The first steps of ether lipid biosynthesis are exclusively localized to peroxisomes and hence some peroxisomal disorders are characterized by a severe deficiency of plasmalogens, the main ether lipids in humans. Here we report on gene defects of plasmalogen biosynthesis, chromosomal localization of the corresponding genes and, as a consequence of plasmalogen deficiency, on structural alterations of caveolae, clathrin-coated pits, endoplasmic reticulum and Golgi cisternae, as well as on the reduced rate of transferrin receptor cycling. The data suggest that plasmalogens, analogous to cholesterol, are essential for correct membrane functioning and their deficiency results in impaired membrane trafficking.

INTRODUCTION

Synthesis of ether phospholipids represents one of the major anabolic capacities of mammalian peroxisomes. The key enzymes involved are dihydroxyacetone phosphate acyltransferase (DAPAT) and alkyl dihydroxyacetone phosphate synthase (ADAPS) which are exclusively localized within peroxisomes (1). The cDNAs of both of these enzymes were recently characterized on a molecular level (2–4) and their intraperoxosomal localization confined to the luminal face of the membrane (4). Peroxisomes synthesize only the ether phospholipid precursor 1-alkylglycerol phosphate. The subsequent steps to complete the phospholipid are identical to those utilized for the synthesis of diacylglycerol phospholipids and hence are localized to the endoplasmic reticulum (ER). The major end product of the ether lipid biosynthetic pathway in most mammalian tissues is plasmalogens (PMs), which predominantly belong to the class of plasmenyl phosphatidyl ethanolamine and plasményl phosphatidylyl choline. However, PMs are not the only molecular species occurring in mammalian tissues that carry an ether-bonded carbon chain. Such chains are also found in ether phospholipids lacking the PM characteristic vinyl ether bond, e.g., platelet-activating factor (PAF), an 1-alkyl-2-acetyl glycerophosphoryl choline and the lipid moiety of distinct glycosyl phosphatidylinositol (GPI)-anchored proteins (for reviews see refs 5 and 6). To date, the physiological role of PMs is not clear, although various important functions have been suggested, such as modulation of membrane fluidity and phase transition temperature (7), protection against oxidative stress (8), participation in signal transduction processes (9,10) and facilitation of membrane fusion (11). Convincing evidence that PMs are indispensable constituents in man is derived from the existence of peroxisomal disorders (for reviews see refs 12 and 13). Except X-linked adrenoleukodystrophy, these disorders are inherited by an autosomal recessive mode and classified into two major categories: disorders of peroxisomal assembly and disorders based on defects of single peroxisomal proteins (12,13). Both categories include disorders accompanied by the biochemical finding of PM deficiency. The most severe assembly disorder, the cerebro-hepato-renal (Zellweger) syndrome (ZS), relies on import defects of peroxisomal matrix proteins caused, for example, by mutations within the PEX5 gene coding for the peroxisomal targeting signal 1 (PTS1) receptor (14). Most matrix proteins, including DAPAT, use this PTS1 import pathway and hence are mislocalized in ZS to the cytoplasm where they are prone to proteolytic digestion. Since peroxisomal membrane proteins are inserted into the membrane by signals different to those found in matrix proteins (15–18), ghostlike peroxisomes are assembled in ZS. Compared with ZS, a much lower incidence is observed with disorders based on isolated defects of DAPAT and ADAPS. Patients suffering from these disorders reveal clinical manifestations indistinguishable from those of rhizomelic chondrodysplasia punctata (RCDP). DAPAT and ADAPS deficiencies are classified as RCDP types 2 and 3, respectively (3), and are differentiated from RCDP type 1 which is based on defects in the PEX7 gene coding for the PTS2 receptor (19). This mobile receptor mediates import of a small subset of peroxisomal matrix proteins including ADAPS, 3-ketoacyl-CoA thiolase, phytanic acid oxidase and mevalonate kinase (for a review see ref. 20). RCDP type 1 patients represent the largest group of the RCDP subgroups which are all distinguished by multiple severe clinical abnormalities usually resulting in death in earliest childhood. In the present paper we report on the genomic organization of the human DAPAT gene. Using this sequence information...
and partial genomic sequences of the ADAPS gene, we defined the chromosomal localization of these genes and characterized on a molecular level the genetic defects in three patients with isolated DAPAT and one patient with isolated ADAPS deficiency. We also detected characteristic phenotypic changes in human skin fibroblasts (hSFs) of these patients, notably a decrease in the number and a change in structure of caveolae, the formation of large, weakly indented clathrin-coated areas at the plasma membrane and a considerable dilation of ER and Golgi cisternae. We also found that the rate of internalization of labeled transferrin was delayed and that both transferrin and cholesterol accumulated in perinuclear structures most likely to belong to the late endosomal/lysosomal compartment.

RESULTS

Organization of human and mouse DAPAT genes

Using specific cDNA probes we screened genomic P1-derived artificial chromosome (PAC) libraries and detected human and mouse DAPAT clones covering the entire genes which were 30 and 26 kb in size, respectively. Both genes revealed a similar organization, consisting of 16 exons and 15 introns (Fig. 1). Although exon sequences vary in size between ∼60 and 300 bp, the largest intron of hsDAPAT contains >5000 bp (Fig. 1A). All exon–intron boundaries follow the GT-AT rule.

Besides the complete sequences of the human and mouse DAPAT genes, we also obtained partial sequences of the human ADAPS gene (data not shown). The human sequences were used to establish the chromosomal localization of the corresponding genes. Whereas hsDAPAT localized to 1q42 (Fig. 2A–C), hsADAPS mapped to 2q33 (Fig. 2D–F) (21). Next to these gene loci important mesenchymal genes are found, some of which relate to severe human disorders.

hsDAPAT and hsADAPS gene defects

hSFs of three patients (patients 1–3) with isolated DAPAT and one (patient 4) with isolated ADAPS deficiency were investigated along with two control cell lines of healthy donors. Patients 1 and 2 were brothers from three siblings whose healthy parents are first cousins. In 1994 when these patients were admitted to the hospital they were at the ages of 1 and 3 years, respectively. Both clinical and biochemical findings suggested that they were affected by the same disorder, pointing to isolated DAPAT deficiency and transmitting RCDP type 2 (3,22). The major presenting clinical features in these patients were developmental delay, hypotonia, failure to thrive, microcephaly, subtle dysmorphic features and marked punctate epiphyseal calcification. Biochemical analysis showed a deficiency of C16:0 and C18:0 PMs and a considerably lowered activity of DAPAT. Phytanic acid oxidase activity was normal and thiolase was present as the mature 41 kDa polypeptide. Patient 3, the daughter of consanguineous parents, also exhibited DAPAT deficiency. Her case has also been reported in detail elsewhere (23). In 1998 she was 6 years old, presenting with a less severe phenotype than that seen in classical RCDP, notable for short stature, microcataracts, mild hypotonia, severe mental retardation and epiphyseal stippling. DAPAT activity in cultured skin fibroblasts was 1.6% that of control activity. Patient 4, the daughter of unrelated parents, was admitted to the hospital at the age of 11 months. Similar to her brother who died at the age of 2 years, patient 4 had short proximal arms, bilateral cataracts, developmental delay, failure to thrive, gastroesophageal efflux, microcephaly and epiphyseal stippling in femoral and humeral bones. Biochemically, a defect in ADAPS activity was noted with concomitant severe deficiency of PMs.

To ensure enzyme deficiency in the mutant cell lines, DAPAT and ADAPS activities were determined and the presence of enzyme protein analyzed by western blotting. Whereas normal specific activities of DAPAT and ADAPS were measured in control cells, DAPAT activity was practically absent in cells from patients 1–3 and, although measurable, it was clearly reduced in cells from patient 4 (Table 1). As expected, no ADAPS activity was detected in ADAPS-deficient fibroblasts of patient 4 which also did not contain detectable amounts of ADAPS protein (Table 1, Fig. 3). The latter, however, was present in quite normal concentrations in all DAPAT-deficient

![Figure 1](image1)

Figure 1. Organization of human and mouse DAPAT genes. The two genes, (A) hsDAPAT and (B) mmDAPAT, revealed quite similar structures, both containing 16 exons. The mutations found in the hsDAPAT gene of three patients were localized to exons 5, 10 and 11, as indicated by the arrows.

![Figure 2](image2)

Figure 2. Localization of hsADAPS and hsDAPAT genes by fluorescence in situ hybridization. (A) Metaphase spread of a human male donor showing hybridization signals of the hsDAPAT gene on the long arm of chromosome 1. Using the inverted DAPI image (C) the signals were mapped to the chromosomal band 1q42 (B). (D) Metaphase spread after hybridization of the hsADAPS gene, hybridizing the signals to chromosome 2q33 (E) with the aid of the inverted counterstain image (F).
cell lines. The fibroblasts of patient 3 carrying an exon 10 deletion were the only ones that did not contain DAPAT protein (Fig. 3).

The DAPAT- and ADAPS-deficient cell lines were then analyzed for their gene defects by RT–PCR and sequencing. Patients 1 and 2 were relatives and exhibited the same two mutations, one base exchange in exon 5 (G632A) and a second one in exon 11 (A1556G), resulting in the exchange of amino acid residues R211H and D519G, respectively. Introduction of these two mutations by site-directed mutagenesis into control DAPAT cDNA resulted in loss (R211H) and an ∼70% reduction (D519G) of enzyme activity (Table 2). Both sequencing and activity measurements suggest that the patients are homozygous for both of these mutations. In patient 3 we detected a deletion of the entire exon 10 of DAPAT, suggesting a splice defect. Sequence analysis of intron 9 indeed revealed an IVS9(–3T→G) mutation resulting in the deletion of 243 bp (Fig. 1).

Two transitions, C926T and T1406C, were found in patient 4, resulting in the exchange of amino acid residues T309I and L469P, respectively. Sequencing of cDNA fragments of both gene segments revealed mixed cDNA populations, derived from two differently mutated alleles (Table 3).

Phenotypic changes in PM-deficient hSFs

Compared with controls showing the typical longitudinal, bipolar appearance, patients’ fibroblasts were frequently enlarged and multipolar. They develop multiple processes most pronounced in cells exhibiting ADAPS deficiency (patient 4). Cholesterol staining using the fluorescent antibiotic filipin revealed that these elongated processes interconnect neighboring hSFs (Fig. 4). Furthermore, about the same plasma membrane cholesterol concentrations are noted in control and mutant cells, but only the mutants show accumulation of cholesterol in perinuclear structures (Fig. 4B–D). Co-staining with LysoTracker and the increased number of multi-vesicular bodies observed by electron microscopy (data not shown) suggest that these structures most likely belong to the late endosomal/lysosomal compartment.

The fact that ether-bonded alkyl chains also occur in many GPI-anchored proteins and these proteins in addition to cholesterol and glycosphingolipids are concentrated in caveolae (for reviews see refs 24 and 25), prompted us to study the cellular distribution of caveolin-1 in control and patients’ fibroblasts. Staining of control cells localized caveolin-1 to highly organized structures forming longitudinally oriented lines running in parallel over the entire plasma membrane (Fig. 5A). In patients’ fibroblasts this organization is largely changed. The parallel lines are only weakly stained, reduced in length and more or less confined to the cell body. Although accumulation of caveolin is seen in small plasma membrane areas, in major parts of the membrane the protein concentration is greatly diminished or even absent (Fig. 5B and C). Correspondingly, an ∼50–60% reduction in the level of caveolin-1 was observed by immunoblotting (data not shown). Electron microscopic analysis provided interesting information on the fine structure of caveolae. Whereas in controls numerous caveolae are densely packed and orderly arranged in line (Fig. 5D and E), the number of caveolar structures is clearly reduced in the mutants, rarely showing more than two to three profiles lying next to each other (Fig. 5F–H). Multiple juxtaposed caveolae,

Table 1. Determination of DAPAT and ADAPS activity in control and mutant hSFs transformed with wild-type and mutated DAPAT cDNA

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Control</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAPAT</td>
<td>5.6</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>3.5</td>
</tr>
<tr>
<td>ADAPS</td>
<td>1.41</td>
<td>1.18</td>
<td>1.39</td>
<td>2.16</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.d., not detectable.

Table 2. DAPAT activity in Escherichia coli lysates transformed with wild-type and mutated DAPAT cDNA

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity (nmol/h mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type cDNA</td>
</tr>
<tr>
<td>DAPAT</td>
<td>48</td>
</tr>
</tbody>
</table>

Table 3. Characterization of mutations in three patients (patients 1–3) with isolated DAPAT and one patient (patient 4) with isolated ADAPS deficiency

<table>
<thead>
<tr>
<th>Patient</th>
<th>Base exchange</th>
<th>Amino acid exchange</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients 1 and 2</td>
<td>G632A</td>
<td>R211H</td>
</tr>
<tr>
<td>A1556G</td>
<td>D519G</td>
<td></td>
</tr>
<tr>
<td>Patient 3</td>
<td>IVS9(–3T→G)</td>
<td>Del(T1281–A1523)</td>
</tr>
<tr>
<td>Del(D427–K507)</td>
<td>Del(D427–K507)</td>
<td></td>
</tr>
<tr>
<td>Patient 4</td>
<td>C926T</td>
<td>T309I</td>
</tr>
<tr>
<td>T1406C</td>
<td>L469P</td>
<td></td>
</tr>
</tbody>
</table>

Note that patients 1 and 2 were brothers and hence carried the same mutations.
as shown in Fig. 5H, are hardly seen in mutant cells in which, in contrast to controls, double-shaped caveolae are regularly visualized (Fig. 5G). Compared with controls, the average diameter of caveolae in mutant cells is reduced by $\sim 30\%$ and the distance between two adjacent caveolae increased by $\sim 250\%$ (Fig. 5F–H).

These striking changes in caveolar structures and the physiological role of caveolae in endocytic processes led us to investigate the distribution of clathrin and the structure of clathrin-coated pits. In contrast to caveolin-1, the concentration of clathrin at the plasma membrane of mutant fibroblasts, compared with control cells, was clearly increased and accompanied by the formation of extended clathrin lattices (Fig. 6A–C). Controls revealed the well-known omega-shaped morphology of clathrin-coated pits at sites of the plasma membrane facing both the plastic dish (Fig. 6D) and the medium (Fig. 6E and F). Moderately indented clathrin-coated regions of normal extension, $\sim 400$ nm in length, were occasionally seen (Fig. 6G). In contrast, clathrin-coated areas in mutant cells were strikingly enlarged and frequently made up by multiple, flat clathrin patches extending into lattices of up to 2 $\mu$m in diameter (Fig. 6H–J).

Phenotypic alterations due to PM deficiency were not restricted to the plasma membrane but were also observed on intracellular membranes, particularly the ER and Golgi cisternae. Using calreticulin and collagen type I as markers for ER and the constitutive secretory pathway, respectively, mutant cells were considerably more intense for these markers compared with control stains, suggesting their accumulation (Fig. 7A–D). In line with this, ER and Golgi cisternae in mutant cells are remarkably dilated (Fig. 7F–H) and the Golgi apparatus strikingly extended, occasionally exceeding 5 $\mu$m in length (Fig. 7F).

The rate of endocytosis is reduced in PM-deficient hSFs

The structural changes observed on the clathrin-dependent endocytic system suggested that, in mutant cells, the rate of endocytosis might be affected. To prove this, we investigated internalization of FITC-labeled transferrin in control cells and cells of patient 3. At 0°C both cell lines bind approximately the same amount of FITC–transferrin (Fig. 8A and B), indicating that they expose about the same number of transferrin receptors. After a 10 min incubation at 37°C, bound transferrin is almost completely taken up by the controls, whereas in mutant cells it is still present on the surface (Fig. 8C and D), suggesting that endocytic uptake is reduced in PM-deficient hSFs. However, not only the rate of uptake but also the rate of transferrin recycling is reduced in the mutants. Whereas controls have recycled most of the FITC–transferrin back to the medium, mutants accumulate the marker in perinuclear structures (Fig. 8D) which, as determined by colocalization with LysoTracker (data not shown), are likely to represent the late endosomal/lysosomal compartment.

DISCUSSION

Characterization of gene defects

The cDNA-derived amino acid sequences of human DAPAT and ADAPS revealed two polypeptide chains consisting of 680
and 659 amino acid residues with molecular masses of 77 and 65 kDa, respectively. Within the peroxisomes the two enzymes localize to the inner aspect of the membrane where they may form a heterotrimeric complex of 210 kDa (4,26). This complex formation might explain the reduced activity of DAPAT in patient 4 in which ADAPS protein is absent. Quite recently it has been established that ADAPS belongs to a newly identified family of flavin adenine dinucleotide (FAD)-binding oxidoreductases (27) with bound FAD being reduced during the catalytic cycle (28). As derived from the structure of fungal vanilyl alcohol oxidase (27), interestingly one of the mutations observed in the ADAPS gene of patient 4 (T309I) maps to the FAD-binding oxidoreductases (27) with bound FAD being reduced during the catalytic cycle (28). As derived from the structure of fungal vanilyl alcohol oxidase (27), interestingly one of the mutations observed in the ADAPS gene of patient 4 (T309I) maps to the FAD-binding site, particularly to the motif -LTVGG-, believed to be involved in binding of the ADP moiety of FAD. In vanilyl alcohol oxidase the FAD-binding domain is followed by the cap domain, presumably related to substrate binding. According to this view, the second mutation observed in patient 4 (L469P) might influence substrate binding. Compared with ADAPS, the functional domains of DAPAT are poorly characterized, thus mutational consequences on enzyme activity are less clearly defined.

A potentially interesting observation was made when we investigated the genes neighboring DAPAT and ADAPS. Close to the gene loci at 1q42 and 2q33, respectively, we found other prominent genes, which also relate to severe human disorders. Interestingly, a striking number of these disorders, such as congenital muscular dystrophy (29), Kenny–Caffey syndrome type 1 (30), hypoparathyroidism–retardation–dysmorphism syndrome (31), mesomelic dysplasia (32), wrinkly skin syndrome (33), cataract (34), lamellar ichthyosis type 2 (35) or amyotrophic lateral sclerosis (36), share common symptoms with RCDP. These symptoms include, for example, growth retardation, bone and ocular anomalies, microcephaly, craniofacial dysmorphism, muscle atrophy and hypocalcemia. Some genes in juxtaposition to the DAPAT and ADAPS genes, such as those coding for parathyroid hormone receptor 2 (37), Indian

Figure 5. Immunofluorescent and ultrastructural visualization of caveolae in control and mutant hSFs. Confocal laser scanning images acquired at exactly the same exposure time show caveolin-1 staining in (A) control, (B) DAPAT-deficient (patient 3) and (C) ADAPS-deficient (patient 4) hSFs. Electron microscopy of control cells (D and E) reveals caveolar structures forming closely packed longitudinal lines which are changed in mutant cells (F–H, patient 3) by containing caveolae decreased in size and number. Double-shaped caveolae (G) occur regularly; however, caveolar clusters (H) are rarely seen in the mutants. Bars: 20 µm (A–C); 200 nm (D); 100 nm (E–H).

Figure 6. Immunofluorescent and ultrastructural visualization of clathrin and clathrin-coated pits in control and mutant hSFs. Confocal laser scanning images acquired at exactly the same exposure time show clathrin staining in (A) control and (B) DAPAT-deficient (patient 3) and (C) ADAPS-deficient (patient 4) hSFs. Electron microscopy of control (D–G) cells reveals omega-shaped and occasionally flat clathrin-coated pits with the normal extension of 300–400 nm. In DAPAT-deficient (patient 3) cells (H–J) flat and weakly indented clathrin-coated areas predominate forming large clathrin lattices sometimes exceeding 2 µm in length. Arrowheads point to intermediate filaments. Bars: 20 µm (A–C); 100 nm (D–H); 200 nm (I and J).
hedgehog protein (38) and the HOXD gene (39), are directly involved in the regulation of endochondral ossification which is severely impaired in patients with isolated DAPAT and ADAPS deficiency. Thus, at least parts of important mesenchymal genes related to connective and muscle tissue as well as patterning, growth and function of skeleton are situated in relative close proximity to the DAPAT and ADAPS genes, suggesting possible common operational interrelations.

PM deficiency affects membrane dynamic processes

In trying to understand the role of PMs and the symptoms observed in RCDP, we have to consider common functional aspects of caveolae, clathrin-coated pits, the ER and the Golgi. Caveolar structures have been implicated in many diverse cellular functions, such as plasma membrane domain organization of cholesterol, glycosphingolipids, various receptors and GPI-anchored proteins. Caveolae were also discussed to play a central role in endocytosis, transcytosis and cholesterol-dependent signal transduction (for reviews see refs 24 and 40). The fact that caveolin-1, -2 and -3 were all found in chondrocytes by immunofluorescence suggests that caveolae are also essential components in chondrocyte function. The upregulation of caveolin-2 and -3 during growth and development of articular cartilage supports this view and indicates a particular role of caveolae in chondrocyte differentiation and endochondral ossification (41).

Studies on the biogenesis of caveolae demonstrated that glycosphingolipid–sphingomyelin–cholesterol-rich domains are initially formed in transitional Golgi elements where phase transition from the liquid-crystallin to a liquid-ordered state takes place. This liquid-ordered state is distinguished by a high degree of acyl chain order favored by interaction of saturated sphingolipid acyl chains and cholesterol (40), thus forming membrane domains characterized by a higher acyl chain melting temperature (Tm) compared with the surroundings. Maintenance of these high Tm domains requires the controlled level of cholesterol and caveolin, as demonstrated by the pharmacologic inhibition of cholesterol transport to the cell surface and the selective oxidation of plasma membrane cholesterol, which result in both disassembly of caveolae and redistribution of caveolin-1 to the Golgi apparatus (42). PM deficiency, which lowers Tm in intact membranes but not in liposomes prepared from these membranes (7), may affect caveolar structures in a similar way to cholesterol, suggesting that adequate membrane concentrations of both PMs and cholesterol are necessary to maintain correct caveolar functions.

Clathrin-coated pits are intimately related to processes of vesicle budding (for a review see ref. 43). These processes require interaction of clathrin with adaptor protein (AP) complexes that are selectively targeted to specific membranes, where they trigger assembly of the clathrin lattice. It is well established that of the four AP complexes known to date, the AP-1 complex principally binds to the trans-Golgi network (TGN), whereas the AP-2 complex is targeted to the plasma membrane. The functions of the recently discovered AP-3 and AP-4 complexes are less clearly defined. However, it has been suggested that both AP-3 and AP-4 are also involved in intracellular protein sorting, the AP-3 complex in sorting of integral membrane proteins to the lysosomal compartment (44,45). The structural alterations seen in PM-deficient cells suggest that several of these clathrin-mediated processes may be affected. Dilations of ER and Golgi cisternae and the accumulation of collagen in these compartments may indicate an impaired exit of secretory proteins from the TGN. In line with this, our studies on the endocytic uptake of transferrin revealed decreased internalization and enhanced accumulation
of labeled transferrin in internal compartments. Since these endocytic processes are distinguished by multiple vesiculation–fusion steps, the fusogenic properties of PMs caused by their marked propensity to form hexagonal phases (11) may be an important parameter in considering functional impairments due to PM deficiency.

Recent studies on cell membranes acutely depleted of cholesterol by β-methyl-cyclodextrin lend further support to the idea that both PMs and cholesterol synergically influence membrane functions (46,47). Strikingly similarly to PM deficiency, acute cholesterol depletion also induces flat clathrin lattices. However, in contrast to PM deficiency, in which the number of transferrin receptors remains quite the same and the plasma membrane concentration of clathrin increases considerably, in acute cholesterol depletion the number of transferrin receptors is ∼2-fold enhanced, whereas the steady-state distribution of clathrin does not change. These data suggest that PMs influence membrane functioning by their own outstanding properties rather than solely affecting membrane cholesterol levels. Filipin staining of control and mutant fibroblasts confirms this view in that it does not show altered plasma membrane cholesterol levels but reveals cholesterol accumulation most likely in late endosomal/lysosomal structures of the mutants.

Cholesterol accumulations confined to late endosomes were also observed in fibroblasts of patients suffering with Niemann–Pick disease type C (NPC), a cellular cholesterol lipidosis. The primary molecular defect has been identified as mutation in the NPC1 protein involved in intracellular cholesterol sorting (48) leading to accumulation of sphingolipids and free cholesterol in liver and spleen and specific glycolipids but not cholesterol and sphingomyelin in brain (49). Comparison of the clinical manifestations of NPC and RCDP shows some similarity, in that hypotonia and delayed motor development were observed in both disorders. Other symptoms, such as development of cataract, proximal limb shortening or punctate epiphyseal calcifications, are unique to RCDP and vertical supranuclear ophthalmoplegia, for example, is a common characteristic only of NPC. In contrast to RCDP children, NPC children usually present in late childhood and die in the second decade (49). Brain PM concentrations, as determined in an NPC mouse model, are only moderately decreased. Thus, although PM deficiency and NPC share common symptoms and both result in an impairment in intracellular cholesterol trafficking, other symptoms and cellular parameters are different, lending support to the concept that PMs and cholesterol have independent functions.

In summary, the results presented in this paper emphasize the importance and outstanding function of PMs and shed new light on their physiological significance. Similarly to cholesterol, PMs may exert fine-tuning effects influencing biophysical and biochemical properties, such as membrane fluidity as well as lipid–lipid and lipid–protein interactions, and therefore seem to play a major role in membrane trafficking processes. Based
on these data, defining the precise role of PMs in membrane functioning will certainly prove interesting.

**MATERIALS AND METHODS**

**Characterization of human and mouse DAPAT genes**

The human genomic library RPCI1, 3-5 Human PAC (library no. 704, Resource Center, Berlin, Germany) and the library RPCI21 Mouse PAC (library no. 711) were screened using the complete cDNAs of hsDAPAT, hsADAPS and mmDAPAT for hybridization (2,4). By this means we found a number of potentially positive clones for hsDAPAT, hsADAPS and mmDAPAT. After rescreening we ended up with 10 clones positive for DAPAT and 6 clones for ADAPS. The clones RPCIP704I221026Q2 (DAPAT) and RPCIP704M04786Q2 (ADAPS) were repetitively sequenced using primers obtained from cDNA sequence information. DNA sequencing was done using the PRISM ready reaction cycle sequencing kit with AmpliTaq FS (PE Applied Biosystems) and the ABI 373 sequencing equipment.

For Southern blotting, cDNA probes corresponding to the coding region of human DAPAT and ADAPS were labeled by random priming (AGS) in the presence of [α-32P]dCTP (Amersham). Human DNA digested with specific endonucleases was hybridized to the labeled probes following standard methods. Blots were washed at high stringency (0.1x SSC, 0.1% SDS, 65°C).

**Fluorescence in situ hybridization**

DNA from the PAC clones RPCIP704I221026Q2 and RPCIP704M04786Q2 was labeled with biotin-16-dUTP (Roche Diagnostics) by standard nick translation. To optimize the hybridization efficiency, metaphase spreads prepared from RPCI1, 3-5 Human PAC (library no. 704, Resource Center, Berlin, Germany) and the library RPCI21 Mouse PAC (library no. 711) were screened using the complete cDNAs of hsDAPAT, hsADAPS and mmDAPAT. After rescreening we ended up with 10 clones positive for DAPAT and 6 clones for ADAPS. The clones RPCIP704I221026Q2 (DAPAT) and RPCIP704M04786Q2 (ADAPS) were repetitively sequenced using primers obtained from cDNA sequence information. DNA sequencing was done using the PRISM ready reaction cycle sequencing kit with AmpliTaq FS (PE Applied Biosystems) and the ABI 373 sequencing equipment.

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**Cell culture and RT–PCR**

Control and mutant hSFs were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 15% fetal calf serum, 100 U/ml penicillin and 100 mg/ml streptomycin. The cell cultures were kept at 37°C in a 5% CO2 atmosphere. Poly(A)+ RNA was isolated from the frozen cells using Poly(A)+ magnet beads (Dynal) and reverse transcribed using d(T)16 primers according to standard protocols (Perkin Elmer). cDNA derived from reverse transcription was analyzed by PCR. Template (200 ng) and an appropriate primer pair was added to a master mix containing PCR buffer, 1.5 mM MgCl2, 0.2 mM dNTPs and 1 U/50 µl Taq/Pwo mix (AGS). The primer pairs used in the RT–PCR reactions, in order to generate the full-length cDNAs were as follows:

**Cholesterol staining, transferrin uptake and immunofluorescence**

Cholesterol staining was performed on cells grown on cover slips by adding the antibiotic filipin (Streptomyces filipinensis; Sigma) to the washed cells at a concentration of 25 µg/ml and incubating the cells for 30 min at room temperature. Cells were washed again, fixed with 3% paraformaldehyde and embedded in Mowiol. Fluorescence was inspected using DAPI filters. For transferrin uptake 5 mg of transferrin (ICN) was labeled with a 10 M excess of FITC (Roche Diagnostics) in 500 µl of phosphate-buffered saline (PBS). After 1 h at room temperature, FITC–transferrin was separated from unreacted FITC by a rapid gel filtration step using MEM without fetal calf serum for elution (Sephadex 50; Amersham Pharmacia). For binding and uptake, cells were incubated with FITC–transferrin at a concentration of 0.2 mg/ml. In case of lysosomal containing, LysoTracker (Molecular Probes) was added to the cell culture 30 min prior to FITC–transferrin at a concentration of 0.2 mg/ml. LysoTracker not taken up by the cells was removed by washing the cells twice with PBS.

Immunofluorescence was performed as previously reported (53). Anti-DAPAT and anti-ADAPS antisera were raised in rabbits using C-terminal peptides of mmDAPAT and mmADAPS coupled to keyhole limpet hemocyanin as previously reported (4,54). Antibodies against caveolin-1 (N-20) and clathrin (C-20) were from Santa Cruz Biotechnology, and those against calreticulin and collagen type I from Dianova and ICN, respectively. They were used at a 100-fold dilution. Cells were permeabilized with 0.1% saponin (caveolin-1) or 1% Triton X-100 (calreticulin). The primary rabbit antibodies were decorated with FITC-labeled goat anti-rabbit IgG.
REFERENCES


