into p3×Flag. The plasmids are referred to as p3×Flag/Δ6 and p3×Flag/Δ6-^{53}HPGG^{56} and allow the expression of N-terminally Flag-tagged desaturases.

The integrity of the constructs and the presence of the desired deletions or mutations were assessed by DNA sequencing.

Cell culture and transfection

COS-7 cells were routinely maintained at ~50% confluence and were cultured in DMEM containing 10% FCS, 50 IU/ml penicillin, and 50 μg/ml streptomycin. The cells were split 1 day before transfection to 30% confluence and transfected the next day using the Easyject Plus electroporator (Equibio, Monchelsea, UK) according to the manufacturer's instructions. Briefly, 10^6 COS-7 cells in 0.8 ml of DMEM were mixed with 30 μg of purified plasmid, electroporated at 250 V and 1,500 μF with unlimited resistance, and seeded on a 10 cm dish containing culture medium.

Immunofluorescence

Coverslips containing the paraformaldehyde-fixed cells transfected with pCMV/Δ6myc were washed in PBS (150 mM NaCl and 5 mM Na phosphate, pH 7.4) and preincubated for 10 min on a drop of blocking buffer containing Triton X-100 (PBS containing 0.5% BSA and 0.1% Triton X-100). The cells were extensively washed with blocking buffer and incubated for 30 min with the primary antibody (monoclonal anti-Myc) in blocking buffer (1:2 dilution). After extensive washes in blocking buffer, the cells were incubated for 30 min with the fluorescent secondary antibody (anti-mouse IgG FITC; Sigma) diluted in blocking buffer (1:200 dilution). The coverslips were again washed extensively in blocking buffer and once in PBS, mounted in Tris-HCl (0.5 M, pH 8.5) containing 70% glycerol, and observed under a Leica DMRB microscope equipped for epifluorescence.

Incubation of transfected COS-7 cells with fatty acid albuminic complex

The functionality of the expressed protein was investigated by incubating the transfected COS-7 cells with different fatty acid albuminic complexes. Each fatty acid was saponified by incubation for 30 min at 70°C with 2 M KOH in ethanol. The resulting fatty

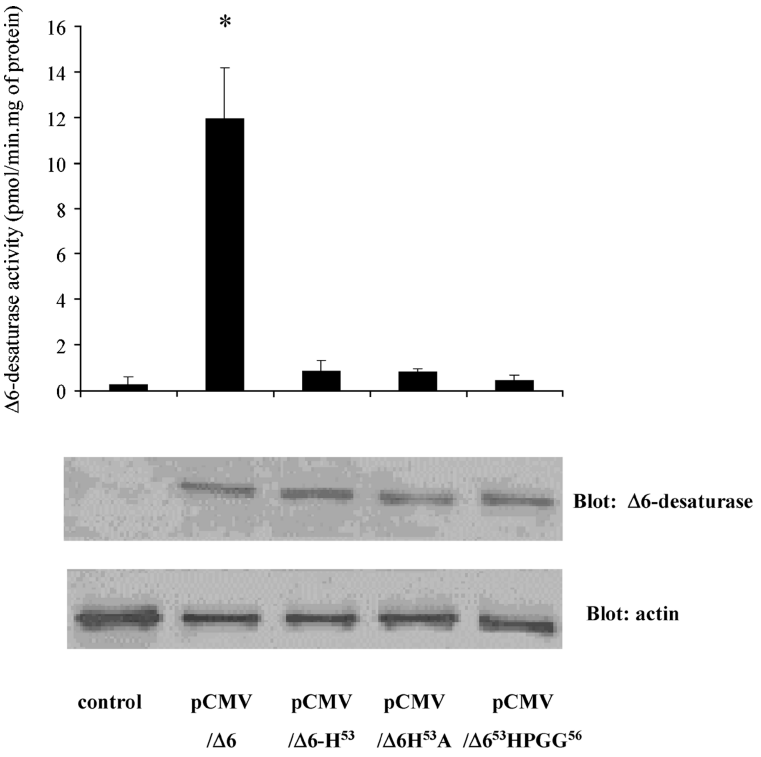

Fig. 2. In vitro desaturation of [1-^{14}C]C18:3n-3 measured in COS-7 cell homogenates not transfected (control) or containing wild-type (transfected with pCMV/Δ6) and mutated forms (transfected with pCMV/Δ6−H^{53}A, pCMV/Δ6−H^{53}, or pCMV/Δ6−^{53}HPGG^{56}) of rat Δ6-desaturase. Desaturase activity was calculated from the level of [1-^{14}C]C18:4n-3 produced, normalized to β-galactosidase activity, and expressed as means ± standard deviations between triplicates. Expression of wild-type and mutated forms of desaturase was assessed by Western blotting of cell lysates. Anti-actin level was assessed by Western blotting as a loading control. This result is representative of three independent experiments. The asterisk indicates a significant difference compared with the control by Student's *t*-test ($P < 0.05$).

acid salt was dissolved at pH 10 in DMEM containing 1% (w/v) BSA. After 15 min of sonication followed by 5 h of shaking, the pH was adjusted to 7.3. FCS was added (10%, v/v), and the final fatty acid concentration of the incubation medium was 0.2 mM unless stated otherwise. At 3 h after transfection, the incubation of COS-7 cells was initiated by replacing the culture medium with 20 ml of the fatty acid-containing medium per 10 cm dish. Incubation was performed for 24 h at 37°C in 5% CO_2 atmosphere.

Fatty acid analysis

COS-7 cells were washed twice with ice-cold PBS (150 mM NaCl and 5 mM Na phosphate, pH 7.4) and scraped into PBS. After centrifugation, the cell pellet was resuspended in PBS and sonicated at 20 W for 5 s. The protein content of the cell homogenate was determined by a modified Lowry procedure (21). Cellular lipids were extracted with hexane-isopropanol (3:2; v/v) as described previously (22). After saponification, fatty acids were methylated with boron trifluoride (14% in methanol) at 70°C for 30 min. Fatty acid methyl esters were extracted with pentane and analyzed by gas chromatography using an Agilent Technologies 6890N (Bios Analytique, Toulouse, France) with a split injector (1:20) at 250°C and a bonded silica capillary column (30 m × 0.25 mm internal diameter; BPX 70; SGE, Villeneuve-St-Georges, France) with a stationary phase of 70% cyanopropylpolysilphenylene-siloxane (0.25 μm film thickness). Helium was used as the gas vector (average velocity, 24 cm/s). The column temperature program started at 150°C, was ramped at 2°C/min to 220°C, and was held at 220°C for 10 min. The flame ionization detector temperature was 250°C. Identification of fatty acid methyl ester peaks was based on retention times obtained for methyl esters prepared from fatty acid standards.

Enzyme assay

Cell homogenates were prepared as described above at 48 h after transfection. Desaturase activity was assayed in a 1 ml mixture containing 100 μl of cell homogenate (5–8 mg protein/ml),

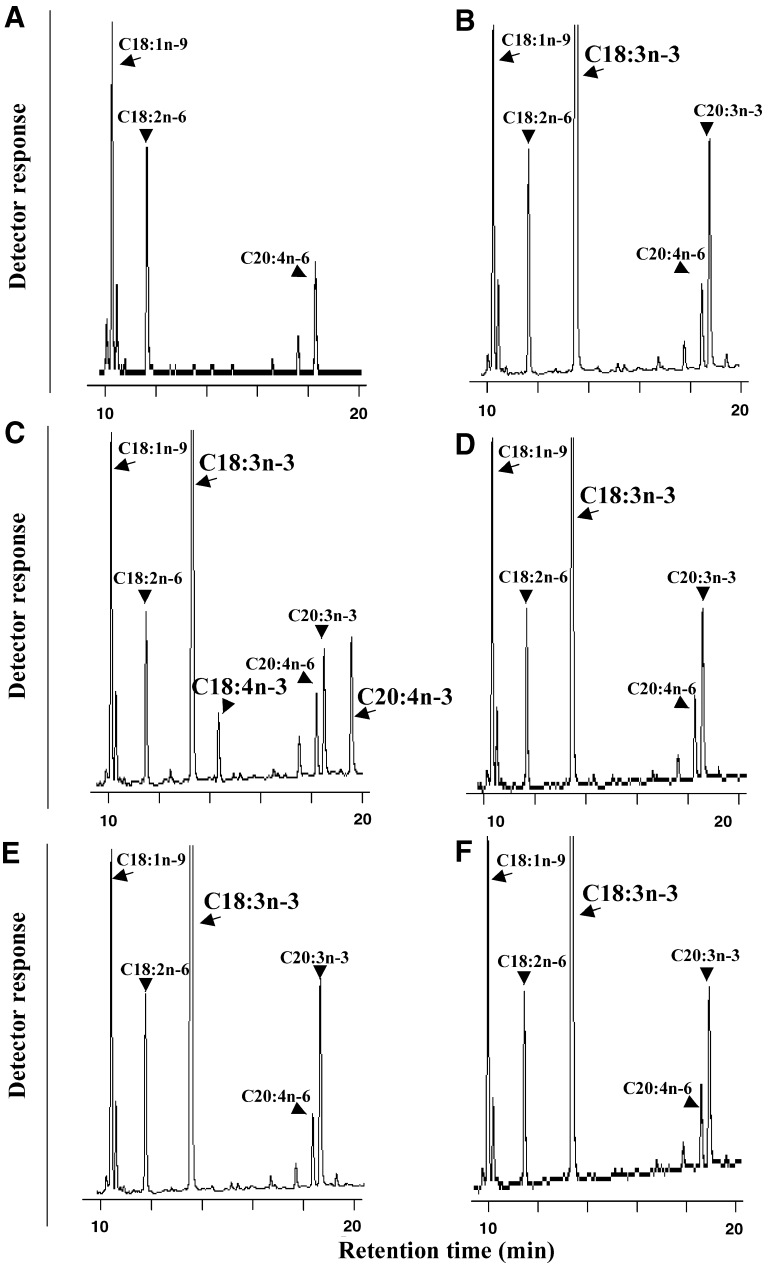

Fig. 3. Gas chromatography(GC) analysis of fatty acid methyl ester from COS-7 cells (A), COS-7 cells incubated with C18:3n-3 (B), or COS-7 cells transiently transfected with pCMV/Δ6 (C), pCMV/Δ6−H^{53} (D), pCMV/Δ6−H^{53}A (E), or pCMV/Δ6−^{53}HPGG^{56} (F) incubated with C18:3n-3. COS-7 cells were transfected or not and subsequently cultivated or not for 24 h with albumin-bound C18:3n-3 (200 μM). Then, the cells were washed extensively with PBS and cellular fatty acids were prepared for GC analysis of fatty acid methyl esters as described in Materials and Methods. The results presented are representative of three independent experiments.

150 mM phosphate buffer (pH 7.2), 6 mM $MgCl_2$, 7.2 mM ATP, 0.54 mM CoA, and 0.8 mM NADH. The reaction was started by adding 60 nmol of [1-^{14}C]18:3n-3 (52 mCi/mmol) and stopped with 1 ml of 2 M KOH in ethanol after 1 h of incubation at 37°C. To assess the substrate quality, a control assay was also run by stopping the reaction before adding the substrate. Fatty acid saponification was performed at 70°C for 30 min. After acidification, the fatty acids were extracted with diethylether, converted to fatty acid naphthacyl esters, and separated by HPLC as described previously (23). Collected fractions were subjected to liquid scintillation counting (Packard Tri-Carb 1600 TR, Meriden, CT). Desaturase activities were normalized for transfection efficiency by measuring the β-galactosidase activity corresponding to 3 μg of a cotransfected β-galactosidase-expressing vector (pCMV/β-Gal).

The β-galactosidase activity was assayed at 37°C in 20 μl of lysate mixed with 142 μl of 0.1 M phosphate buffer (pH 7.5), 2.5 μl of 0.1 M $MgCl_2$, 4.5 M β-mercaptoethanol, and 55 μl of *O*-nitrophenyl β-D-galactopyranoside (Sigma) (4 mg/ml in 0.1 M phosphate buffer).

Immunoprecipitation and immunoblotting

Two days after transfection, cells were lysed at 4°C in lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM EDTA, 10% glycerol, 1% Nonidet P-40, 1 mM PMSF, and 10 μg/ml aprotinin). Lysates were subjected to 12 h of immunoprecipitation with 1 μg of either monoclonal anti-Flag M2 (Sigma) or polyclonal anti-HA Y11 (Santa Cruz Biotechnologies, Le Perray-en-Yvelines, France) followed by adsorption to Sepharose-coupled protein G (Sigma) for 3 h. Immunoprecipitates were separated by SDS-PAGE and analyzed by immunoblotting. For determination of total protein levels, aliquots of cell lysates were also subjected to direct immunoblotting.

Reduced protein samples were analyzed by SDS-PAGE and blotted onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). To measure wild-type and mutant Δ6-desaturase expression, the anti Δ6-desaturase serum S1 targeting the C-terminal region of the protein was used at a 1:2,000 dilution, as described previously (13). To measure cytochrome b5 expression, anti-cytochrome b5 (24) was used at a 1:200 dilution. Anti-HA was used at a 1:200 dilution, anti-Flag was used at a 1:300 dilution, and anti-actin (Sigma) was used at a 1:100 dilution. The secondary antibody was a peroxidase-conjugated anti-rabbit IgG (Sigma) or a peroxidase-conjugated anti-mouse IgG (Sigma). Saturation and incubation with antibodies were performed for 90 min in TBS (20 mM Tris-HCl and 150 mM NaCl, pH 7.4) containing 0.05% Tween-20 and 5% nonfat dry milk. Washes were performed in TBS containing 0.05% Tween-20. Peroxidase activity was revealed using ECL Plus reagent according to the manufacturer's instructions (Amersham Biosciences, Uppsala, Sweden) and scanned with the Molecular Dynamics Storm (Amersham Biosciences).

RESULTS

Immunolocalization of rat Δ6-desaturase expressed in COS-7 cells

A C-terminally myc-tagged rat Δ6-desaturase was expressed in COS-7 cells. Myc-tagged Δ6-desaturase has a molecular mass of 47 kDa as detected by Western blot using a serum targeting rat Δ6-desaturase (**Fig. 1A**). Using anti-myc antibody, we determined the subcellular localization of C-terminally myc-tagged rat Δ6-desaturase expressed in COS-7 cells. In transiently transformed COS-7 cells, a perinuclear network was observed (Fig. 1B). This typical pattern suggests that rat Δ6-desaturase localizes in the endoplasmic reticulum.

Expression and activity of wild-type and mutated rat Δ6-desaturases in COS-7 cells

The wild-type (Δ6) and the three mutated forms of rat Δ6-desaturases with deletion of the ^{53}HPGG^{56} motif (Δ6−^{53}HPGG^{56}), deletion of H^{53} (Δ6−H^{53}), or substitution of an alanine for H^{53} (Δ6−H^{53}A) expressed in COS-7 cells were analyzed by Western blotting. A serum targeting the C-terminal region of the protein (13), which has not been modified by site-directed mutagenesis, was used to probe the membranes. Western blotting revealed that the wild type and the three mutated forms of rat Δ6-desaturase were similarly expressed in COS-7 cells (**Fig. 2**). The deletion of the ^{53}HPGG^{56} motif or of H^{53}, or the substitution of an alanine for H^{53} did not alter rat Δ6-desaturase expression in this cell line.

The in vitro Δ6-desaturase assay performed on the COS-7 cell lysates corresponding to the samples used for Western blotting showed a dramatic increase in Δ6-desaturase activity only in cells expressing the wild-type enzyme, compared with nontransfected cells (Fig. 2). The deletion of the ^{53}HPGG^{56} motif or of H^{53}, or the substitution of an alanine for H^{53} suppressed the activity of Δ6-desaturase as measured in vitro.

TABLE 2. Desaturation index of COS-7 cells not transfected (control) or transfected with pCMV/Δ6, pCMV/Δ6−H^{53}, pCMV/Δ6−H^{53}A, or pCMV/Δ6−^{53}HPGG^{56} and incubated with different Δ6-desaturase substrates

Incubated Fatty Acid	C16:0	C18:2n-6	C22:5n-3
Δ6-Desaturase substrate	C16:0	C18:2n-6	C24:5n-3
Desaturation index (%)	C16:1n-10/C16:0 × 100	(C18:3n-6 + C20:3n-6)/C18:2n-6 × 100	C24:6n-3/C24:5n-3 × 100
Transfection			
Control	0.72	1.27	nd
pCMV/Δ6	15.31	37.22	9.58
pCMV/Δ6−H^{53}	0.78	2.04	nd
pCMV/Δ6−H^{53}A	0.81	1.62	nd
pCMV/Δ6−^{53}HPGG^{56}	0.55	2.08	nd

COS-7 cells were transfected or not and subsequently cultivated for 24 h with distinct albumin-bound fatty acids (200 μM). Then, the cells were washed extensively with PBS and cellular fatty acids were prepared for gas chromatography analysis as described in Materials and Methods. nd, the Δ6-desaturation product was not detectable.

Fatty acid analysis of COS-7 cells expressing wild-type and mutated rat Δ6-desaturases

We analyzed the fatty acid profiles of COS-7 cells transfected or not with pCMV/Δ6, pCMV/Δ6−^{53}HPGG^{56}, pCMV/Δ6−H^{53}, or pCMV/Δ6−H^{53}A and incubated or not with C18:3n-3 for 24 h (**Fig. 3**). In each case, the presence of recombinant desaturases was assessed by Western blotting (data not shown). The presence of C20:3n-3 in cells incubated with C18:3n-3 shows that these cells have incorporated and elongated the C18:3n-3 (Fig. 3B–F). In COS-7 cells that have not been incubated with C18:3n-3, the presence of C20:3n-3 was not detected (Fig. 3A). Two additional peaks were observed in cells expressing the wild-type rat Δ6-desaturase (Fig. 3C), whereas these peaks were absent in nontransfected COS-7 cells (Fig. 3A, B) and in cells expressing the mutated forms of Δ6-desaturase (Fig. 3D–F). These two additional fatty acids were identified as C18:4n-3, produced by Δ6-desaturation of C18:3n-3, and C20:4n-3, which corresponds to newly synthesized C18:4n-3 subsequently elongated in COS-7 cells. Together with our in vitro analyses, these results indicate that only the wild-type form of Δ6-desaturase confers to this cell line the capacity of C18:3n-3 Δ6-desaturation.

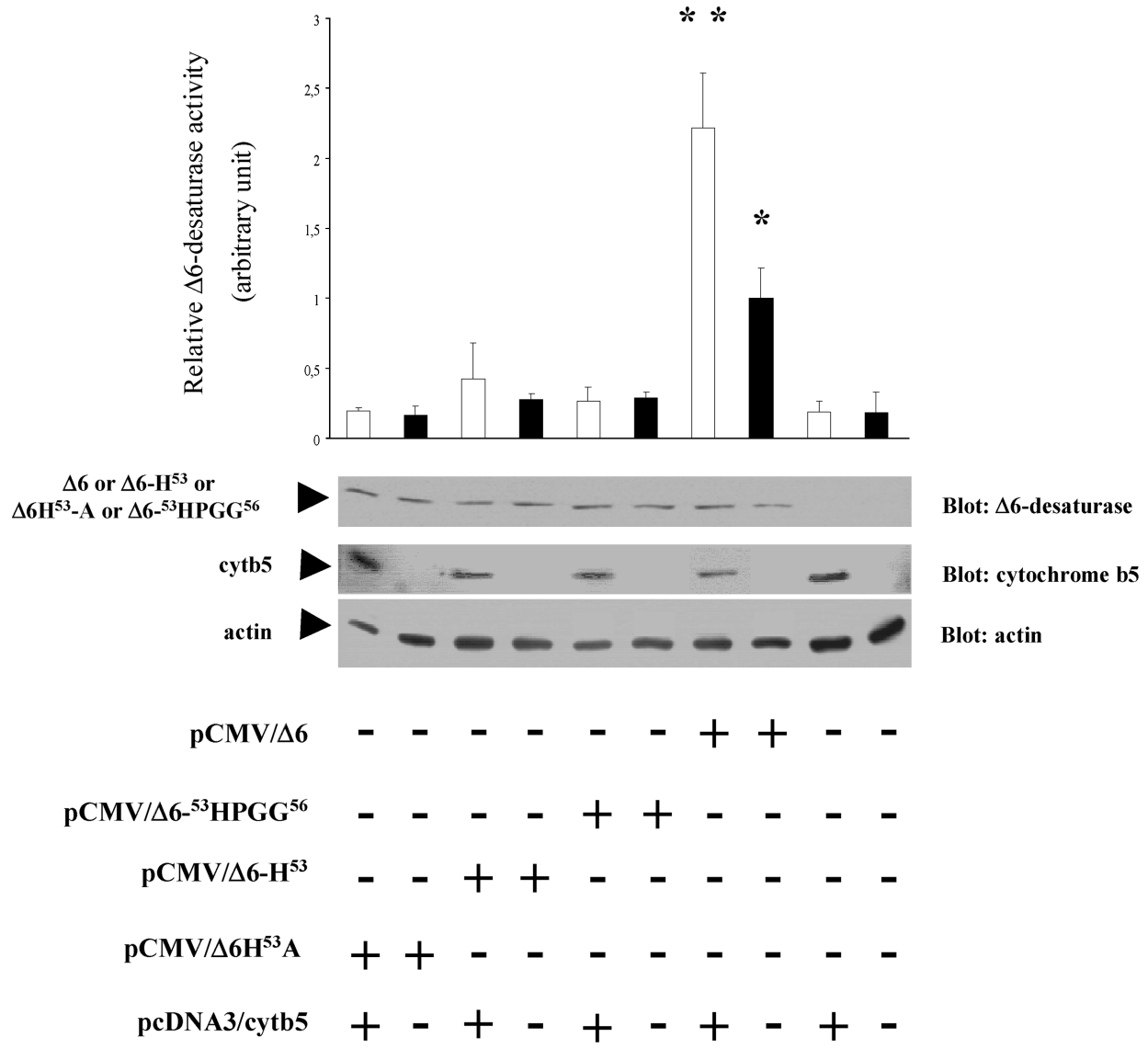

Fig. 4. Effect of coexpression of rat cytochrome b5 on the activity of wild-type and mutant Δ6-desaturases. COS-7 cells were transfected or not with pCMV/Δ6, pCMV/Δ6−H^{53}, pCMV/Δ6−H^{53}A, or pCMV/Δ6−^{53}HPGG^{56} in the absence or presence of pcDNA3/cytb5. Cell lysates were subjected to Δ6-desaturase assay in vitro, the desaturase activity was calculated from the level of [1-^{14}C]C18:3n-3 desaturated to [1-^{14}C]C18:4n-3, and the activity was normalized to β-galactosidase activity. Results are presented relative to the activity measured in COS-7 cells transfected with pCMV/Δ6 alone. As controls, cell lysates were also subjected to immunoblotting with anti-Δ6-desaturase (Blot: Δ6-desaturase), anti-cytochrome b5 (Blot: cytochrome b5), and anti-actin (Blot: actin). The results are presented as means of three independent transfections, because each transfection activity was assayed in triplicate. Error bars indicate SEM. The asterisk indicates a significant difference, compared with nontransfected cells ($P < 0.05$), and the double asterisk indicates a significant increase compared with nontransfected cells ($P < 0.05$) and with cells transfected with pCMV/Δ6 alone ($P < 0.05$).

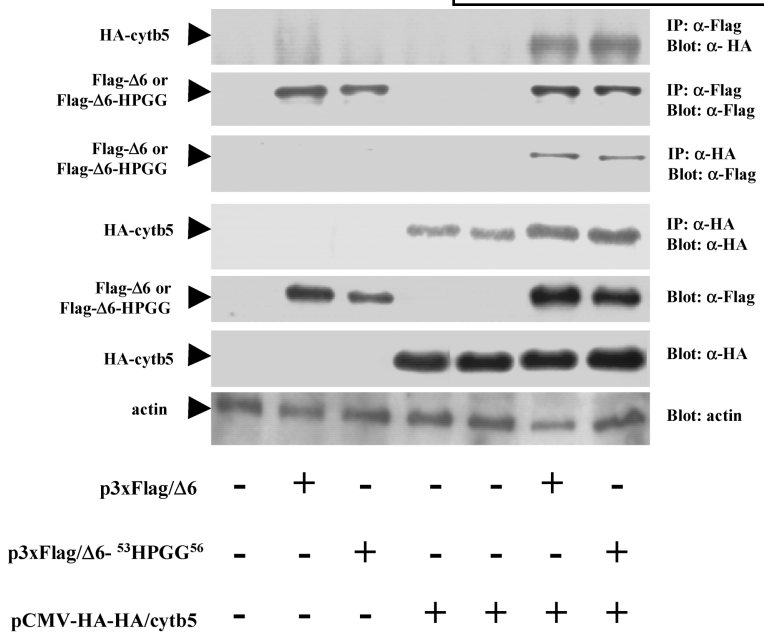

Fig. 5. The rat Δ6-desaturase ^{53}HPGG^{56} motif is not required for association with cytochrome b5. COS-7 cells were transfected with p3×Flag/Δ6 or p3×Flag/Δ6-HPGG in the absence or presence of pCMV-HAHA/cytb5. Cell lysates were subjected to immunoprecipitation with anti-Flag antibody and anti-hemagglutinin (HA) antibody. The Flag-tagged desaturase immunoprecipitation was visualized with Flag immunoblotting (IP: α-Flag, Blot: α-Flag), and the HA-tagged cytochrome b5 immunoprecipitation was visualized with HA immunoblotting (IP: α-HA, Blot: α-HA). The association of HA-tagged cytochrome b5 with Flag-tagged desaturases was visualized with HA immunoblotting (IP: α-Flag, Blot: α-HA) and with Flag immunoblotting (IP: α-HA, Blot: α-Flag). As controls, cell lysates were also subjected to immunoblotting with anti-Flag (Blot: α-Flag), anti-HA (Blot: α-HA), and anti-actin (Blot: actin).

We first used α-linolenic acid (C18:3n-3) for functional analysis of wild-type and mutated forms of Δ6-desaturase. However, it has been shown that the gene encoding animal Δ6-desaturase (FADS2) encodes a protein that acts not only on 18-carbon polyunsaturated substrates (4, 5) but also on 24-carbon PUFA (13, 14) and palmitic acid (15). Therefore, we investigated the activity of the wild-type and mutated forms of Δ6-desaturases in several other substrates. For this, COS-7 cells transfected with pCMV/Δ6, pCMV/Δ6−^{53}HPGG^{56}, pCMV/Δ6−H^{53}, or pCMV/Δ6−H^{53}A were incubated in the presence of palmitic acid (C16:0), linoleic acid (C18:2n-6), or docosapentaenoic acid (C22:5n-3). After 24 h of incubation, the cellular fatty acid methyl ester profiles were analyzed and used to determine the level of Δ6-desaturation. The expression of each form of recombinant desaturase was systematically assessed by Western blotting (data not shown). As shown in **Table 2**, regardless of which fatty acid was incubated with COS-7 cells, only the wild-type form of rat Δ6-desaturase conferred to the cells the capacity to act on C16:0, C18:2n-6, and C24:5n-3 (produced by cellular elongation of incubated C22:5n-3), compared with nontransfected cells. The level of C16:1n-10, C18:3n-6, and C24:6n-3 detectable in transfected cells expressing mutated forms of Δ6-desaturase was similar to that in nontransfected cells (see supplementary data).

Effect of rat cytochrome b5 on the activity of wild-type and mutated rat Δ6-desaturases in COS-7 cells

The microsomal form of rat cytochrome b5 was cotransfected with the wild-type or mutated forms of Δ6-desaturase in COS-7 cells. The expression of Δ6-desaturases and cytochrome b5 was controlled by Western blotting (**Fig. 4**). Then, relative Δ6-desaturase activities were measured in vitro on the cell lysates. This assay showed that overexpression of rat microsomal cytochrome b5 cannot compensate for the effect of the deletions or the mutation in the ^{53}HPGG^{56} motif of the rat Δ6-desaturase cytochrome b5 domain (Fig. 4). Interestingly, coexpression of rat endoplasmic reticulum cytochrome b5 with wild-type Δ6-desaturase markedly increased (2.2-fold) the Δ6-desaturase activity, compared with Δ6-desaturase alone.

Interaction between wild-type or mutated Δ6-desaturase and cytochrome b5

The possibility that Δ6-desaturase may interact with cytochrome b5 was investigated in COS-7 cells. Immunoprecipitation of lysates from transfected COS-7 cells with an antibody directed against Flag-tagged Δ6-desaturase revealed the presence of HA-tagged cytochrome b5 (**Fig. 5**). Similarly, when immunoprecipitation was performed with an antibody directed against HA-tagged cytochrome b5, the Flag-tagged Δ6-desaturase was coimmunoprecipitated. Interestingly, Flag-tagged Δ6-desaturase lacking the HPGG motif also coimmunoprecipitated with HA-tagged cytochrome b5, suggesting that the ^{53}HPGG^{56} motif of the desaturase is not required for interaction between Δ6-desaturase and cytochrome b5.

DISCUSSION

This study presents the localization in an animal cell line of rat Δ6-desaturase in the endoplasmic reticulum (Fig. 1) and provides the first evidence for the requirement of the ^{53}HPGG^{56} motif, and at least H^{53} in this motif, in the cytochrome b5-like domain of this enzyme. As the mutated proteins had no activity when expressed at levels similar to those in the wild type, we concluded that the domain is important for the protein activity but not for its expression or stability (Fig. 2). Moreover, our data showed that this motif, and particularly H^{53}, plays a critical role in Δ6-desaturase activity in its different substrates (Fig. 3, Table 2). This observation is consistent with the previously

reported essentialness of H^{41} in borage Δ6-desaturase (11) and with the role of the cytochrome b5-like domain in yeast Δ9-desaturase activity (12).

The COS-7 cells are unlikely to be deficient in cytochrome b5. For example, rat Δ9-desaturase, which requires cytochrome b5 to function, is active when expressed in COS7 cells (15). This suggests that endogenous cytochrome b5, constitutively present in COS-7 cells, cannot rescue the activity of a Δ6-desaturase whose cytochrome b5-like domain has been mutated or deleted. To further address this hypothesis, we measured Δ6-desaturase activities in COS-7 cells transiently transfected with distinct forms of Δ6-desaturase in the presence or absence of coexpressed rat cytochrome b5. Similarly, we showed that rat cytochrome b5 coexpressed in this cell line did not rescue the activity of mutated forms of Δ6-desaturase (Fig. 4). Thus, neither endogenous microsomal cytochrome b5 nor coexpressed rat cytochrome b5 could rescue the activity of mutated Δ6-desaturases.

The major role of the rat Δ6-desaturase cytochrome b5-like domain may have led to the speculation that the enzyme can function independently of free microsomal cytochrome b5. However, coexpression of microsomal rat cytochrome b5 with rat Δ6-desaturase is necessary for an optimal PUFA desaturation in yeast (17). Thus, the role of microsomal cytochrome b5 in the process of Δ6-desaturation could not be dismissed. Consistent with this proposal, we showed that microsomal cytochrome b5 stimulated Δ6-desaturase activity when coexpressed in a mammalian cell line (Fig. 4).

Because the ^{53}HPGG^{56} region of the rat Δ6-desaturase cytochrome b5-like domain may represent an important motif for the structure of the protein and its putative interaction with other proteins, we tested whether Δ6-desaturase or Δ6-desaturase with complete deletion of the ^{53}HPGG^{56} sequence interacts with cytochrome b5 in COS-7 cells. When coexpressed in COS-7 cells, wild-type Δ6-desaturase interacted with cytochrome b5 (Fig. 5). This protein-protein interaction may contribute to the effect of cytochrome b5 on Δ6-desaturase activity. Interestingly, we observed that the complete deletion of the ^{53}HPGG^{56} motif did not alter the interaction between Δ6-desaturase and cytochrome b5 (Fig. 5), providing evidence that this motif is not necessary for interaction between these two proteins, whereas cytochrome b5 could not rescue the activity of mutated forms of Δ6-desaturases (Fig. 4).

Therefore, the different results described here assess the important role of both the Δ6-desaturase cytochrome b5-like domain and the microsomal cytochrome b5 in the process of Δ6-desaturation. This study also shows that microsomal cytochrome b5 cannot compensate for the essential role of the highly conserved ^{53}HPGG^{56} motif in the rat Δ6-desaturase cytochrome b5-like domain. The precise role of free cytochrome b5 in Δ6-desaturase activity should be further defined. It would be interesting to investigate further the cytochrome b5-Δ6-desaturase interaction and the stimulatory effect of cytochrome b5 using models with greater physiological expression of both proteins. Whether cytochrome b5 contributes to an electron transfer required for Δ6-desaturase activity remains to be elucidated. As has been shown for cytochrome P450 monooxygenase (25), the possibility that cytochrome b5 may not function as an electron transfer component in the Δ6-desaturase enzymatic system could be considered. Together, these results suggest essential and distinct roles for free cytochrome b5 and the fused cytochrome b5-like domain in governing Δ6-desaturase activity.

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