Subcellular localization of the hum isoprenoid biosynthesis pathway

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SUMMARY

The importance of the cholesterol/isoprenoid biosynthetic pathway for human development and health is emphasized by the multiple morphogenic, developmental and neurological abnormalities associated with inborn errors in this pathway. The subcellular localization of the pathway is a controversial subject. In the past decade several reports have suggested that peroxisomes play a critical role in the pathway since several of the enzymes involved have been reported to be localized predominantly in these organelles. However, other reports do not support this peroxisomal localization. In this study, the subcellular localization of one of the enzymes, human mevalonate pyrophosphate decarboxylase, was studied by conventional subcellular fractionation and digitonin permeabilisation studies, immunofluorescence microscopy and immunoelectron microscopy. An exclusive cytosolic localization was found for both endogenous human mevalonate pyrophosphate decarboxylase (in human fibroblasts, liver and HEK293 cells) and overexpressed mevalonate pyrophosphate decarboxylase (in human fibroblasts, HEK293, CV1 cells). We did not obtain any indication for a peroxisomal localization. Our results do not support a central role of peroxisomes in the cholesterol/isoprenoid biosynthetic pathway.

INTRODUCTION

Mevalonate pyrophosphate decarboxylase (MPD; E.C. 4.1.1.33) is one of the early enzymes of the cholesterol/isoprenoid biosynthetic pathway which provides the cell with the isoprene unit isopentenylpyrophosphate (IPP). IPP is the building block of a wide variety of sterols and non-sterol isoprenoids that are involved in diverse cellular processes, including cell growth and differentiation, glycosylation, signal transduction, and electron transport (1).

The prevailing view with respect to the subcellular distribution of the enzymes involved in the cholesterol/isoprenoid biosynthesis is that peroxisomes, subcellular organelles
Mevalonate pyrophosphate decarboxylase is a cytosolic enzyme

...implicated in a variety of metabolic processes, play a central role in the early, pre-squalene part of the pathway (2). This view is based on various reports indicating that several enzymes involved in the conversion of acetyl-CoA into IPP are partly or even predominantly located in peroxisomes (3, 4, 5, 6, 7).

MPD catalyzes the sixth reaction of the isoprenoid/cholesterol biosynthetic pathway which is the decarboxylation and hydration of mevalonate pyrophosphate to produce IPP. The postulation that MPD would be located in peroxisomes is based on the following observations. Firstly, in some livers of patients suffering from Zellweger syndrome, which do not contain peroxisomes, a 60% decrease in MPD activity was found (8). Since peroxisomal deficiency often leads to degradation and/or inactivation of peroxisomal enzymes as a result of the mislocalization to the cytosol, this decreased activity has been explained to be indicative of a peroxisomal localization of MPD. Secondly, latency of MPD activity was found in monkey kidney (CV1) cells after selective permeabilisation of cellular membranes with digitonin (6). This latency was similar to the latency of the peroxisomal catalase, suggesting that both enzymes are located in the same subcellular compartment. Thirdly, immunofluorescence microscopy performed with CHO cells and peroxisomal targeting sequence (PTS) 1 protein import-deficient human fibroblasts, transiently transfected with an amino-terminal fragment (47 amino acids) of human MPD tagged with an HA epitope revealed a punctate pattern similar to the distribution of the peroxisomal catalase suggesting that the amino terminus harbours a PTS2-like PTS (9). Indeed, transient transfection of this construct in PTS2 protein import-deficient fibroblasts resulted in a cytosolic localization, suggesting that the construct is imported into peroxisomes via the PTS2 receptor protein PEX7 (9). Finally, transient transfection of the same construct lacking the SVX5QL sequence resulted in a cytosolic localization indicating that this sequence might be a novel PTS2-like signal (9).

Other data, however, do not support a peroxisomal localization of MPD. Firstly, we measured normal MPD activity and MPD protein levels in fibroblasts and liver homogenates of patients with a peroxisome biogenesis defect and in liver homogenates of PEX5 knock-out mice (10, 11). Moreover, also in PEX2 protein-import deficient cells from patients affected with rhizomelic chondrodysplasia punctata no deficient activity was observed (unpublished data). Secondly, selective permeabilization with digitonin of rat hepatocytes, normal rat kidney cells or mouse melanoma (B16F10) cells resulted in a complete release of MPD protein similar to the release of the cytosolic lactate dehydrogenase, suggesting that both enzymes are in the same subcellular compartment. Under these conditions peroxisomal catalase activity was completely retained in the cells (12, 13). Thirdly, after subcellular fractionation of B16F10 cells, the MPD protein was recovered in fractions which contained only cytosolic (and no peroxisomal) proteins indicating that MPD is a cytosolic protein (13). Furthermore, immunofluorescence microscopy in rat hepatocytes using purified antibodies directed against rat MPD showed a cytosolic localization of the MPD protein (12). Finally, in conventional subcellular fractionation studies performed with HepG2 cells, we never have been able to demonstrate a peroxisomal localization of MPD activity (unpublished data).

In summary, from the combined data one may conclude that MPD is a cytosolic protein in rat and mouse cells while in human cells the subcellular localization is not clear. So far, the subcellular distribution of human MPD has been studied mostly in overexpression...
systems using constructs containing only the amino-terminal part of MPD. However, it remains to be determined whether the authentic human protein is a peroxisomal or cytosolic protein or both. This prompted us to initiate a thorough study to conclusively determine the subcellular localization of human MPD both under normal conditions and when overexpressed in cell lines. Using a variety of biochemical and microscopical techniques, we found a cytosolic localization of both endogenously expressed and overexpressed MPD 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 patient who suffered from Zellweger syndrome and who was a homozygote for an insertion mutation in the *PEX19* gene (14), and from a patient affected with homozygous familial hypercholesterolemia (FHC) (GM00701, Coriell cell repositories). The fibroblasts were cultured in HAM F-10 containing 10% FCS and 1% penicillin/streptomycin in a temperature and humidity controlled incubator (95% air, 5% CO₂ as the gas phase) at 37°C. Prior to experiments the cells were grown until 70-80% confluency after which the medium was substituted with HAM containing 10% lipoprotein (cholesterol)-depleted fetal calf serum. Experiments were performed after 72 hours of culturing in lipoprotein (cholesterol)-depleted medium.

For MPD expression studies, the HEK293 Flp-In and CV1 Flp-In cell lines (Invitrogen) were used and cultured in DMEM containing 10% FCS, 1% penicillin/streptomycin and 100 μg/ml hygromycin in a temperature and humidity controlled incubator (95% air, 5% CO₂ as the gas phase) at 37°C. Prior to experiments the cells were grown until 70-80% confluency after which the medium was substituted for DMEM containing 10% lipoprotein (cholesterol)-depleted fetal calf serum. Experiments were performed after 24 hours of culturing in lipoprotein (cholesterol)-depleted medium.

**Generation of cell lines stably overexpressing human MPD**

The open reading frame (ORF) of human MPD cDNA was amplified by PCR from cDNA prepared from human skin fibroblasts and ligated as a *BamH1-XhoI* fragment under 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.

HEK293 Flp-In cells or CV1 Flp-In cells were cultured in Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal calf serum (FCS), 1% penicillin/streptomycin. Stable MPD-expressing cell lines were generated by co-transfection of the pOG44 and pcDNA5/FRT-MPD (CV1 and HEK293) 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 co-transfection of the pOG44 with the empty pcDNA5/FRT vector. For expression studies the HEK293 Flp-In cell lines, stably expressing human MPD (HEK-MPD), CV1 Flp-In cell
lines stably expressing human MPD (CV1-MPD) 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 activity in cells overexpressing human MPD was 5 times higher compared to the control cell lines.

Subcellular fractionation
For subcellular fractionation studies, cells were cultured in 162-cm² Falcon flasks, harvested, washed three times with PBS and two times with fractionation buffer (0.25 M sucrose, 1 mM EDTA, 10 mM HEPES, 1 mM phenylmethylsulfonyl fluoride, pH=7.4). Next, the cells were homogenized using a ball bearing cell cracker (EMBL, Germany), after which the post nuclear supernatant (PNS, 10', 500 x 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 MOPS, 1 mM EDTA, and 2 mM KCl (pH=7.3). Gradients were centrifuged for 2.5 hours in a vertical rotor (MSE 8x35) at 19,000 rpm 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 essentially as described by Biardi et al. (6) with a few modifications. HEK293 and CV-1 cells were seeded in 60 mm plates at a density of 3.0 x 10⁶ cells/plate and fibroblast cells at a density of 2.0 x 10⁶ cells/plate. After culturing for one or three days in DMEM or HAM containing 10% lipoprotein (cholesterol)-depleted fetal calf serum, cells were washed twice with ice-cold KH buffer (50 mM HEPES, 110 mM KOAc, pH=7.2). The plates were then transferred on ice and incubated in KHM buffer (20 mM HEPES, 110 mM KOAc, 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 as pellet fractions and kept on ice. Enzyme measurements were done immediately in both fractions.

Enzyme assays
MPD activity was measured radiochemically as described previously (10). Phospho gluco isomerase (PGI) (15) and catalase (CAT) (8) activities were measured spectrophotometrically as described.

Immunoblot analysis
Proteins were separated by SDS-PAGE and transferred onto nitrocellulose by semidry blotting (16). The highly specific affinity-purified antibody directed against human MPD (10) was used at a 1:250 dilution. Antigen-antibody complexes were visualized 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 prior to the incubation with antibodies.
Figure 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 analyzed for the cytosolic marker PGI (black bars) and the peroxisomal marker CAT (gray bars). Relative activities were expressed as a percentage of total gradient activity present in each fraction. The pattern of distribution of MPD protein as determined by immunoblot analysis with an affinity purified antibody raised against human MPD is 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 (closed symbols) and pellet (open symbols) fractions were analyzed for the activities of the cytosolic marker PGI (square) and the peroxisomal marker CAT (triangle). Relative activities were expressed as a percentage of total activity (supernatant + pellet) present in each fraction. The pattern of latency of MPD protein as determined by immunoblot analysis with an affinity purified antibody raised against human MPD is similar to the pattern of PGI activity.

Immunofluorescence
Cells were seeded on cover slides in 6 wells-plates and cultured as indicated in Cell lines and culture conditions. Immunofluorescence was performed as described (17). Cells were double labeled with antibodies directed against human MPD (10) and the peroxisomal marker catalase (18) or the cytosolic marker metallo matrix protein 7 (MMP7) (MMP-7 Ab-1 (Clone 1D2), Labvision). MPD antibodies were visualized using biotinylated Donkey-anti-Rabbit Ig (Amersham) and streptavidin-labeled fluorescein isothiocyanate (Strep-Fite). Catalase and MMP7 were visualized using Goat-anti-Mouse-labeled Alexa568 (Molecular Probes). Pictures were taken using a confocal laser scanning microscope (Leica).
Liver immunoelectron microscopy

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 as described (19). Ultra thin sections of Unicryl embedded samples were immunostained with polyclonal antibodies against MPD (10) or the peroxisomal alanine/glyoxylate aminotransferase (AGT) (20) as previously described (19). Negative controls were incubated with normal rabbit serum.

RESULTS

Subcellular fractionation of MPD in human fibroblasts

To determine whether human MPD is localized 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. Previous studies have demonstrated that in this particular cell line no peroxisomal remnants are present due to a homozygous insertion in the PEX19 gene (14). After growth of the cells in lipoprotein-depleted medium to assure optimal induction of the isoprenoid biosynthetic pathway, we prepared a post-nuclear 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 colocalize as expected from the absence of peroxisomes, which leads to the cytosolic localization of peroxisomal enzymes (Fig. 1B). Immunoblot analysis of the fractions from the same density gradients using affinity-purified antisera against human MPD revealed a similar distribution pattern for MPD protein as for PGI activity (Fig. 1A, B).

Digitonin permeabilization studies in human fibroblasts

As an alternative approach to study the subcellular localization of MPD in human fibroblasts we exposed the cells to increasing concentrations of digitonin. Digitonin permeabilizes cellular membranes by complexing with cholesterol. Since the membranes of most cellular organelles contain lower levels of cholesterol than the plasma membrane, cells will lose their cytosolic components at lower concentrations of digitonin than the organelar 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 to PGI (Fig. 1C). This indicated that the plasma membrane was disrupted first, resulting in the release of cytosolic PGI. The peroxisomal membranes, however, are only permeabilized 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 determined MPD protein by immunoblot analysis in all pellet and supernatant fractions, we found that the release of MPD from the normal fibroblasts into the supernatant fractions occurs at the same concentration of digitonin as cytosolic PGI (Fig. 1C). In the ZS fibroblasts, PGI, CAT and MPD were released...
from the cells at the same digitonin concentration (Fig. 1D). Thus, also in digitonin permeabilization studies human MPD behaves similar as cytosolic PGI and clearly different than peroxisomal CAT.

**Immunofluorescence studies in fibroblasts**

To study the subcellular localization of human MPD further, we performed immunofluorescence microscopy making use of the highly specific affinity-purified polyclonal antibodies directed against human MPD. To this end, we performed double labeling of fibroblasts cultured in lipoprotein-depleted medium using the anti-MPD 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 immunolabeling of MPD 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 localization of MPD. Moreover, there was no co-localization of MPD and CAT in the normal fibroblasts while in the ZS fibroblasts the distribution pattern of CAT is superimposable to that of MPD indicating a co-localization of CAT and MPD in the cytosol. Also when we compared the fluorescent signals obtained with anti-MPD and anti-MMP7 we found a clear colocalization in the cytosol both in normal fibroblasts and ZS fibroblasts.

![Figure 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 MPD (A, E) and the peroxisomal marker CAT (B, F) or with antibodies directed against MPD (C, G) and the cytosolic marker MMP7 (D, H). MPD shows the same pattern as the cytosolic MMP7 in both cell lines. MPD shows co-localization with CAT in the ZS fibroblasts in which CAT is localized in the cytosol but no co-localization is observed between MPD and the peroxisomal CAT in control fibroblasts.](i)
Subcellular localization of human MPD in overexpressing cell lines

The results of the various localization studies in human fibroblasts all indicate that endogenous MPD is at least predominantly, if not exclusively, located in the cytosol and not in peroxisomes. These results are in apparent contrast to the reported peroxisomal localization of an overexpressed amino-terminal fragment of 47 amino acids of the human MPD fused with an HA epitope upon transient transfection into human fibroblasts and CHO (9). To determine whether this discrepancy in localization might be due to a difference in expression levels, we decided to also investigate the subcellular localization of overexpressed MPD in various cell types. These include CV1 and HEK293 cells stably transfected with human MPD cDNA under control of the CMV promoter and human

Figure 3. Subcellular fractions of human fibroblasts derived from an FH C patient (A), HEK293 (B) cells or CV1 cells (C) overexpressing full length human MPD were obtained by nycodenz equilibrium density gradient centrifugation as described in materials and methods. Fractions were analyzed for the cytosolic marker PGI (black bars) and the peroxisomal marker CAT (gray bars). Relative activities were expressed as a percentage of total gradient activity present in each fraction. The pattern of distribution of MPD protein as determined by immunoblot analysis with an affinity purified antibody raised against human MK is similar to the pattern of PGI activity.

Human fibroblasts derived from an FH C patient (D), HEK293 cells (E) or CV1 cells (F) overexpressing full length human MPD were incubated with increasing concentrations of digitonin as described in materials and methods. Supernatant (closed symbols) and pellet (open symbols) fractions were analyzed for the activities of the cytosolic marker PGI (square) and the peroxisomal marker CAT (triangle). Relative activities were expressed as a percentage of total activity (supernatant + pellet) present in each fraction. The pattern of latency of MPD protein as determined by immunoblot analysis with an affinity purified antibody raised against human MPD is similar to the pattern of PGI activity.
fibroblasts from a patient homozygous for familial hypercholesterolemia, which exhibit five times higher MPD activity when compared to control fibroblasts (Hogenboom et al., unpublished results).

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

Figure 4. Human fibroblasts derived from an FHC patient (A-D), HEK293 cells (E-H) or CV1 cells (I-L) overexpressing full length human MPD were labeled with antibodies as described in materials and methods. Cells were double labeled using antibodies directed against MPD (A, E, I) and the peroxisomal marker CAT (B, F, J) or with antibodies directed against MPD (C, G, K) and the cytosolic marker MMP7 (D, H, L). The diffuse distribution pattern of MPD differs from the punctated pattern of CAT but MPD shows the same pattern as the cytosolic MMP7 in all cell lines.
**Mevalonate pyrophosphate decarboxylase is a cytosolic enzyme**

**Immunoelectron microscopy studies in human liver**

While our combined data show that at least in humans, MPD is predominantly a cytosolic protein, they cannot exclude the possibility that a minor amount of MPD is localized in peroxisomes. Therefore, we also performed immunogold labelling on ultrathin sections of human liver, the organ displaying 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 MPD we found only occasional labelling in the cytoplasm of the liver parenchyal cells. Although we carefully checked a large number of peroxisomes we were unable to detect any labelling of MPD in these peroxisomes (Fig. 5A). 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 sections of the same samples with antibodies against peroxisomal AGT. This revealed a distinct label which was found in the peroxisomal matrix (Fig. 5B), while no label was observed in negative controls.

![Figure 5](image)

**Figure 5.** Electron microscopy of human control liver. (A) Ultrathin Unicryl sections of human liver were immunostained with the affinity purified antibodies against MPD. The peroxisomes (P) remain unlabelled. (B) Ultrathin Unicryl sections immunostained with antibodies against AGT reveals a clear localisation in the peroxisomal matrix. Scale bar = 500 nm; M, mitochondria.

**DISCUSSION**

The cholesterol/isoprenoid biosynthetic pathway has been the subject of extensive studies in the last decades. The importance of the pathway for human development and health is underlined by the identification of patients with inborn errors in this pathway, which are often characterized by multiple morphogenic, developmental and neurological
abnormalities. However, the subcellular localization of the enzymes involved in the pathway is still a very controversial subject. Various studies have suggested that, in addition to enzymes involved in a variety of other metabolic pathways, peroxisomes predominantly harbor most of the enzymes involved in the presqualene part of the cholesterol/isoprenoid biosynthetic pathway including 3-hydroxy-3-methylglutaryl CoA reductase (3), mevalonate kinase (4), phosphomevalonate kinase (5), MPD (6), isopentenyl pyrophosphate isomerase (7) and farnesylpyrophosphate synthase (8, 21).

The peroxisomal localization of the authentic MPD, however, has not been demonstrated in humans, although a reporter construct consisting of an amino terminal fragment of MPD fused to an HA tag was reported to end up in peroxisomes. In contrast, full-length MPD has been shown to be cytosolic in rat and mice (12, 13). To conclusively determine the subcellular localization of human MPD and the relevance of peroxisomes in cholesterol/isoprenoid biosynthesis, we used a variety of techniques to study the localization of the enzyme, including conventional subcellular fractionation and cell permeabilization techniques, immunofluorescence and electron microscopy techniques. Our results unequivocally demonstrate that both endogenous (in human fibroblasts, human liver and HEK293 cells) and overexpressed full-length human MPD (in human fibroblasts, HEK293 and CV1 cells) have an exclusive cytosolic but no peroxisomal localization.

This conclusion is in line with our recent studies in which normal MPD activities and MPD protein were found in patients suffering from peroxisome biogenesis disorders (10, 11) and with the cytosolic localization of MPD in rat and mouse (12, 13). The fact that human MPD is a cytosolic enzyme raises questions about the postulated central role of peroxisomes in cholesterol/isoprenoid biosynthesis. In fact, using a similar approach as used for our localization studies of MPD, we also could demonstrate a cytosolic localization of human mevalonate kinase and human phosphomevalonate kinase, two enzymes that had been postulated to be predominantly peroxisomal (manuscripts in preparation).

These combined results imply that peroxisomes may not play a central role in cholesterol/isoprenoid biosynthesis.

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