Mevalonate kinase is a cytosolic enzyme in humans

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Summary

In the past decade several reports have appeared which suggest that peroxisomes play a central role in isoprenoid/cholesterol biosynthesis. These suggestions were based primarily on the reported finding of several of the enzymes of the presqualene segment of the biosynthetic pathway in peroxisomes. More recently, however, conflicting results have been reported raising doubt about the postulated role of peroxisomes in isoprenoid biosynthesis, at least in humans. In this study we have studied the subcellular localisation of human mevalonate kinase (MK) using a variety of biochemical and microscopical techniques. These include conventional subcellular fractionation studies, digitonin permeabilisation studies, immunofluorescence microscopy and immunocytochemistry. We exclusively found a cytosolic localisation of both endogenous human MK (human fibroblasts, liver and HEK293 cells) and overexpressed human MK (human fibroblasts, HEK293 cells and CV1 cells). No indication of a peroxisomal localisation was obtained. Our results do not support a central role for peroxisomes in isoprenoid biosynthesis.

Key words: Cholesterol biosynthesis, Isoprenoid, Peroxisomes, Mevalonate kinase

Introduction

The isoprenoid biosynthetic pathway plays a central role in cellular metabolism in that it produces a range of isoprenoids, which are vital for diverse cellular functions. In addition to sterol isoprenoids, such as cholesterol, these isoprenoids include, among others, the side chains of haem A and ubiquinone, and farnesyl and geranylgeranyl moieties used for the isoprenylation of proteins that function in intracellular signalling (Goldstein and Brown, 1990). The synthesis of the nonsterol and sterol isoprenoids starts with three molecules of acetyl-CoA, which in a series of six different enzyme reactions are converted into isopentenylpyrophosphate (IPP), the basic C5 isoprene unit used for the synthesis of all subsequent isoprenoids (Goldstein and Brown, 1990). Several reports have indicated that several if not most of the enzymes involved in the conversion of acetyl-CoA into IPP may be located partly or even predominantly in peroxisomes – subcellular organelles that are implicated in a variety of metabolic processes (Biardi et al., 1994; Biardi and Krisans, 1996; Keller et al., 1985; Olivier et al., 1999; Paton et al., 1997). This had led to the postulation that peroxisomes play a central role in isoprenoid biosynthesis (Kovacs et al., 2002). One of the enzymes that has been studied more thoroughly in this respect is mevalonate kinase (MK; E.C. 2.7.1.36). MK is the first enzyme to follow the highly regulated HMG-CoA reductase and catalyses the phosphorylation of mevalonate to produce phosphomevalonate (for reviews, see Houten et al., 2000; Houten et al., 2003).

The claim that MK might be located predominantly in peroxisomes is based on several observations. First, immunoblot analysis of gradient fractions obtained by equilibrium density centrifugation of rat liver homogenates revealed immunoreactive material in both cytosolic and peroxisomal fractions (Stamellos et al., 1992). Second, immunofluorescence microscopy performed with CV1 cells overexpressing rat *MK* cDNA revealed a colocalisation of rat MK with peroxisomal catalase (Biardi et al., 1994). Third, immunogold labelling experiments using MK-specific antisera and performed on liver tissue from rats treated with cholestyramine and statins revealed specific labelling mainly in the peroxisomal matrix (Biardi et al., 1994). Furthermore, selective permeabilisation with digitonin of CV1 cells showed latency of MK similar to that of peroxisomal catalase, suggesting that both enzymes are in the same subcellular compartment (Biardi and Krisans, 1996). Furthermore, in some livers and fibroblast cells of patients suffering from Zellweger syndrome, a markedly deficient MK activity was found. Because the loss of peroxisomes, which occurs in Zellweger cells, leads to the mislocalisation of peroxisomal enzymes to the cytosol, often followed by inactivation and/or degradation of these enzymes, this finding has been interpreted as indicative for a peroxisomal localisation of MK (Biardi et al., 1994; Krisans et al., 1994; Wanders and Romeijn, 1998). Finally, the amino acid sequence of MK harbours an N-terminal stretch with a putative consensus sequence of the peroxisomal targeting sequence type 2 (PTS2) and thus may be involved in peroxisomal targeting of MK via the PTS2 receptor protein PEX7 (Olivier and Krisans, 2000).

More recent data, however, do not support a peroxisomal localisation of MK. First, selective permeabilisation with digitonin of rat hepatoma H35 cells resulted in a total release of MK activity, similar to the release of the cytosolic marker lactate dehydrogenase, suggesting that both enzymes are in the same subcellular compartment. Peroxisomal catalase activity was completely retained in the cells after permeabilisation

(Gupta et al., 1999). Second, we measured normal MK activity and MK protein levels in fibroblasts and liver homogenates of patients with a peroxisome biogenesis defect and in liver homogenates of *PEX5* knockout mice (Hogenboom et al., 2002) (Hogenboom et al., 2003). Moreover, we showed that the deficient MK activities found in some livers of diseased Zellweger patients are a result of the bad preservation of the livers, rather than a result of the presumed mislocalisation of the protein (Hogenboom et al., 2002). Finally, in conventional subcellular fractionation studies performed with rat tissue and cultured human fibroblasts, and digitonin permeabilisation experiments with cultured human fibroblasts, we have never been able to demonstrate a peroxisomal localisation of MK activity (S.H. et al., unpublished).

In summary, one should conclude that from the combined data it remains unclear whether MK is peroxisomal, at least in humans. This prompted us to initiate a thorough study to determine conclusively the subcellular localisation of human MK both under normal conditions and when overexpressed in cell lines. We used a variety of biochemical and microscopical techniques and found a cytosolic localisation of both endogenously expressed and overexpressed MK and no indication of a peroxisomal localisation.

Materials and Methods

Cell lines and culture conditions

Primary skin fibroblasts were obtained from a healthy control subject, from a patient who suffered from Zellweger syndrome and who was a homozygote for an insertion mutation in the *PEX19* gene (Matsuzono et al., 1999), and from a patient affected with homozygous familial hypercholesterolaemia (FHC) (GM00701, Coriell cell repositories). The fibroblasts were cultured in HAM F-10 containing 10% fetal calf serum (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 the experiments the cells were grown until 70-80% confluency, after which the medium was substituted for HAM containing 10% lipoprotein (cholesterol)-depleted FCS. Experiments were performed after 72 hours of culturing in lipoprotein (cholesterol)-depleted medium.

For MK expression studies, the HEK293 Flp-In and CV1 Flp-In cell lines (Invitrogen) were used and cultured in Dulbecco's modified Eagle's medium (DMEM) 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 the experiments the cells were grown until 70-80% confluency, after which the medium was substituted for DMEM containing 10% lipoprotein (cholesterol)-depleted FCS. Experiments were performed after 24 hours of culturing in lipoprotein (cholesterol)-depleted medium.

Generation of cell lines stably overexpressing human MK

The open reading frame (ORF) of control human *MVK* cDNA was amplified by PCR from cDNA prepared from human skin fibroblasts using primer set $MK_{1\text{-}39}$ (5′-cg ata gga tcc ATG TTG TCA GAA GTC CTA CTG GTG TCT GC-3′) and $MK_{1195\text{-}1177}$ (5′-cga tag gta CCT CTC AGA GGC CAT CCA G-3′). The primers introduce a 5′ *Bam*HI site and a 3′ *Kpn*I site (underlined). The PCR product was ligated into the pGEM-T vector (Promega) and sequenced to exclude PCR-introduced mutations. Subsequently, the ORF was released as a *Bam*HI-*Sal*I fragment and ligated as a *Bam*HI-*Xho*I fragment, under the control of the CMV promoter, in the pcDNA5/FRT vector (Invitrogen). The entire insert was sequenced to assure the absence of taq polymerase-introduced errors. To construct an SKL-tagged MK, the ORF of MK was amplified by PCR using primer set $MK\text{-}SKL_{1\text{-}29}$ (5′-cg ata gga tcc ATG TTG TCA GAA GTC CTA CTG GTG TCT GC-3′) and $MK\text{-}SKL_{1188\text{-}1170}$ (5′-ctc gag *tca aag cct tga* GAG GCC ATC CAG GGC TTG C-3′). The primers introduce a 5′ *Bam*HI site and a 3′ *Xho*I site (underlined) and the SKL sequence (italics). The MK-SKL amplicon was ligated as a *Bam*HI-*Xho*I fragment in the pcDNA5/FRT vector (Invitrogen) and verified by sequencing of the insert.

HEK293 Flp-In cells or CV1 Flp-In cells were cultured in DMEM, containing 10% FCS, 1% penicillin/streptomycin. Stable MK-expressing cell lines were generated by cotransfection of pOG44 (Invitrogen) and pcDNA5/FRT-MK (CV1 and HEK293) or pcDNA5/FRT-MK-SKL (CV1) 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 media were changed every 3-4 days until Hygromycin-resistant colonies were evident. Control Hygromycin-resistant cell lines were generated by cotransfection of pOG44 with the empty pcDNA5/FRT vector. For expression studies, the HEK293 Flp-In cell lines stably expressing human MK (HEK-MK), CV1 Flp-In cell lines stably expressing human MK (CV1-MK) or MK-SKL (CV1-MK-SKL) 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 MK activity in cells overexpressing human MK was 30-35 times higher compared with the activity in control cell lines.

Subcellular fractionation

For subcellular fractionation studies, cells were cultured in 162 cm^2 Falcon flasks, harvested, washed three times with PBS and twice with fractionation buffer (0.25 M sucrose, 1 mM EDTA, 10 mM HEPES, 1 mM phenylmethylsulfonyl fluoride, pH 7.4). Next, the cells were homogenised using a ball bearing cell cracker (EMBL, Germany), after which the post nuclear supernatant (PNS, 10 minutes, 500 ***g***) was layered on top of a continuous Nycodenz gradient (15-35%), with a cushion of 1 ml 50% Nycodenz in 0.25 mM sucrose, 5 mM 3-[N-morpholino]propanesulfonic acid (MOPS), 1 mM EDTA, and 2 mM KCl (pH 7.3). Gradients were centrifuged for 2.5 hours 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 permeabilisation with digitonin

Cell permeabilisation experiments were performed with cells attached to plates, essentially as described by Biardi and Krisans (Biardi and Krisans, 1996) with a few modifications. HEK293 and CV-1 cells were seeded in 60 mm plates at a density of 3.0×10^5 cells/plate and fibroblast cells at a density of 2.0×10^5 cells/plate. After culturing for 1 or 3 days in DMEM or HAM containing 10% lipoprotein (cholesterol)-depleted FCS, cell were washed twice with ice-cold KH buffer (50 mM HEPES, 110 mM KOAc, pH 7.2). The plates were transferred on ice and incubated in KHM buffer (110 mM KOAc, 20 mM HEPES, 2 mM MgOAc, pH 7.2) containing various concentrations of digitonin (0, 20, 50, 150, 500 or 1000 μg/ml) or, as a control, 0.1% (w/v) Triton X-100. After 5 minutes, the buffer was collected as supernatant fractions and kept on ice. Subsequently, cells were incubated in KH buffer containing 1000 μg/ml digitonin. After 30 minutes, the buffer was collected and kept on ice. These latter fractions are referred to as pellet fractions. Enzyme measurements were done immediately in both fractions.

Enzyme assays

MK activity was measured radiochemically as described previously

(Hoffmann et al., 1992; Wanders and Romeijn, 1998). Phospho gluco isomerase (PGI) (Wanders et al., 1989) and catalase (CAT) (Krisans et al., 1994) activities were measured spectrophotometrically as described.

Immunoblot analysis

Proteins were separated by SDS-PAGE and transferred onto nitrocellulose by semidry blotting (Kyhse-Andersen, 1984). The highly specific affinity-purified antibody directed against human MK (Hogenboom et al., 2002) was used at a 1:1000 dilution. Antigen-antibody complexes were visualised with goat anti-rabbit IgG-alkaline phosphatase conjugate and CDP-star. As a control for transfer of protein, each blot was reversibly stained with Ponceau S before the incubation with antibodies.

Immunofluorescence

Cells were seeded on cover slides in 6-well plates and cultured as indicated in *Cell lines and culture conditions*. Immunofluorescence was performed as described (van Grunsven et al., 1999). Cells were double labelled with antibodies directed against human MK (Hogenboom et al., 2002) and the peroxisomal marker catalase (Wiemer et al., 1992) or the cytosolic marker metallo matrix protein 7 (MMP7) (MMP-7 Ab-1 (Clone 1D2), Labvision). MK antibodies were visualised using biotinylated donkey-anti-rabbit Ig (Amersham) and streptavidin-labelled fluorescein isothiocyanate (Strep-FITC). Catalase and MMP7 were visualised using goat-anti-mouse-labelled Alexa568 (Molecular Probes). Pictures were taken using a confocal laser scanning microscope (Leica).

Liver immunocytochemistry

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 previously (Espeel et al., 1990; Espeel and Van Limbergen, 1995). Ultrathin sections of Unicryl-embedded samples were immunostained with polyclonal antibodies against MK (Hogenboom et al., 2002) or the peroxisomal alanine/glyoxylate aminotransferase (AGT) (Wanders et al., 1987) as previously described (Espeel and Van Limbergen, 1995). Cryostat sections (6 μm) were immunostained against MK or AGT as previously described (Espeel et al., 1990). Negative controls were incubated with normal rabbit serum.

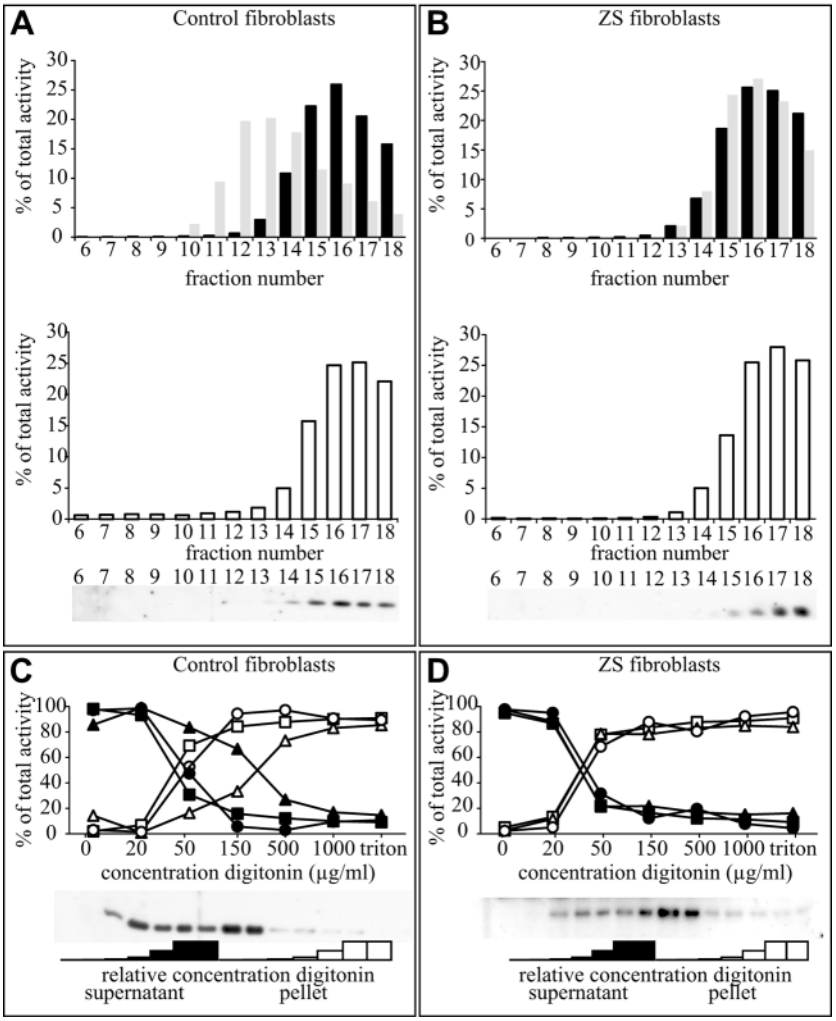

Fig. 1. Subcellular fractions of human fibroblasts derived from a control subject (A) or a ZS patient (B) were obtained by Nycodenz equilibrium density gradient centrifugation as described in Materials and Methods. Fractions were analysed for the cytosolic marker PGI (black bars), the peroxisomal marker CAT (grey bars) and MK (open bars). Relative activities were expressed as a percentage of total gradient activity present in each fraction. The pattern of distribution of MK activity and MK protein as determined by immunoblot analysis with an affinity purified antibody raised against human MK was similar to the pattern of PGI activity. 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 analysed for the activities of the cytosolic marker PGI (square), the peroxisomal marker CAT (triangle) and MK (circle). Relative activities were expressed as a percentage of total activity (supernatant + pellet) present in each fraction. The pattern of latency of MK activity and MK protein as determined by immunoblot analysis with an affinity purified antibody raised against human MK were similar to the pattern of PGI activity.

Results

Subcellular fractionation of MK in human fibroblasts

To determine whether in human cells MK is localised in the cytosol or the peroxisomes, or both, we first performed subcellular fractionation studies with human skin fibroblasts. As a control we included fibroblasts from a patient who suffered from Zellweger syndrome (ZS). Previous studies have shown that in this particular cell line no peroxisomal remnants are present because of a homozygous insertion in the *PEX19* gene (Matsuzono et al., 1999). After growth of the cells in lipoprotein-depleted medium to assure optimal induction of the isoprenoid biosynthetic pathway, we prepared a postnuclear supernatant (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 catalase and the cytosolic marker enzyme PGI (Fig. 1A). In the ZS fibroblasts, both marker enzymes colocalise as expected from the absence of

peroxisomes, which leads to the cytosolic localisation of peroxisomal enzymes (Fig. 1B). When MK activity was measured in the gradient fractions, the activity showed the same distribution as the cytosolic marker PGI both in the normal fibroblasts (Fig. 1A) and in the ZS fibroblasts (Fig. 1B). Immunoblot analysis of the fractions from the same density gradients using affinity-purified antiserum against human MK revealed a similar distribution pattern for the MK protein as for the MK activity (Fig. 1A,B).

Digitonin permeabilisation studies in human fibroblasts

As an alternative approach to study the subcellular localisation of MK in human fibroblasts we exposed the cells to increasing concentrations of digitonin. Digitonin permeabilises cellular membranes by complexing with cholesterol. Because the membranes of most cell organelles contain lower levels of cholesterol than the plasma membrane, cells will lose their cytosolic components at lower concentrations of digitonin than the organellar contents. Indeed, when we measured the enzyme activities of CAT and PGI in supernatant and pellet fractions of normal fibroblasts we found a clearly increased latency for CAT compared with PGI (Fig. 1C). This indicated that the plasma membrane was disrupted first, resulting in the release of cytosolic PGI. The peroxisomal membranes, however, were only permeabilised at higher concentrations of digitonin, resulting in the release of the peroxisomal matrix content, including CAT, into the supernatant fraction. As expected, in the ZS fibroblasts lacking peroxisomes, no difference in latency between PGI and CAT was observed (Fig. 1D). When we measured MK activity in all pellet and supernatant fractions, we found that the release of MK from the normal fibroblasts into the supernatant fractions occurred at the same concentration of digitonin as cytosolic PGI (Fig. 1C). In the ZS fibroblasts, PGI, CAT and MK were released from the cells at the same digitonin concentration (Fig. 1D). Immunoblot analysis of the various fractions using the affinity-purified antiserum against human MK revealed a similar distribution pattern for the MK protein as for its activity (Fig. 1C,D). Thus, in digitonin permeabilisation studies, human MK behaves similarly to cytosolic PGI and clearly differently than peroxisomal CAT.

Immunofluorescence studies in fibroblasts

To study the subcellular localisation of human MK further, we performed immunofluorescence microscopy, making use of the highly specific affinity-purified polyclonal antibodies directed against human MK. We performed double labelling of fibroblasts cultured in lipoprotein-depleted medium using the anti-MK antiserum and a monoclonal antibody directed against human peroxisomal CAT or a monoclonal antibody directed against human MMP7, a cytosolic marker (Fig. 2). When we compared the immunolabelling of MK in the 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 localisation of MK. Moreover, there was no colocalisation of MK and CAT in the normal fibroblasts, whereas in the ZS fibroblasts the distribution pattern of CAT was the same as that of MK, indicating a colocalisation of CAT and MK in the cytosol. Also, when we compared the fluorescent signals obtained with anti-MK and anti-MMP7 we found a clear colocalisation in the cytosol both in normal fibroblasts and ZS fibroblasts.

Subcellular localisation of human MK in overexpressing cell lines

The results of the various localisation studies in human

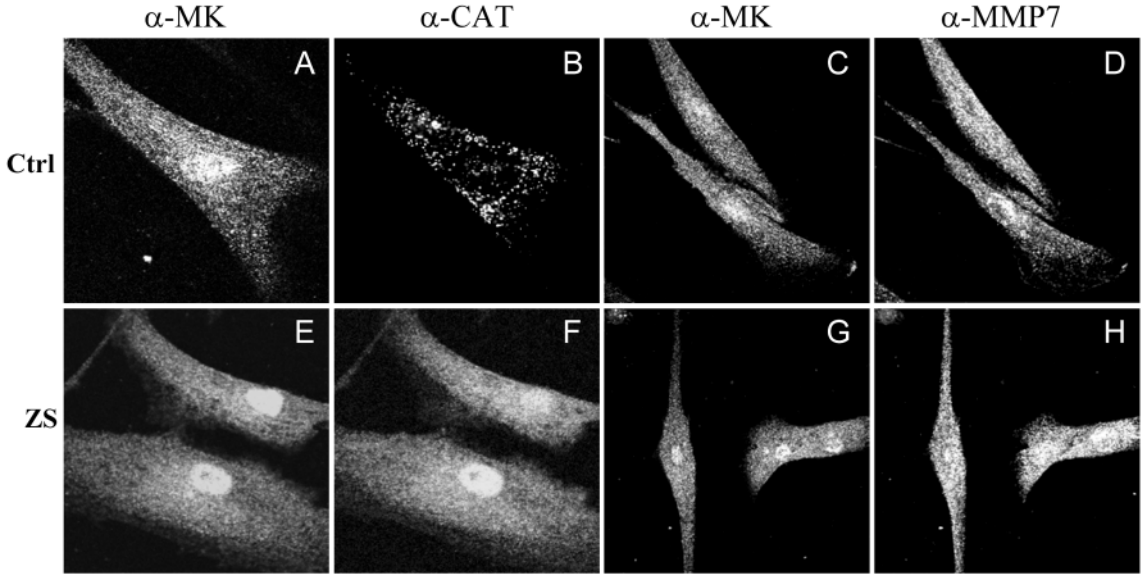

Fig. 2. Human fibroblasts derived from a control subject (A-D) or a ZS patient (E-H) were labelled with antibodies as described in Materials and Methods. Cells were double labelled using antibodies directed against MK (A,E) and the peroxisomal marker CAT (B,F) or with antibodies directed against MK (C,G)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

fibroblasts all indicate that endogenous MK is at least predominantly, if not exclusively, located in the cytosol and not in peroxisomes. These results are in contrast to the reported peroxisomal localisation of MK in the liver of cholestyramine plus mevinolin-treated rats and in CV1 cells overexpressing rat MK (Biardi et al., 1994). To determine whether this discrepancy in localisation might be due to the specific cell type studied and/or the result of overexpression of MK, we decided to investigate the subcellular localisation of overexpressed MK in various cell types. These include CV1 and HEK293 cells stably transfected with human MK cDNA under the control of the CMV promoter and human fibroblasts from a patient homozygous for familial hypercholesterolaemia. The fibroblasts of such patients exhibit a five times higher MK activity when compared with control fibroblasts (S.H. et al., unpublished).

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 MK activities and MK protein content in all fractions, we found again a distribution pattern of MK similar to cytosolic PGI and clearly distinct from peroxisomal CAT in all cell lines (Fig. 3A-C). This was the case for endogenously overexpressed human MK (FHC, Fig. 3A), constitutively overexpressed human MK (HEK-MK, Fig. 3B; CV1-MK, Fig. 3C), endogenously expressed human MK (HEK– cells; not shown) and monkey

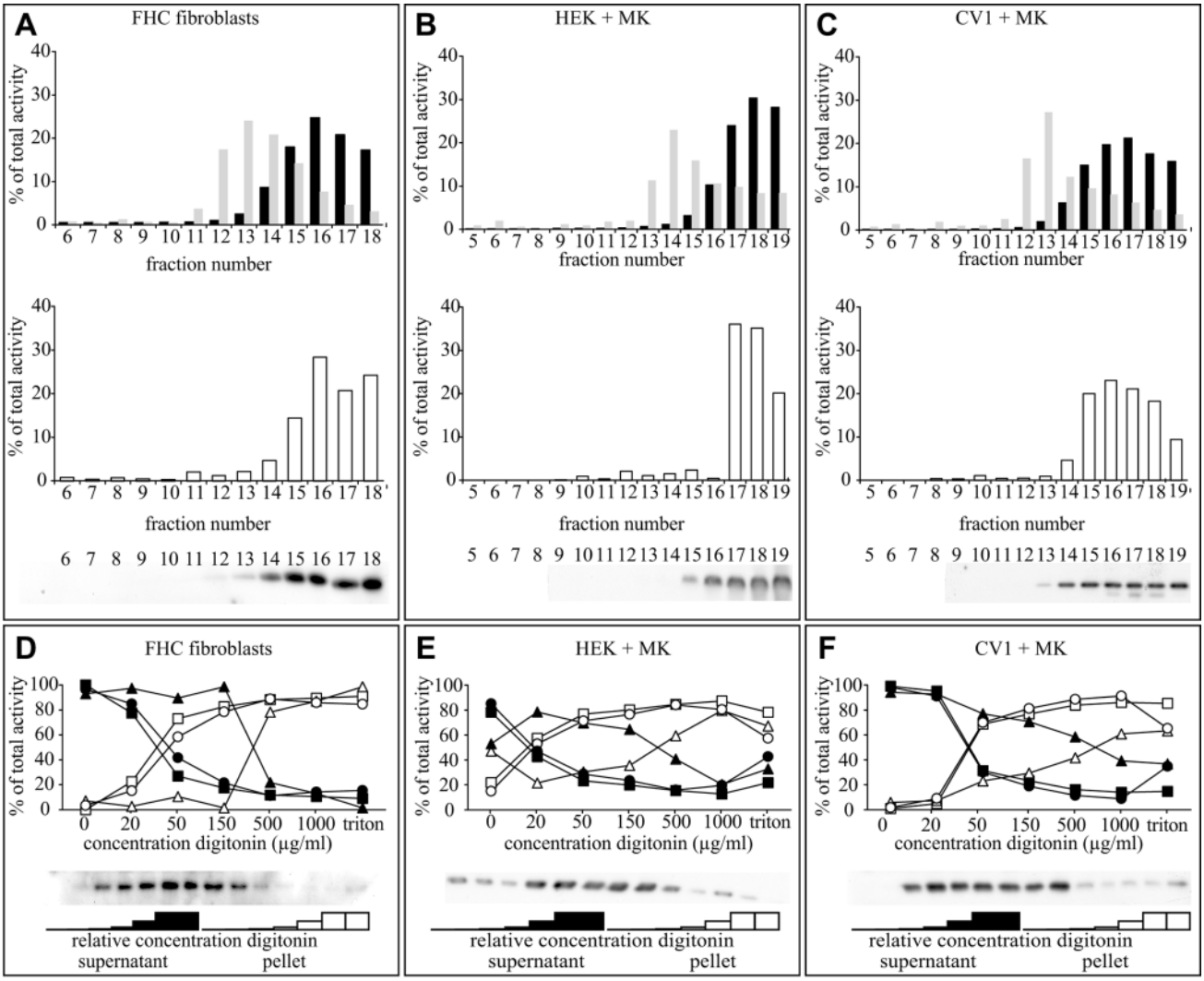

Fig. 3. Subcellular fractions of human fibroblasts derived from an FHC patient (A), HEK (B) cells or CV1 cells (C) overexpressing full-length human MK were obtained by Nycodenz equilibrium density gradient centrifugation as described in Materials and Methods. Fractions were analysed for the cytosolic marker PGI (black bars), the peroxisomal marker CAT (grey bars) and MK (open bars). Relative activities were expressed as a percentage of total gradient activity present in each fraction. The patterns of distribution of MK activity and MK protein as determined by immunoblot analysis with an affinity purified antibody raised against human MK were similar to the pattern of PGI activity. Human fibroblasts derived from an FHC patient (D), HEK cells (E) or CV1 cells (F) overexpressing full-length human MK were incubated with increasing concentrations of digitonin as described in Materials and Methods. Supernatant (open symbols) and pellet (closed symbols) fractions were analysed for the activities of the cytosolic marker PGI (square), the peroxisomal marker CAT (triangle) and MK (circle). Relative activities were expressed as a percentage of total activity (supernatant + pellet) present in each fraction. The patterns of latency of MK activity and MK protein as determined by immunoblot analysis with an affinity purified antibody raised against human MK were similar to the pattern of PGI activity.

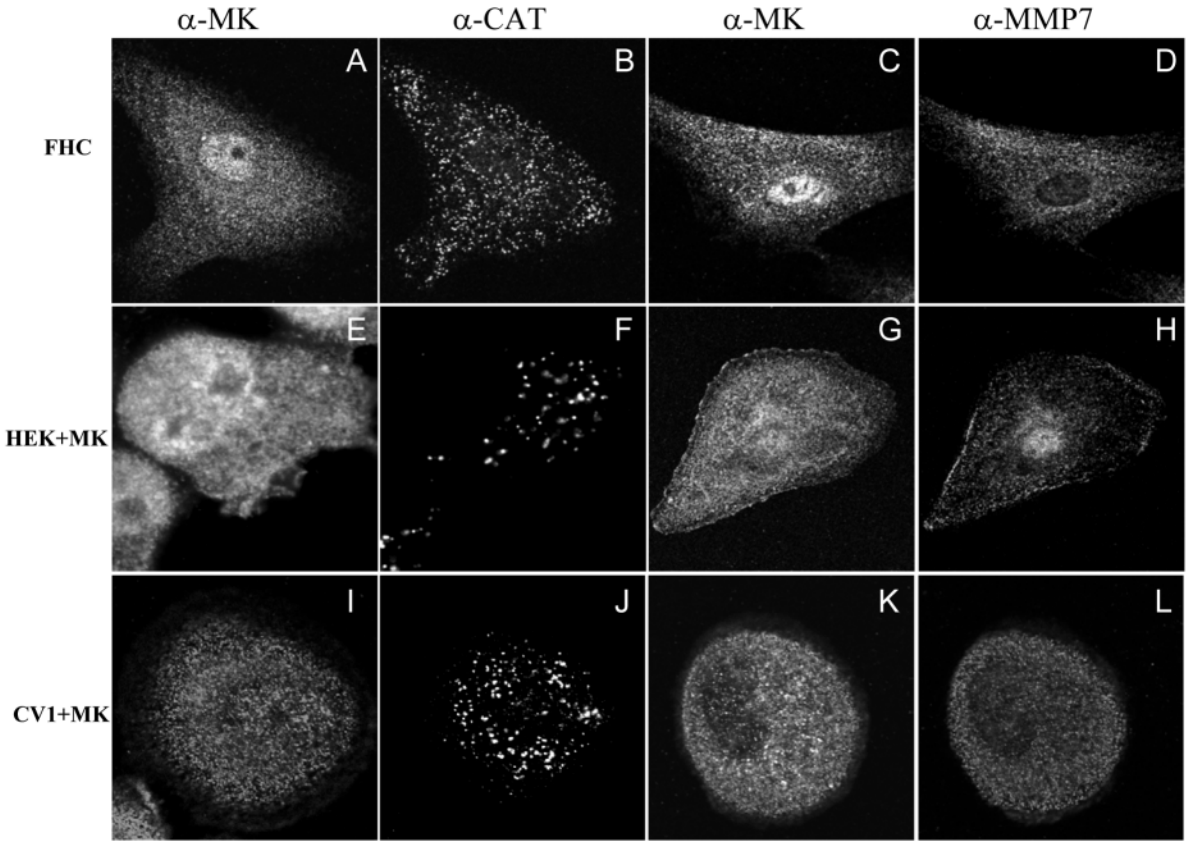

Fig. 4. Human fibroblasts derived from an FHC patient (A-D), HEK cells (E-H) or CV1 cells (I-L) overexpressing full-length human MK were labelled with antibodies as described in Materials and Methods. Cells were double labelled using antibodies directed against MK (A,E,I) and the peroxisomal marker CAT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

MK (CV1– cells; not shown). Also, after selective permeabilisation of the cellular membranes using increasing concentrations of digitonin, we found that both endogenously and constitutively overexpressed human MK behaved similar to cytosolic PGI (Fig. 3D-F). Moreover, immunofluorescent labelling of the endogenously and constitutively overexpressed MK showed a cytosolic localisation that was the same as that of cytosolic MMP7 protein, and clearly different from the localisation of CAT in these cell lines (Fig. 4).

SKL-tagged MK localises to peroxisomes

To exclude the possibility that our antibody is only able to detect MK when it is located in the cytosol but not when it is localised in peroxisomes, we constructed a full-length human MK to which an artificial PTS1 signal (SKL) was added at the carboxy terminus, and we expressed this construct in CV1-FlpIn cells. Although some cytosolic localisation was observed, there also was a peroxisomal localisation of MK as evident from the punctated pattern, which colocalised with peroxisomal catalase as visualised by immunofluorescence microscopy (Fig. 5).

Immunocytochemical studies in human liver

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

In the immunogold labelling experiments using the highly specific affinity-purified polyclonal antibodies directed against human MK, we found only occasional labelling in the cytosol of the human liver. Although we carefully checked a large number of peroxisomes we were unable to detect any labelling of MK in these peroxisomes (Fig. 6A). Moreover, even after incubation with higher concentrations of antibodies, as a result of which aspecific labelling strongly increased, no peroxisomal labelling could be observed. As a control we performed immunogold labelling experiments on the same sections with antibodies against peroxisomal AGT. This revealed a distinct label over the peroxisomal matrix, whereas no label was observed in negative controls (Fig. 6B).

Because a cytosolic localisation of a nonabundant antigen is hard to detect in ultrathin sections, we also performed immunocytochemistry on cryostat sections of human liver using the antibodies against MK and AGT. Light microscopy revealed a clear diffuse staining reaction in the cytosol of the

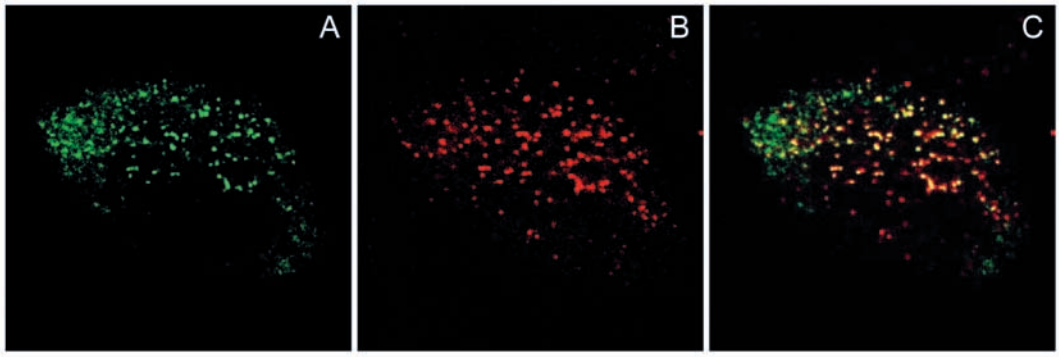

Fig. 5. CV1 cells overexpressing full-length human MK with an artificial PTS1 signal (SKL) were labelled with antibodies as described in Materials and Methods. Cells were double labelled using antibodies directed against MK (A) and a peroxisomal marker CAT (B). The overlay image of both signals (C) shows a clear colocalisation of MK and CAT in the majority of peroxisomes. In addition, cytosolic labelling of MK can be observed (upper left corner).

hepatocytes (Fig. 6C) with the antibodies against MK, following a similar pattern as typically found for the localisation of catalase in Zellweger livers (not shown). By contrast, a distinct punctated pattern of peroxisomes was obtained with antibodies against AGT (Fig. 6D).

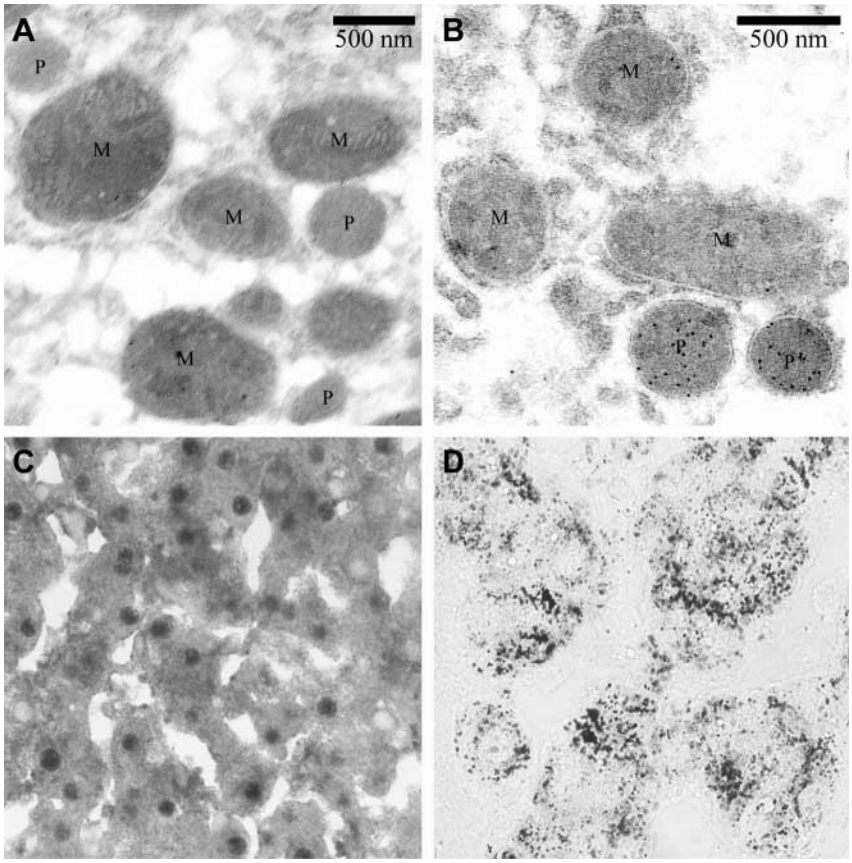

Fig. 6. Electron microscopy (A,B) and light microscopy (C,D) of human control liver. (A) Ultrathin Unicryl sections of human liver were immunostained with the affinity purified antibodies against MK. The peroxisomes (P) remain unlabelled. (B) Ultrathin Unicryl sections immunostained with antibodies against against AGT reveals a clear localisation in the peroxisomal matrix. Bar, 500 nm; M, mitochondria. (C) Cryostat sections immunostained with affinity purified antibodies against MK (silver enhancement of colloidal gold) reveals a diffuse reaction in the cytosol of the hepatocytes. For comparison, immunostaining with antibodies against AGT reveals a distinct granular pattern, reflecting a peroxisomal localisation of AGT (D).

Discussion

Since their discovery in the late 1960s, an increasing number of important metabolic pathways has been attributed to peroxisomes, making it clear that these organelles are essential cell constituents, the absence of which is incompatible with human life and survival. Most intriguing among these pathways, and claimed to be predominantly peroxisomal in the 1990s, has been the isoprenoid/cholesterol biosynthesis pathway, the components of which for several decades had been thought to have a shared cytosolic/endoplasmic reticulum localisation. More specifically, a predominant peroxisomal localisation has been reported for several enzymes functioning in the presqualene segment of the pathway, including 3-hydroxy-3-methylglutaryl CoA reductase (Keller et al., 1985), MK (Biardi et al., 1994), phosphomevalonate kinase (Olivier et al., 1999), mevalonate pyrophosphate decarboxylase (Biardi and Krisans, 1996), isopentenyl pyrophosphate isomerase (Paton et al., 1997) and farnesylpyrophosphate synthase (Gupta et al., 1999; Krisans et al., 1994).

We have sought confirmation for the claim that MK would be predominantly peroxisomal and, as a consequence, that peroxisomes would play a central role in the biosynthesis of isoprenoids, including cholesterol. We used a variety of biochemical and microscopical experimental approaches but in all cases we only found evidence for a cytosolic localisation and no evidence for a peroxisomal localisation. This is true for both endogenous and for overexpressed human MK. These results are in contrast to those published by Krisans and co-workers, who reported a predominant peroxisomal localisation of rat MK in the case of high expression (Biardi et al., 1994). Although we cannot exclude a different localisation of the enzyme in humans and rat, it should be noted that our subcellular fractionation studies of rat liver also revealed a cytosolic localisation of MK (S.H.

et al., data not shown). Moreover, Gupta et al. reported that MK in rat hepatoma H35 cells behaves like cytosolic lactate dehydrogenase in digitonin permeabilisation studies similar to the ones we have reported (Gupta et al., 1999). Thus, it remains unclear how Krisans and co-workers determined that MK would be predominantly peroxisomal. Indeed, immunogold labelling experiments using highly specific, affinity-purified antibodies directed against human MK did not even reveal a minor peroxisomal localisation of human MK. Importantly, we were able to show a peroxisomal localisation of human MK when the protein was tagged with a carboxyterminal PTS1 sequence, hence our inability to show a peroxisomal localisation of MK was not due to a technical shortcoming.

Our results are also in line with our recent finding of a normal MK activity in cells of patients who suffered from Zellweger syndrome (Hogenboom et al., 2002; Hogenboom et al., 2003). In addition, Ghys et al. recently reported that the human PTS2 receptor was not retained by Ni-nitrilotriacetic acid (NTA) resin loaded with $(His)_6$-MK (Ghys et al., 2002), which corroborated with our own finding that human MK is not recognised by human and yeast *PEX7* in a yeast two-hybrid screen (S.H., unpublished). Although the putative PTS2 motif in the MK sequence (KVX_5HA) has a good match to the postulated consensus sequence, it is very different from the RLX_5HL sequence found in the three other known PTS2-targeted proteins in humans (phytanoyl-CoA 2-hydroxylase (Jansen et al., 1997), 3-ketoacyl-CoA thiolase (Bout et al., 1988) and alkyl-dihydroxyacetonephosphate synthase (de Vet et al., 1997). Furthermore, the recently published crystal structure of rat MK complexed with ATP (Fu et al., 2002) and the mutation analysis of rat MK (Potter et al., 1997) showed that the N-terminal lysine of the putative PTS2 motif is part of the ATP binding site. Finally, the motif is part of a conserved domain, which is also present in MKs of archeabacteria and eubacteria, which do not posses peroxisomes, and in the related nonperoxisomal galactokinases (Houten et al., 2000).

Now that we have shown that, at least in humans, MK is not localised in peroxisomes but in the cytosol, one can raise questions on the supposed peroxisomal localisation of other enzymes functioning in the presqualene segment of the isoprenoid biosynthetic pathway. In fact, Michihara et al. recently reported a cytosolic localisation of rat and mouse mevalonate pyrophosphate decarboxylase, which was also thought to be peroxisomal (Michihara et al., 2001; Michihara et al., 2003). Moreover, using a similar approach as for human MK in this study, we found that human phosphomevalonate kinase and mevalonate pyrophosphate decarboxylase are localised in the cytosol and not in peroxisomes (S.H., unpublished). These combined data strongly suggest that peroxisomes in humans may not play a central role in isoprenoid/cholesterol biosynthesis and corroborate well with our previous findings that functional peroxisomes are not required for isoprenoid biosynthesis (Hogenboom et al., 2002; Hogenboom et al., 2003).

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