Phosphomevalonate kinase is a cytosolic protein in humans

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Abstract **In the past decade, a predominant peroxisomal localization has been reported for several enzymes functioning in the presqualene segment of the cholesterol/isoprenoid biosynthesis pathway. More recently, however, conflicting results have been reported raising doubts about the postulated role of peroxisomes in isoprenoid biosynthesis, at least in humans. In this study, we have determined the subcellular localization of human phosphomevalonate kinase using a variety of biochemical and microscopic techniques, including conventional subcellular fractionation studies, digitonin permeabilization studies, immunofluorescence, and immunoelectron microscopy. We found an exclusive cytosolic localization of both endogenously expressed human phosphomevalonate kinase (in human fibroblasts, human liver, and HEK293 cells) and overexpressed human phosphomevalonate kinase (in human fibroblasts, HEK293 cells, and CV1 cells). No indication of a peroxisomal localization was obtained. Our results do not support a central role of peroxisomes in isoprenoid biosynthesis.**—Hogenboom, S., J. J. M. Tuyp, M. Espeel, J. Koster, R. J. A. Wanders, and H. R. Waterham. **Phosphomevalonate kinase is a cytosolic protein in humans. *J. Lipid Res.* 2004.** 45: **697–705.**

Supplementary key words cholesterol biosynthesis • isoprenoid • peroxisomes • Zellweger syndrome

In the past 10 years, several reports have appeared suggesting a central role of peroxisomes in isoprenoid biosynthesis (1). These reports indicated that many if not most of the enzymes involved in the presqualene segment of the isoprenoid biosynthesis pathway may be located partly or even predominantly in peroxisomes, subcellular organelles implicated in a variety of metabolic processes (2–6). The isoprenoid biosynthesis pathway supplies cells with intermediates for the biosynthesis of a variety of compounds with important functions in cellular processes. These compounds include, among others, the side chains of heme A and ubiquinone, dolichol, isopentenyl tRNA, and farnesyl and geranylgeranyl moieties used for the isoprenylation of proteins that function in intracellular signaling. In addition to these nonsterol isoprenoids, the pathway produces sterol isoprenoids such as cholesterol, a structural component of membranes and precursor for bile acids and steroid hormones (7).

Isoprenoid biosynthesis starts with three molecules of acetyl-CoA, which in a series of six different enzyme reactions are converted to isopentenyl pyrophosphate, the basic C5 isoprene unit used for the synthesis of all isoprenoids (7). Phosphomevalonate kinase (PMK; EC 2.7.4.2.) catalyzes the fifth reaction of the pathway, which is the phosphorylation of phosphomevalonate to produce pyrophosphomevalonate. Several observations have led to the claim that PMK would be located predominantly in peroxisomes. First, selective permeabilization with digitonin of monkey kidney (CV1) cells revealed latency of endogenous PMK activity similar to that of peroxisomal catalase (CAT), suggesting that both enzymes are localized in the same subcellular compartment (5). Second, (immuno)fluorescence microscopy performed with CHO cells overexpressing a 200-amino acid carboxyl-terminal fragment of human PMK fused to the carboxyl terminus of green fluorescent protein (GFP) revealed a colocalization of this fusion protein with peroxisomal CAT (4). Third, human PMK contains a carboxyl-terminal serine-arginine-leucine (SRL), which matches the consensus peroxisomal targeting sequence type 1 (PTS1), suggesting that PMK may be targeted to peroxisomes via the PTS1-mediated protein import pathway (4, 8). This suggestion was reinforced by the finding that the above-mentioned GFP-PMK fusion protein remained in the cytosol when expressed in PTS1 pro-

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Abbreviations: CAT, catalase; FHC, familial hypercholesterolemia; GFP, green fluorescent protein; MMP7, metallo-matrix protein 7; PGI, phosphoglucoisomerase; PMK, phosphomevalonate kinase; PTS, peroxisomal targeting sequence; ZS, Zellweger syndrome.

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tein import-deficient fibroblasts (4). Transient expression of this fusion protein in fibroblasts deficient in the import of PTS2-containing proteins revealed a punctate (peroxisomal) pattern in immunofluorescence experiments (4). Finally, in some livers of patients with Zellweger syndrome (ZS), a markedly deficient PMK activity was found. Because the loss of peroxisomes, which occurs in ZS cells, leads to mislocalization of peroxisomal enzymes to the cytosol often followed by the inactivation and/or degradation of these enzymes, this latter finding has been interpreted as indicative of a peroxisomal localization of PMK (9).

More recent data, however, do not support a peroxisomal localization of PMK. First, selective permeabilization of rat hepatoma H35 cells with digitonin resulted in a 91% release of PMK activity, similar to the release of the cytosolic marker lactate dehydrogenase, whereas peroxisomal CAT activity was completely retained in the cells after permeabilization, suggesting that PMK is cytosolic (10). Second, we recently found completely normal PMK activity and PMK protein levels in fibroblasts and liver homogenates of patients with a peroxisome biogenesis defect and in liver homogenates of *PEX5* knockout mice (11, 12). Moreover, we demonstrated that the deficient PMK activities reported in some livers of ZS patients are a result of the bad condition and/or preservation of the livers, rather than of the presumed mislocalization of the protein (11). Finally, in conventional subcellular fractionation studies that we performed with rat liver tissue, cultured human fibroblasts, and HepG2 cells, and in digitonin permeabilization experiments with cultured human fibroblasts, we never were able to demonstrate a peroxisomal localization of PMK activity (our unpublished data).

In summary, one has to conclude from the combined data that it remains unclear whether PMK is a true peroxisomal enzyme under physiological conditions. This prompted us to initiate a thorough study to determine the subcellular localization of human PMK. To avoid inconclusive results with tagged and reporter proteins, we generated highly specific antibodies that recognize the authentic human PMK, enabling localization studies in cells under normal conditions as well as in cells overexpressing human PMK. Using a variety of biochemical and microscopic techniques, we found a cytosolic localization of both endogenous and overexpressed human PMK and no indication of a peroxisomal localization.

MATERIALS AND METHODS

Cell lines and culture conditions

Primary skin fibroblasts were obtained from a healthy control subject, from a ZS patient who was a homozygote for an insertion mutation in the *PEX19* gene (13), and from a patient homozygous for familial hypercholesterolemia (FHC) (GM00701; Coriel cell repositories). The fibroblasts were cultured in HAM F-10 medium (GIBCO) containing 10% FCS and 1% penicillin/streptomycin in a temperature- and humidity-controlled incubator (95% air, 5% CO_2 as the gas phase) at 37°C. Before experiments, the cells were grown to 70–80% confluence, after which the medium was substituted with HAM F-10 medium containing 10% lipoprotein (cholesterol)-depleted FCS. Experiments were performed after 72 h of culturing in lipoprotein (cholesterol)-depleted medium.

For PMK expression studies, the human embryonic kidney (HEK293) Flp-In and CV1 Flp-In cell lines (Invitrogen) were used and cultured in DMEM (GIBCO) containing 10% FCS, 1% penicillin/streptomycin, and 100 μg/ml hygromycin in a temperature- and humidity-controlled incubator (95% air, 5% CO_2 as the gas phase) at 37°C. Before experiments, the cells were grown to 70–80% confluence, after which the medium was substituted with DMEM containing 10% lipoprotein (cholesterol)-depleted FCS. Experiments were performed after 24 h of culturing in lipoprotein (cholesterol)-depleted medium.

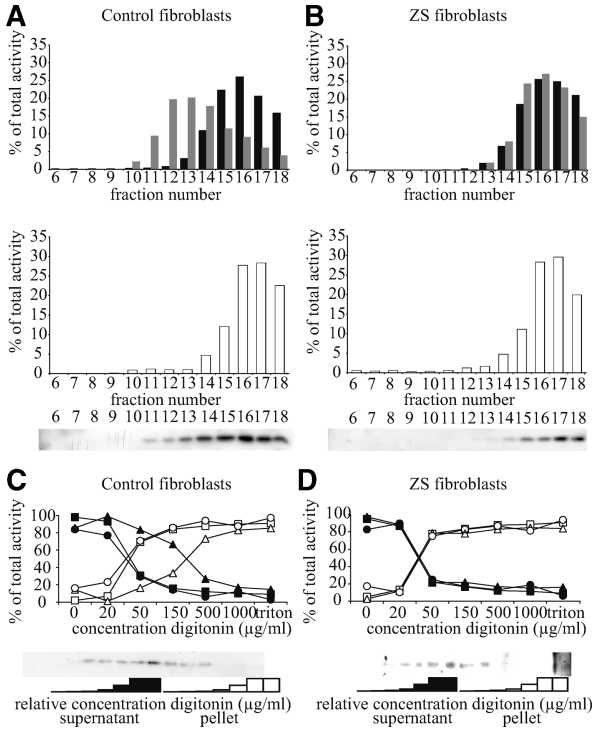

Fig. 1. A and B: Subcellular fractions of human fibroblasts derived from a control subject (A) or a Zellweger syndrome (ZS) patient (B) were obtained by Nycodenz equilibrium density gradient centrifugation as described in Materials and Methods. Fractions were analyzed for the cytosolic marker phosphoglucoisomerase (PGI; black bars), the peroxisomal marker catalase (CAT; gray bars), and phosphomevalonate kinase (PMK; white bars). Relative activities were expressed as the percentage of total gradient activity present in each fraction. The pattern of distribution of PMK activity and PMK protein as determined by immunoblot analysis with an affinity-purified antibody raised against human PMK is similar to the pattern of PGI activity. C and D: Human fibroblasts derived from a control subject (C) or a ZS patient (D) were incubated with increasing concentrations of digitonin as described in Materials and Methods. Supernatant (open symbols) and pellet (closed symbols) fractions were analyzed for the activities of the cytosolic marker PGI (squares), the peroxisomal marker CAT (triangles), and PMK (circles). Relative activities were expressed as the percentage of total activity (supernatant plus pellet) present in each fraction. The pattern of latency of PMK activity and PMK protein as determined by immunoblot analysis with an affinity-purified antibody raised against human PMK is similar to the pattern of PGI activity. The bars below the immunoblots indicate the relative digitonin concentrations.

Generation of cell lines stably overexpressing human PMK

The open reading frame of control human PMK cDNA was amplified by PCR from cDNA prepared from human skin fibroblast RNA and ligated as a *Bam*HI-*Xho*I fragment under transcriptional control of the cytomegalovirus (CMV) promoter in the pcDNA5/FRT vector (Invitrogen). The entire insert was sequenced to ensure the absence of Taq polymerase-introduced errors.

HEK293 Flp-In cells or CV1 Flp-In cells were cultured in DMEM containing 10% FCS and 1% penicillin/streptomycin. Stable PMK-expressing cell lines were generated by cotransfection of CV1 and HEK293 cells with pOG44 and pcDNA5/FRT-PMK using Lipofectamine Plus reagent in growth medium without Zeocin according to the manufacturer's recommendations (Invitrogen). Forty-eight hours after transfection, hygromycin B was added to the medium to a final concentration of 100 μg/ml, and the medium was changed every 3–4 days until hygromycin-resistant colonies were evident. Control hygromycin-resistant cell lines were generated by cotransfection with pOG44 and the empty pcDNA5/FRT vector. For expression studies, the HEK293 Flp-In cell lines stably expressing human PMK (HEK-PMK), the CV1 Flp-In cell lines stably expressing human PMK (CV1-PMK), and the control cell lines transfected with empty pcDNA5/FRT (HEK− or CV1−) were cultured in DMEM containing 10% FCS, 1% penicillin/streptomycin, and 100 μg/ml hygromycin. The PMK activity in cells overexpressing human PMK was approximately five times higher than that in the control cell lines.

Subcellular fractionation

For subcellular fractionation studies, cells were cultured in 162 cm^2 Falcon flasks, harvested, and washed three times with PBS and two times with fractionation buffer (0.25 M sucrose, 1 mM EDTA, 10 mM HEPES, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4). Next, the cells were homogenized using a ball-bearing cell cracker (EMBL), after which the postnuclear supernatant (PNS; 10 min, 500 *g*) was layered on top of a continuous Nycodenz gradient (15–35%) with a cushion of 1 ml of 50% Nycodenz in 0.25 mM sucrose, 5 mM MOPS, 1 mM EDTA, and 2 mM KCl, pH 7.3. Gradients were centrifuged for 2.5 h in a vertical rotor (MSE; 8 × 35) at 19,000 rpm (~32,000 *g*) at 4°C. After centrifugation, 16–19 fractions were collected from the bottom of the gradient.

Cell permeabilization with digitonin

Cell permeabilization experiments were performed with cells attached to plates essentially as described by Biardi and Krisans (5) with a few modifications. HEK293 and CV1 cells were seeded on 60 mm plates at a density of 3.0×10^5 cells/plate and fibroblast cells were seeded at a density of 2.0×10^5 cells/plate. After culturing for 1 or 3 days in DMEM or HAM F-10 medium, respectively, containing 10% lipoprotein (cholesterol)-depleted FCS, cells were washed twice with ice-cold KH buffer (50 mM HEPES and 110 mM KOAc, pH 7.2). The plates were then transferred to ice and incubated in KHM buffer (20 mM HEPES, 110 mM KOAc, and 2 mM MgOAc, pH 7.2) containing various concentrations of digitonin (0, 20, 50, 150, 500, or 1,000 μg/ml) or, as a control, 0.1% (w/v) Triton X-100. After 5 min, the buffer was collected as "supernatant" fractions and kept on ice. Subsequently, cells were incubated in KH buffer containing 1,000 μg/ml digitonin, which results in total permeabilization. After 30 min, the buffer was collected and kept on ice. These latter fractions were referred to as "pellet" fractions. Enzyme measurements were performed immediately in both fractions.

Enzyme assays

PMK activity was measured by a radiochemical assay as described previously (11). Phosphoglucoisomerase (PGI) (14) and CAT (9) activities were measured by spectrophotometric assays as described.

Immunoblot analysis

Proteins were separated by SDS-PAGE and transferred onto nitrocellulose by semidry blotting (15). The highly specific affinity-purified antibody directed against human PMK (11) was used at a 1:500 dilution. Antigen-antibody complexes were visualized with goat anti-rabbit IgG-alkaline phosphatase conjugate and CDP-star (Roche Chemicals). As a control for the transfer of protein, each blot was reversibly stained with Ponceau S before incubation with antibodies.

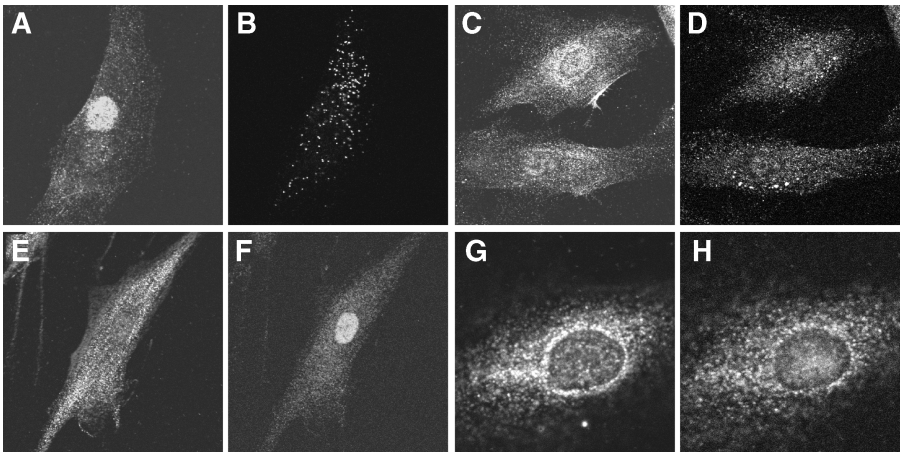

Fig. 2. Human fibroblasts derived from a control subject (A–D) or a ZS patient (E–H) were labeled with antibodies as described in Materials and Methods. Cells were double labeled using antibodies directed against PMK (A and E) and the peroxisomal marker CAT (B and F) or with antibodies directed against PMK (C and G) and the cytosolic marker metallo-matrix protein 7 (MMP7; D and H). PMK shows the same pattern as the cytosolic MMP7 in both cell lines. PMK shows colocalization with CAT in the ZS fibroblasts in which CAT is localized in the cytosol, but no colocalization is observed between PMK and the peroxisomal CAT in control fibroblasts.

Immunofluorescence

Cells were seeded on coverslips in six-well plates and cultured as indicated above. Immunofluorescence was performed as described (16). Cells were double labeled with polyclonal antibodies directed against human PMK (11) and monoclonal antibodies directed against the peroxisomal marker CAT (17) or the cytosolic marker metallo-matrix protein 7 (MMP7) (Ab-1, clone 1D2; Labvision). PMK antibodies were visualized using biotinylated donkey anti-rabbit Ig (Amersham) and streptavidin-labeled fluorescein isothiocyanate (DAKO). CAT and MMP7 were visualized using goat anti-mouse-labeled Alexa 568 (Molecular Probes). Photographs were taken using a confocal laser scanning microscope (Leica).

Immunocytochemistry of liver samples

Human liver biopsies were fixed in 4% formaldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, containing 1% calcium chloride and processed for Unicryl embedding or for cryostat sectioning as described (18, 19). Ultrathin sections of Unicryl embedded samples were incubated with polyclonal antibodies against PMK (11) or the peroxisomal alanine/glyoxylate aminotransferase (AGT) (20), decorated with colloidal gold, and examined by electron microscopy as previously described (18). Cryostat sections (7 μm) were immunostained with colloidal gold against PMK or AGT followed by silver enhancement and examined by light microscopy as previously described (19). Negative controls were incubated with normal rabbit serum.

Localization of the GFP-PMK fusion protein

Using the same strategy as Olivier et al. (4), a 600 bp *Pst*I-*Apa*I fragment of human PMK cDNA was subcloned in frame with the coding sequence of GFP by means of insertion into the *Pst*I-*Apa*I sites of pEGFP-C3 (Clontech). The GFP-PMK expression plasmid was transfected into cultured fibroblasts [control (Ctrl) and ZS] and CV1 cells using Lipofectamine Plus (Gibco BRL). The transfection medium consisted of 1 ml of DMEM, 1 μg of plasmid DNA, and 6 μl of Lipofectamine Plus reagent in which the cells were incubated for 2 h at 37°C. After this 2 h incubation period, 2 ml of DMEM containing 20% FBS and 1% penicillin/streptomycin was added to the culture medium. After 24 h, cells were prepared for immunofluorescence as described above using monoclonal antibodies against CAT or against a peroxisomal membrane protein, deficient in X-linked adrenoleukodystrophy (ALDP), and goat anti-mouse Alexa 568 (Molecular Probes). The localization of the GFP-PMK fusion protein was determined by examining the intrinsic fluorescence of GFP.

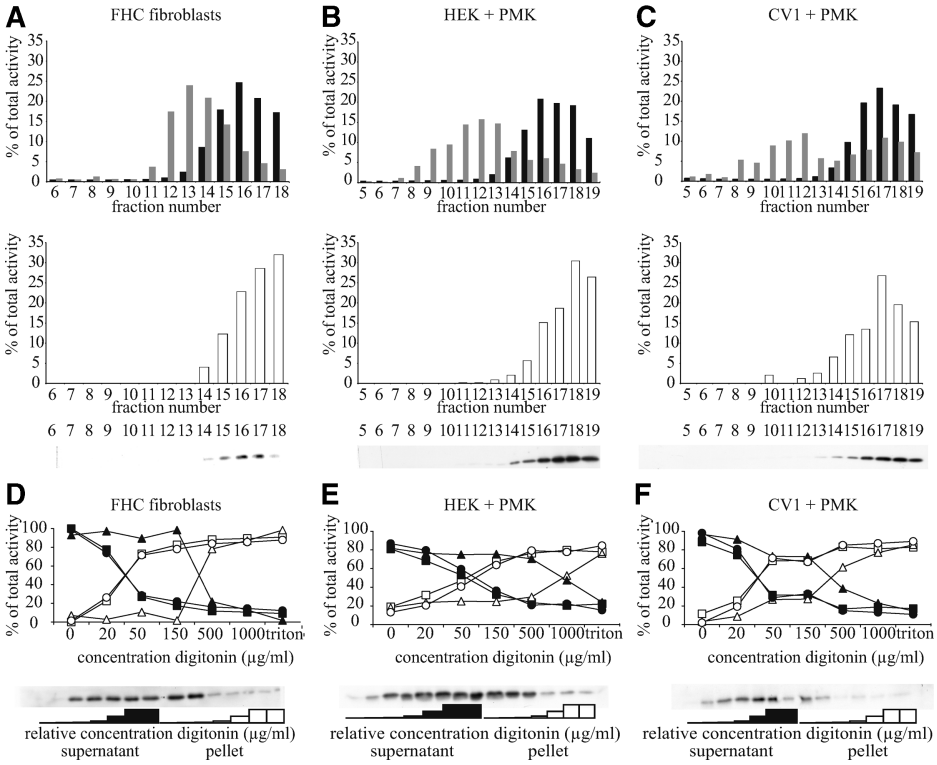

Fig. 3. A–C: Subcellular fractions of human fibroblasts derived from a familial hypercholesterolemia (FHC) patient (A) or HEK293 cells (B) and CV1 cells (C) overexpressing full-length human PMK were obtained by Nycodenz equilibrium density gradient centrifugation as described in Materials and Methods. Fractions were analyzed for the cytosolic marker PGI (black bars), the peroxisomal marker CAT (gray bars), and PMK (white bars). Relative activities were expressed as the percentage of total gradient activity present in each fraction. The pattern of distribution of PMK activity and PMK protein as determined by immunoblot analysis with an affinity-purified antibody raised against human PMK is similar to the pattern of PGI activity. D–F: Human fibroblasts derived from an FHC patient (D) or HEK293 cells (E) and CV1 cells (F) overexpressing full-length human PMK were incubated with increasing concentrations of digitonin as described in Materials and Methods. Supernatant (open symbols) and pellet (closed symbols) fractions were analyzed for the activities of the cytosolic marker PGI (squares), the peroxisomal marker CAT (triangles), and PMK (circles). Relative activities were expressed as a percentage of total activity (supernatant plus pellet) present in each fraction. The pattern of latency of PMK activity and PMK protein as determined by immunoblot analysis with an affinity-purified antibody raised against human PMK is similar to the pattern of PGI activity. The bars below the immunoblots indicate the relative digitonin concentrations.

RESULTS

Subcellular fractionation of PMK in human fibroblasts

To determine whether in human cells PMK is localized in the cytosol, the peroxisomes, or both, we first performed subcellular fractionation studies with human skin fibroblasts. As a control, we included fibroblasts from a ZS patient lacking any peroxisomal remnants (13). After growth of the cells in lipoprotein-depleted medium to ensure a good induction of the isoprenoid biosynthetic pathway, we prepared a PNS, which was further fractionated by Nycodenz equilibrium density gradient centrifugation. In the normal fibroblasts, this resulted in a clear separation of peroxisomes and cytosol, as reflected by the distribution of the peroxisomal marker enzyme CAT and the cytosolic marker enzyme PGI (**Fig. 1A**). In the ZS fibroblasts, both marker enzymes colocalize, as expected from the absence of peroxisomes that leads to the cytosolic localization of peroxisomal enzymes (Fig. 1B). When PMK activity was measured in the gradient fractions, the activity showed the same distribution as that of the cytosolic marker PGI in both the normal fibroblasts (Fig. 1A) and the ZS fibroblasts (Fig. 1B). Immunoblot analysis of the fractions from the same density gradients using affinity-purified antiserum against human PMK revealed a similar distribution pattern for PMK protein as for PMK activity (Fig. 1A, B).

Digitonin permeabilization studies in human fibroblasts

As an alternative approach to study the subcellular localization of PMK in human fibroblasts, we exposed the cells to increasing concentrations of digitonin. When we measured the enzyme activities of CAT and PGI in the supernatant and pellet fractions of normal fibroblasts, we found a clearly different enzyme-release profile for CAT compared with PGI (Fig. 1C). This indicates that the plasma membrane was disrupted at a lower concentration of digitonin, resulting in the release of cytosolic PGI, whereas the peroxisomal membranes were permeabilized only at higher concentrations of digitonin, resulting in the release of the peroxisomal matrix content, including CAT. As expected, in the ZS fibroblasts lacking peroxisomes, no difference was observed in the release of PGI and CAT by digitonin (Fig. 1D). When we measured PMK activity in all pellet and supernatant fractions, we found that the release of PMK from the normal fibroblasts into the supernatant fractions occurs at the same concentration of digitonin as that of cytosolic PGI (Fig. 1C). In the ZS fibroblasts, PGI, CAT, and PMK were released from the cells at the same digitonin concentration (Fig. 1D). Immunoblot analysis of the various fractions using the antiserum against human PMK revealed a similar distribution pattern for PMK protein as for its activity (Fig. 1C, D). Thus, also in digitonin permeabilization studies, human PMK behaves similar to cytosolic PGI and clearly different from peroxisomal CAT.

Immunofluorescence studies in fibroblasts

We also studied the subcellular localization of human PMK by immunofluorescence microscopy. To this end, we performed double labeling of fibroblasts cultured in lipo-

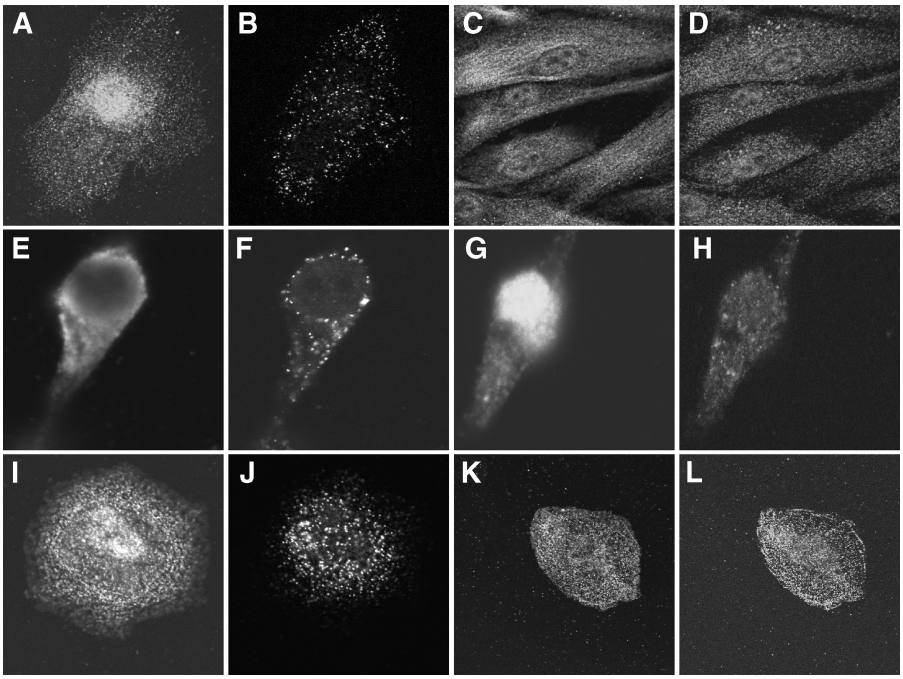

Fig. 4. Human fibroblasts derived from an FHC patient (A–D) or HEK293 cells (E–H) and CV1 cells (I–L) overexpressing full-length human PMK were labeled with antibodies as described in Materials and Methods. Cells were double labeled using antibodies directed against PMK (A, E, and I) and the peroxisomal marker CAT (B, F, and J) or with antibodies directed against PMK (C, G, and K) and the cytosolic marker MMP7 (D, H, and L). The diffuse distribution pattern of PMK differs from the punctate pattern of CAT, but PMK shows the same pattern as the cytosolic MMP7 in all cell lines.

protein-depleted medium using the polyclonal anti-PMK antiserum and monoclonal antibodies directed against human peroxisomal CAT or against human cytosolic MMP7, a cytosolic marker (**Fig. 2**). When we compared the immunolabeling of PMK in normal fibroblasts and ZS fibroblasts, we observed a similar cytosolic distribution pattern of the fluorescent signal in both cell lines, indicating that the presence or absence of peroxisomes does not affect the localization of PMK. Moreover, there was no colocalization of PMK and CAT in the normal fibroblasts, whereas in the ZS fibroblasts, the distribution pattern of CAT is superimposable on that of PMK, indicating colocalization of CAT and PMK in the cytosol. Also, when we compared the fluorescent signals obtained with anti-PMK and anti-MMP7, we found clear colocalization in the cytosol in both normal fibroblasts and ZS fibroblasts.

Subcellular localization of human PMK in overexpressing cell lines

The results of our various localization studies in human fibroblasts all indicate that endogenous PMK is predominantly, if not exclusively, located in the cytosol and not in peroxisomes. These results are in contrast to the reported peroxisomal localization of the GFP-PMK fusion protein upon overexpression in CHO cells (4). To determine whether this discrepancy in localization might be attributable to the overexpression, we also studied the subcellular localization of overexpressed PMK in various cell types. These include CV1 and HEK293 cells stably transfected with human PMK cDNA under the control of the CMV promoter and human FHC fibroblasts.

After fractionation of the various PNS fractions of these cell lines by Nycodenz equilibrium density gradient centrifugation followed by the measurement of PGI, CAT, and PMK activities and PMK protein content in all fractions, we again found a distribution pattern of PMK similar to that of cytosolic PGI and clearly distinct from that peroxisomal CAT in all cell lines (**Fig. 3A–C**). This was the case for endogenously overexpressed human PMK (Fig. 3A, FHC), constitutively overexpressed human PMK (Fig. 3B, HEK + PMK, and Fig. 3C, CV1 + PMK), and endogenously expressed human PMK (HEK− cells; data not shown) and monkey PMK (CV1− cells; data not shown). Also, after selective permeabilization of the cellular membranes using increasing concentrations of digitonin, we found that both endogenously and constitutively overexpressed human PMKs behave similar to cytosolic PGI (Fig. 3D—F). Moreover, immunofluorescence labeling of the endogenously and constitutively overexpressed human PMK shows a cytosolic localization superimposable on that of cytosolic MMP7 protein and clearly different from the localization of CAT in these cell lines (**Fig. 4**).

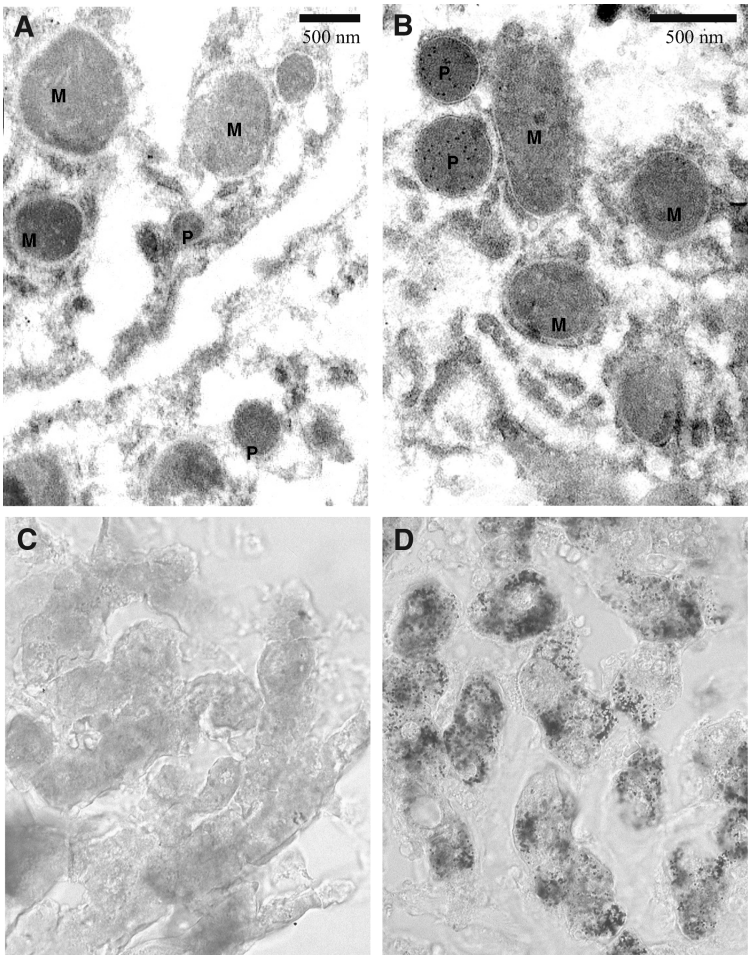

Fig. 5. Immunocytochemistry of human control liver. A: Ultrathin Unicryl sections of human liver were immunolabeled with the affinity-purified antibodies against PMK. The peroxisomes (P) remain unlabeled. M, mitochondria. B: Ultrathin Unicryl sections immunolabeled with antibodies against alanine/glyoxylate aminotransferase (AGT) reveal a clear localization in the peroxisomal matrix. Scale bars in A and B = 500 nm. C: Cryostat sections immunolabeled with affinity-purified antibodies against PMK (silver enhancement of colloidal gold labeling) reveal a diffuse reaction in the cytosol of the hepatocytes. D: For comparison, sections immunolabeled with antibodies against AGT reveal a distinct granular pattern, reflecting a peroxisomal localization of AGT.

Immunocytochemical studies in human liver

Although our combined data show that, at least in humans, PMK is predominantly a cytosolic protein, they cannot exclude the possibility that a minor amount of PMK is localized in peroxisomes. Therefore, we also performed immunocytochemical studies with ultrathin sections and cryostat sections of human liver, the organ with the highest expression of the enzymes of the presqualene segment of the isoprenoid biosynthesis pathway.

In immunogold labeling experiments using antibodies directed against human PMK, we found only occasional labeling in the cytoplasm of liver parenchymal cells. Although we carefully checked a large number of peroxisomes, we were unable to detect any labeling of PMK in these peroxisomes (**Fig. 5A**). Moreover, even after incubation with higher concentrations of antibodies, as a result of which nonspecific labeling strongly increased, no peroxisomal labeling could be observed (data not shown). As a control, we also performed immunogold labeling experiments on liver sample sections with antibodies against peroxisomal AGT. This revealed a distinct label in the peroxisomal matrix (Fig. 5B), whereas no label was observed in negative controls.

Because cytosolic localization of a nonabundant protein is difficult to demonstrate in ultrathin sections, we also performed immunocytochemistry on cryostat sections of human liver using antibodies against PMK and AGT using the sensitive silver enhancement technique. Although overall staining with the PMK antibodies was rather weak, we observed only a diffuse staining in the cytosol of hepatocytes (Fig. 5C). This pattern is similar to the pattern typically found for the localization of CAT in ZS livers (data not shown). In contrast, a distinct punctate pattern of peroxisomes was obtained when the sections were incubated with antibodies against AGT (Fig. 5D).

Subcellular localization of the GFP-PMK fusion protein

Our results are in marked contrast to those of Olivier et al. (4), who postulated a predominant peroxisomal localization of human PMK based on expression studies with a GFP-PMK fusion protein. We repeated their experiment and expressed the same GFP-PMK fusion protein in different cell lines. Transient expression in human control fibroblasts (control) and CV1 cells revealed a cytosolic localization of the GFP-PMK fusion protein in ~60% of the GFP-positive cells as determined by GFP fluorescence (data not shown). The other GFP-positive cells displayed a punctate fluorescence suggesting a peroxisomal localization (**Fig. 6A**, **E**). Surprisingly, however, immunofluorescence labeling of peroxisomes in these cells using monoclonal antibodies directed against peroxisomal CAT (Fig. 6B, F) or the peroxisomal membrane protein ALDP (data not shown) revealed a punctate pattern that clearly differed from the punctate fluorescence of GFP-PMK (Fig. 6A, E). Indeed, in overlays of the different fluorescence patterns, we observed no colocalization of GFP-PMK with CAT or ALDP (data not shown). Moreover, expression of GFP-PMK in human ZS fibroblasts revealed the same fluorescence patterns as in the control and CV1 cells, including the punctate fluorescence of GFP-PMK in ~40% of the cells (Fig. 6C). These results show that the punctate fluorescence of GFP-PMK is not the result of peroxisomal localization. The punctate fluorescence also does not reflect a lysosomal localization, as determined by subse-

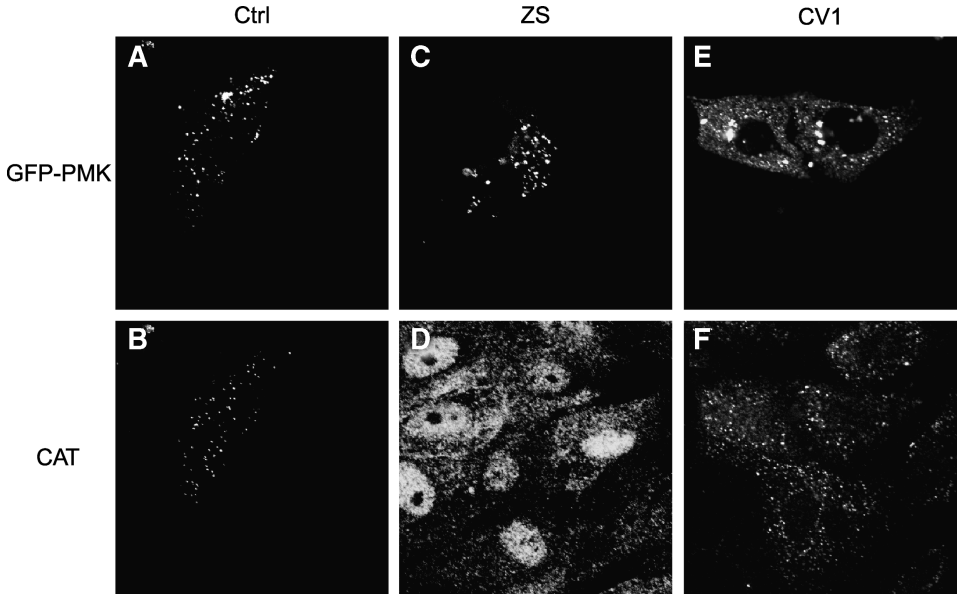

Fig. 6. Human fibroblasts derived from a control (Ctrl) subject (A and B) and a ZS patient (C and D) and CV1 cells (E and F) were transfected with green fluorescent protein (GFP)-PMK and subsequently labeled with antibodies directed against the peroxisomal CAT as described in Materials and Methods. Both GFP-PMK (A and E) and CAT (B and F) show a punctate fluorescent pattern in control fibroblasts and CV1 cells, which do not colocalize. Although CAT is localized in the cytosol in ZS fibroblasts (D), the GFP-PMK is still observed in a punctate fluorescent pattern in these cells (C).

quent studies with Lysotracker (Molecular Probes) (data not shown). In addition to the punctate fluorescence, most transfected cells also displayed relatively large fluorescent GFP signals, which might reflect protein aggregates (Fig. 6A, C, E).

DISCUSSION

Compartmentalization of cellular processes into different subcellular compartments is one of the major characteristics of eukaryotic cells. Since their discovery in the 1960s, an increasing number of important metabolic pathways have been attributed to peroxisomes. In the past decade, a predominant peroxisomal localization has also been reported for several enzymes functioning in the presqualene segment of the cholesterol/isoprenoid biosynthesis pathway, including 3-hydroxy-3-methylglutaryl CoA reductase (2), mevalonate kinase (3), PMK (4), mevalonate pyrophosphate decarboxylase (5), isopentenyl pyrophosphate isomerase (6), and farnesyl pyrophosphate synthase (9, 10). However, conflicting results have been reported, raising doubts about the postulated role of peroxisomes in isoprenoid biosynthesis, at least in humans. In this study, we have sought confirmation for the claim that PMK would be predominantly peroxisomal and, as a consequence, that peroxisomes would play a central role in the biosynthesis of isoprenoids, including cholesterol. To this end, we studied the subcellular localization of human PMK using a variety of biochemical and microscopic techniques. In all cases, we found only a cytosolic localization of both endogenously expressed human PMK (in human fibroblasts, human liver, and HEK293 cells) and overexpressed human PMK (in human FHC fibroblasts, HEK293 cells, and CV1 cells). Indeed, no indication of a peroxisomal localization of human PMK was obtained.

Our results are in agreement with our recent finding of normal PMK activity in cells of patients who suffered from ZS (11, 12) but are in contrast to those published by Olivier et al. (4), who postulated a predominant peroxisomal localization of human PMK based primarily on expression studies with a GFP-PMK fusion protein. One plausible explanation for the fact that the authentic nonmodified human PMK is localized in the cytosol and the GFP-PMK appeared peroxisomal could be that the fusion of PMK to GFP alters the protein conformation of PMK, thereby exposing its carboxyl-terminal PTS1-like SRL sequence and leading to peroxisomal import. When we tested this possibility by expressing the same GFP-PMK fusion protein in different cell lines, however, we observed a punctate pattern that did not colocalize with the punctate pattern of the peroxisomal CAT or ALDP. The fact that this punctate pattern of GFP-PMK was even observed in peroxisome-deficient ZS cells implies that the punctate pattern is not attributable to a peroxisomal localization of the protein. We have no explanation for the punctate pattern observed with GFP-PMK, but we found that GFP-PMK is also not localized in the lysosomes. Our combined data imply that one should be very careful in drawing definite conclusions from studies with overexpressed reporter proteins when these are not confirmed by studies with the authentic nonmodified protein under physiological conditions. Another observation arguing against a peroxisomal localization of PMK is the fact that several organisms, including yeast, contain a PMK that has no similarity to mammalian PMKs and, moreover, do not possess a putative PTS signal, although the peroxisomal import machinery is well conserved among yeast and mammals (21).

Now that we have shown that, at least in human cells, PMK is not localized in peroxisomes but in the cytosol, one can raise questions regarding the supposed peroxisomal localization of other enzymes functioning in the presqualene segment of the isoprenoid biosynthetic pathway. In fact, Michihara et al. (22, 23) recently reported a predominant cytosolic localization of rat and mouse mevalonate pyrophosphate decarboxylase, which also had been postulated to be peroxisomal. Moreover, using an approach similar to that used for human PMK in this study, we found that human mevalonate kinase (24) and mevalonate pyrophosphate decarboxylase (our unpublished observations) are localized in the cytosol and not in peroxisomes. All of these data strongly suggest that peroxisomes in humans are not involved in isoprenoid/cholesterol biosynthesis and corroborate our previous findings that functional peroxisomes are not required for isoprenoid biosynthesis (11, 12).

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