Identification of peroxisomal targeting signals in cholesterol biosynthetic enzymes: AA-CoA thiolase, HMG-CoA synthase, MPPD, and FPP synthase

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Abstract **At least three different subcellular compartments, including peroxisomes, are involved in cholesterol synthesis. The peroxisomal targeting signals for phosphomevalonate kinase and isopentenyl diphosphate isomerase have been identified. In the current study we identify the peroxisomal targeting signals required for four other enzymes of the cholesterol biosynthetic pathway: acetoacetyl-CoA (AA-CoA) thiolase, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase, mevalonate diphosphate decarboxylase (MPPD), and farnesyl diphosphate (FPP) synthase. Data are presented that demonstrate that mitochondrial AA-CoA thiolase contains both a mitochondrial targeting signal at the amino terminus and a peroxisomal targeting signal (PTS-1) at the carboxy terminus. We also analyze a new variation of PTS-2 sequences required to target HMG-CoA synthase and MPPD to peroxisomes. In addition, we show that FPP synthase import into peroxisomes is dependent on the PTS-2 receptor and identify at the amino terminus of the protein a 20-amino acid region that is required for the peroxisomal localization of the enzyme. These data provide further support for the conclusion that peroxisomes play a critical role in cholesterol biosynthesis.**—Olivier, L. M., W. Kovacs, K. Masuda, G-A. Keller, and S. K. Krisans. **Identification of peroxisomal targeting signals in cholesterol biosynthetic enzymes: AA-CoA thiolase, HMG-CoA synthase, MPPD, and FPP synthase.** ***J. Lipid Res.*** **2000.** 41: **1921–1935.**

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Peroxisomal proteins are posttranslationally translocated into the matrix of the organelle by specific targeting signals. Currently two peptides for peroxisomal matrix protein targeting have been identified. Peroxisomal targeting signal 1 (PTS-1), the first to be characterized, is a tripeptide with the consensus sequence (S/A/C)(K/H/R)(L/M) found at the extreme carboxy terminus of most peroxisomal proteins (1, 2). The second, peroxisomal targeting signal 2 (PTS-2), is a nine-amino acid sequence found within the amino-terminal region of a smaller set of peroxisomal matrix proteins (3). PTS-2 sequences are found at variable distances from the amino terminus and, in some proteins, are cleaved after import. The consensus PTS-2 is a nine-amino acid, bipartite sequence (R/K)(L/V/I)X_5(H/Q)(L/A), where X indicates any amino acid. Although a consensus sequence has been identified, there is evidence that the PTS-2 may not only be a required sequence of amino acids, but that the targeting information may consist of a structural or charge-based motif (4). It is interesting to note that while most known peroxisomal matrix proteins have either a PTS-1 or a PTS-2 there are other peroxisomal proteins that do not contain either targeting sequence. For such proteins it is hypothesized that import may occur through the use of nonconventional PTS-1 or PTS-2 sequences that are not currently recognized, may use a third type of targeting sequence that has yet to be identified or may multimerize in the cytosol with PTS-containing proteins and gain import into the matrix by "piggybacking" in a heteromeric complex (5, 6).

Receptors for PTS-1 sequences (Pex5p) and PTS-2 sequences (Pex7p) have been identified and protein import studies indicate that these receptors are essential for peroxisomal protein import. Mutations in the receptors are responsible for several of the fatal human peroxisomal biogenesis disorders (PBD) such as Zellweger syndrome, infantile Refsum disease, neonatal adrenoleukodystrophy, and rhizomelic chondrodysplasia punctata (7, 8). Human cell fusion studies have shown that there are at least 16 genetic complementation groups for PBD (9). Other studies demonstrate that the genetic defects result in either altered protein import into peroxisomes or faulty peroxi-

Abbreviations: AA-CoA thiolase, acetoacetyl-CoA thiolase; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; FBS, fetal bovine serum; FPP, farnesyl diphosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; IPP, isopentenyl diphosphate; MPPD, mevalonate diphosphate decarboxylase; MvK, mevalonate kinase; PBD, peroxisomal biogenesis disorders; PMP, peroxisomal membrane protein; PMP70, 70-kDa peroxisomal membrane protein; PMvK, phosphomevalonate kinase; PTS-1, peroxisomal targeting signal 1; PTS-2, peroxisomal targeting signal 2; RSV, Rous sarcoma virus; SRE, sterol response element.

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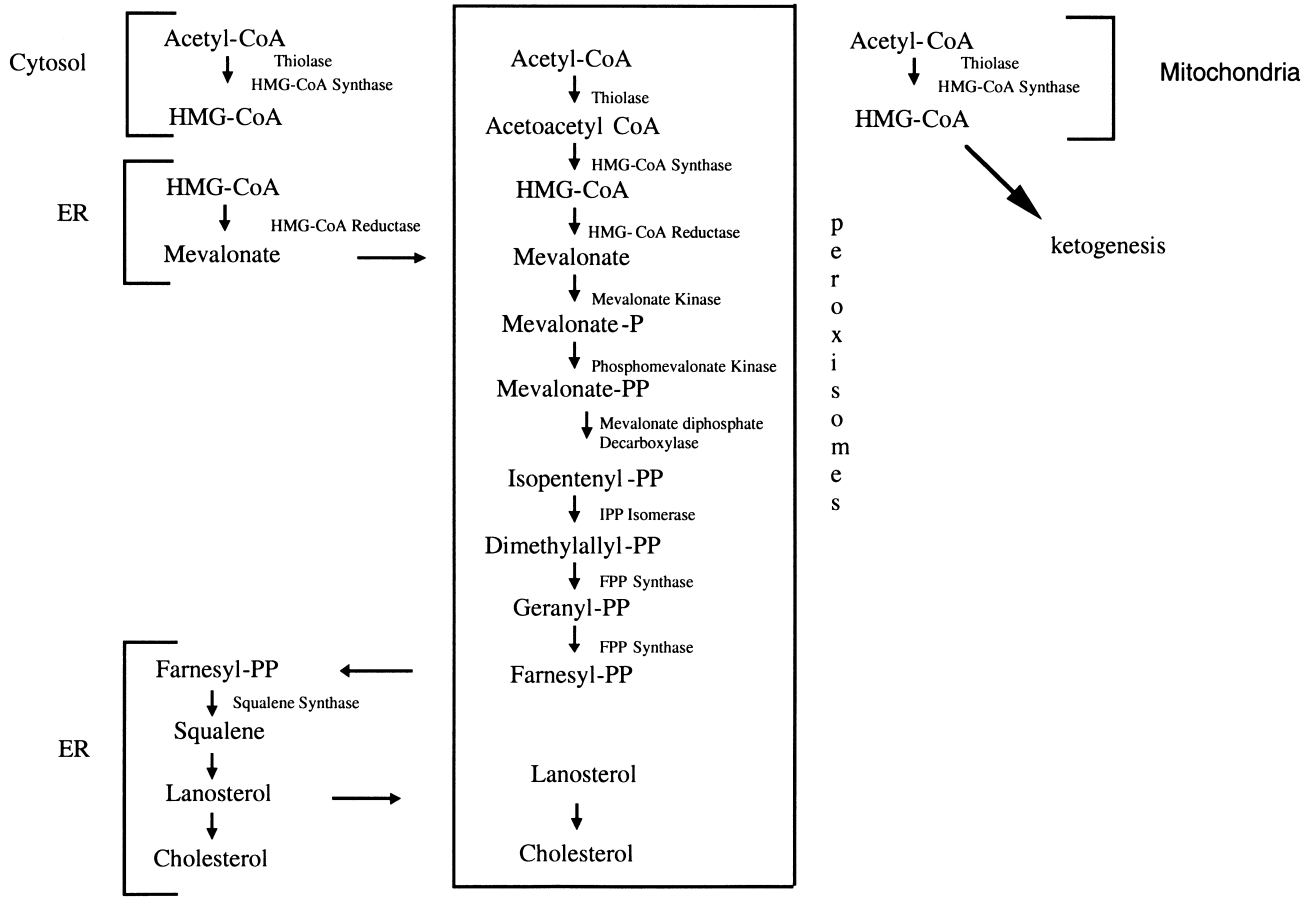

Fig. 1. Current model of the subcellular compartmentalization of cholesterol biosynthesis. Conversion of acetyl-CoA to HMG-CoA occurs in the cytosol, peroxisomes, and mitochondria. The further conversion of HMG-CoA to mevalonate occurs both in the ER and peroxisomes. However, the conversion of mevalonate to FPP occurs predominantly in the peroxisomes. The further metabolism of FPP to squalene proceeds exclusively in the ER, and the final conversion of lanosterol to cholesterol occurs in the ER and may also be localized to peroxisomes.

some biogenesis (10), which may affect import of proteins containing PTS-1 sequences only, with defects in Pex5p, affect PTS-2 sequences only, with defects in Pex7p, or affect the import of both PTS-1 and PTS-2 sequences with defects in other components in the import apparatus.

At least three different subcellular compartments, including peroxisomes, are involved in cholesterol synthesis (**Fig. 1**). The enzyme that catalyzes the initial reaction of the cholesterol biosynthetic pathway, acetoacetyl-CoA thiolase (AA-CoA thiolase), has been reported to be cytosolic, mitochondrial, and peroxisomal (11, 12). The presence of a peroxisomal AA-CoA thiolase has been suggested through studies in which either isolated peroxisomes (12) or partially purified AA-CoA thiolase from peroxisomes (11) was shown to condense acetyl-CoA into AA-CoA.

The next reaction in the cholesterol biosynthetic pathway converts AA-CoA into 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) by HMG-CoA synthase, which has been found in mitochondria, peroxisomes, and cytosolic fractions (13, 14). The third reaction of the pathway catalyzed by HMG-CoA reductase, the rate-limiting step in cholesterol biosynthesis, has been localized to both the endoplasmic reticulum (ER) and peroxisomes (15–17). Both mevalonate kinase (MvK) (18, 19) and farnesyl diphosphate (FPP) synthase (20, 21) have been shown by immunofluorescence and immunoelectron microscopy to be predominantly peroxisomal. Furthermore, the enzymes catalyzing reactions between MvK and FPP synthase, phosphomevalonate kinase (PMvK), mevalonate diphosphate decarboxylase (MPPD), and isopentenyl diphosphate (IPP) isomerase, have been suggested to be peroxisomal on the basis of studies utilizing permeabilized cells in which the cytosol has been removed (22). Interestingly, the activity of squalene synthase, the next enzyme in the pathway after FPP synthase and the first committed enzyme for cholesterol biosynthesis, has been reported to be exclusively in the ER (23, 24). Finally, enzyme activities for the subsequent conversion of lanosterol to cholesterol have been found both in peroxisomes and in the ER (25).

While the studies demonstrating activities of AA-CoA thiolase, HMG-CoA synthase, PMvK, MPPD, and IPP isomerase (11–14, 22) and the immunofluorescence and immunoelectron microscopy studies with MvK and FPP synthase (19, 20, 23) were suggestive of a peroxisomal localization of these enzymes, the specific peroxisomal targeting signals had yet to be identified. However, we have shown that PMvK contains a consensus PTS-1 (SRL), which is required for its localization to peroxisomes (26). Similarly, our group has shown that a

consensus PTS-1 (HRM) in IPP isomerase is responsible for its peroxisomal localization (27). Through amino acid sequence analysis of AA-CoA thiolase, HMG-CoA synthase, MvK, and MPPD we have identified putative peroxisomal targeting signals contained within these enzymes. AA-CoA thiolase contains a putative PTS-1, while HMG-CoA synthase, MvK, and MPPD contain putative PTS-2s. However, no targeting signal was identified in the sequence for FPP synthase, suggesting that FPP synthase might gain import into the peroxisomal matrix through an alternative mechanism. In this study we have identified the sequences required by AA-CoA thiolase, HMG-CoA synthase, MPPD, and FPP synthase to target the proteins to peroxisomes.

MATERIALS AND METHODS

Materials

Restriction enzymes were purchased from New England BioLabs (Beverly, MA) and all biochemicals were purchased from Sigma (St. Louis, MO). Adobe (San Jose, CA) PhotoShop was utilized for making the photographs.

Cell culture

Chinese hamster ovary (CHO) cells and JD15 cells (a gift from R. Davis, Dept. of Biology, San Diego State University, San Diego, CA) were maintained in Dulbecco's modified Eagle's medium (DMEM)-F12 containing 5% fetal bovine serum (FBS; Life Technologies, Gaithersburg, MD), penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml) at 37°C in a 5% CO_2 incubator. The peroxisomal deficient (PTS-1, PTS-2, and PTS-1 and PTS-2 deficient) transformed fibroblasts were a generous gift from S. Subramani (Department of Biology, University of California at San Diego, La Jolla, CA). All fibroblast cell lines were maintained in DMEM-F12 containing 10% FBS, penicillin (100 units/ml) and streptomycin sulfate (100 μg/ml) at 37°C in a 5% CO_2 incubator.

Vector construction

cDNA for AA-CoA thiolase was reverse transcribed from rat liver total RNA using the primers 5′-gacaagcttttccggtctccatggct gccctggc and 5′-aaaacaagatgtctacagcttctcaatcagcacggcgga. Similarly, the partial cDNA for HMG-CoA synthase was also reverse transcribed from rat liver total RNA using the primers 5′-acaca aagctttcaccatgcctgggtcacttcct and 5′-tccttggatccatgtattcccagactcc tcaaa. FPP synthase, AA-CoA thiolase, and HMG-CoA synthase fusion vectors were subcloned into a pcDNA3 vector containing the Myc epitope immediately before the stop codon [pcDNA3-Myc was a gift from S. Gould (Department of Pediatrics, Johns Hopkins University, School of Medicine, Baltimore, MD)]. This vector contains in-frame *Hin*dIII and *Bam*HI sites between the Rous sarcoma virus (RSV) promoter and the Myc epitope, which were utilized for subcloning. To construct the various fusion vectors, template DNA and specific primers (**Table 1**) were used in polymerase chain reactions (PCRs) (94°C for 15 s, 55°C for 30

TABLE 1. Vector construction

Plasmid	Template	Primers
pcDNA3-Myc FPS	pGemFPPS	5′-agcaagcttgtcagaatg 5′-gcttgtggatcctgtttg
pcDNA3-Myc FPS-N20	pcDNA3-Myc FPS	5′-aatctggatccagtgctggatgaaa 5′-agcaagcttgtcagaatg
pcDNA3-Myc FPS-C-terminal	pcDNA3-Myc FPS	5′-ccaggaagatggatgggggca 5′-tatcagaagcttagaatggggcttggatgc
pcDNA3-Myc FPS-N16	pcDNA3-Myc FPS-N20	5′-agaattaagcttagaatgaaacatg 5′-aatctggatccagtgctggatgaaa
pcDNA3-Myc FPS-N14	pcDNA3-Myc FPS-N20	5′-aatgggaagcttagaatggatgttcat 5′-aatctggatccagtgctggatgaaa
pcDNA3-Myc FPS-QEΔGA	pcDNA3-Myc FPS-N20	a. 5′-taatacgactcactataggg 5′-gtgctggatgaaattctgcttagctccgttctgaac b. 5′-gttcataacggagctaagcagaatttcatc 5′-ggttctttctcctttcacaagtcgagc
pCR3.1 MPD HA	pET-14bMPD	a. 5′-tacccatacgacgttccggactacgcctcggagaagccgctggcggcagtcact 5′-ctgcaccaggaccagttataaggtaccac b. 5′-ggcggccccatggcctacccatacgacgtaccagac 5′-ctgcaccaggaccagttataaggtaccac
pCR 3.1 MPD HA w/o PTS-2	pET-14bMPD	a. 5′-tacccatacgacgttccggactacgcctcggagaagccgctggcggcagtcact 5′-cccatcaactcctccctgtgaggatccct b. 5′-ggcggccccatggcctacccatacgacgtaccagac 5′-cccatcaactcctccctgtgaggatccct
pcDNA3-Myc AACoAT-FL	pCR2.1 AACoA T	a. 5′-gacaagcttttccggtctccatggctgccctggc 5′-aaaacaagatgtctacagcttctcaatcagcacggcgga b. 5′-actattgaggaggacctgctggccattgaaaaggcagggattcca 5′-tccctggtcctcctcggagatcagcttctgctctggctgagaggcaag
pcDNA3-Myc AACoAT-T 1	pcDNA3-Myc AACoAT-FL	5′-gcatccaagctttccatggctgatgtggtta 5′-actattgaggaggacctgctggccattgaaaaggcagggattcca
pcDNA3-Myc AACoAT-T2	pcDNA3-Myc AACoAT-FL	5′-gcatccaagctttccatggctgatgtggtta 5′-agatgtctatctagactaaatcagcacggcggaagc
pcDNA3-Myc H Syn-minus PTS-2	pCR2.1 Myc H Synth	5′-acacaaagctttcaccatgcctgggtcacttcct 5′-agactggatccattttgatttgtcgatgattgt
pcDNA3-Myc H Syn-plus PTS-2	pCR2.1 Myc H Synth	5′-acacaaagctttcaccatgcctgggtcacttcct 5′-tccttggatccatgtattcccagactcctcaaa

sec, and 72°C for 1 min). PCR products were restriction digested with the indicated endonucleases (New England BioLabs), cleaned on QIAquick columns (Qiagen, Valencia, CA), and ligated into the pRSV-Myc vector. Ligation products were transformed into DH5α cells (GIBCO-BRL, Gaithersburg, MD). Subcloning and reading frame were verified by both restriction digest and DNA sequencing. MPD HA fusion vectors underwent PCR and subsequently were TA ligated into the pCR 3.1 vector from InVitrogen (Carlsbad, CA).

Transfection and immunofluorescence

Expression vectors were transfected into cultured fibroblasts and processed for immunofluorescence as described (26). The Myc epitope was detected with a mouse anti-Myc antibody (purchased from InVitrogen) at 1:100 dilution, and the HA epitope was detected using a mouse anti-HA antibody at 1:100 dilution. Catalase was detected with a rabbit anti-catalase antibody at 1:100 dilution and the 70-kDa peroxisomal membrane protein (PMP70) was detected using a rabbit anti-PMP70 antibody at 1:50 dilution. As secondary antibodies, goat anti-rabbit conjugated with Texas red and goat anti-mouse conjugated with fluorescein were used. Control experiments were routinely included to verify that no bleedthrough of primary and secondary antibodies was detected in either channel. Cells were examined with a Nikon Microphot fluorescence microscope and with an Olympus confocal microscope.

Cryoultramicrotomy and immunolabeling

Sample preparation and immunolabeling were carried out as described (20) with the following changes; sections of liver tissue were from rats fed a normal diet and were immunolabeled with an affinity purified antibody to cytoplasmic HMG-CoA synthase, a generous gift from Dr. P. Edwards.

RESULTS

Mitochondrial AA-CoA thiolase contains both an amino-terminal mitochondrial targeting signal and a carboxy-terminal PTS-1

AA-CoA thiolase catalyzes the conversion of acetyl-CoA to AA-CoA in the first reaction of the cholesterol biosynthetic pathway. Two genes have been identified for AA-CoA thiolase. One of the genes reportedly encodes a mitochondrially localized AA-CoA thiolase (28) while the other is believed to encode a cytosolic AA-CoA thiolase (29). However, data generated by our group and others indicate that peroxisomes also contain significant AA-CoA thiolase activity (11, 12). Analysis of the protein sequences of both AA-CoA thiolases revealed a consensus PTS-1 (QKL) at the carboxy terminus of the mitochondrial AA-CoA thiolase, in addition to its amino-terminal mitochondrial targeting sequence, whereas no peroxisomal targeting sequence was detected in the cytosolic AA-CoA thiolase. Therefore, studies were designed to determine if the putative PTS-1 identified in the mitochondrial AA-CoA thiolase is responsible for the targeting of the protein to peroxisomes. A Myc epitope tag was added within the coding region of mitochondrial AA-CoA thiolase, between the mitochondrial targeting signal and the peroxisomal targeting signal. Three vectors of rat mitochondrial AA-CoA thiolase were made that encoded the full-length protein (AA-CoA thiolase), a protein with an amino-terminal truncation, deleting the mitochondrial targeting sequence (AA-CoA thiolase T1), and a protein with an amino- and carboxy-terminal truncation, in which both the mitochondrial targeting signal (35 amino acids) and the 3-amino acid putative peroxisomal targeting signal were removed (AA-CoA thiolase T2). The eukaryotic expression vectors were transiently transfected into CHO cells and processed for double-label immunofluorescence, using antibodies to the Myc epitope and to the peroxisomal marker catalase. Expression of the full-length mitochondrial AA-CoA thiolase construct resulted in strong mitochondrial labeling (**Fig. 2a**). The immunofluorescent pattern obtained with the Myc antibody is completely superimposable onto the pattern of the mitochondrial marker protein, cytochrome oxidase (Fig. 2a and b). However, expression of the mitochondrial AA-CoA thiolase T1 construct revealed a punctate distribution with the Myc antibody that was similar to that for catalase (Fig. 2c and d). This punctate distribution did not colocalize with mitochondrial labeling (data not shown). The staining of the two cells at the right in Fig. 2c does not indicate a cytosolic localization. On occasion staining such as this is observed and is due to autofluorescence, as a result of the antibody. Our conclusion that the transfection of the T1 construct resulted in peroxisomal localization is based on the fact that the punctate labeling, as is seen in the larger cell in Fig. 2c, colocalized with the peroxisomal marker enzyme and was repeatedly observed to be the major fluorescent pattern through many transfections.

Finally, expression of the mitochondrial AA-CoA thiolase T2 construct (which has both the mitochondrial and peroxisomal targeting sequences removed) resulted in cytosolic labeling (Fig. 2e) for the Myc antibody and a punctate pattern for catalase (Fig. 2f). As an additional control, the mitochondrial AA-CoA thiolase T1 construct, was transfected into two peroxisomal import-deficient cell lines: a cell line deficient in PTS-1 protein import only, and a cell line deficient in PTS-2 protein import only. Peroxisomal import-deficient cell lines have been derived from individuals affected with PBD and have become a significant tool in the identification of the peroxisomal import pathways utilized by individual peroxisomal enzymes. Immunofluorescence for the Myc epitope in cells deficient in the PTS-1 import pathway revealed cytosolic labeling, which is the predicted result if the protein depends on the PTS-1 receptor, whereas, as expected, cells deficient in PTS-2 protein import displayed a punctate pattern similar to that obtained with PMP70 (data not shown). From these data we conclude that the mitochondrial AA-CoA thiolase contains both an amino-terminal mitochondrial targeting signal and a carboxy-terminal PTS-1. Similarly, HMG-CoA lyase also contains both a mitochondrial and a peroxisomal targeting signal (CKL) (30, 31).

HMG-CoA synthase is a peroxisomal protein

HMG-CoA synthase catalyzes the second reaction in the cholesterol biosynthetic pathway, converting AA-CoA to HMG-CoA. Similar to AA-CoA thiolase, two genes for HMG-CoA synthase have been identified. Again, one gene encodes a mitochondrial enzyme required for ketogenesis

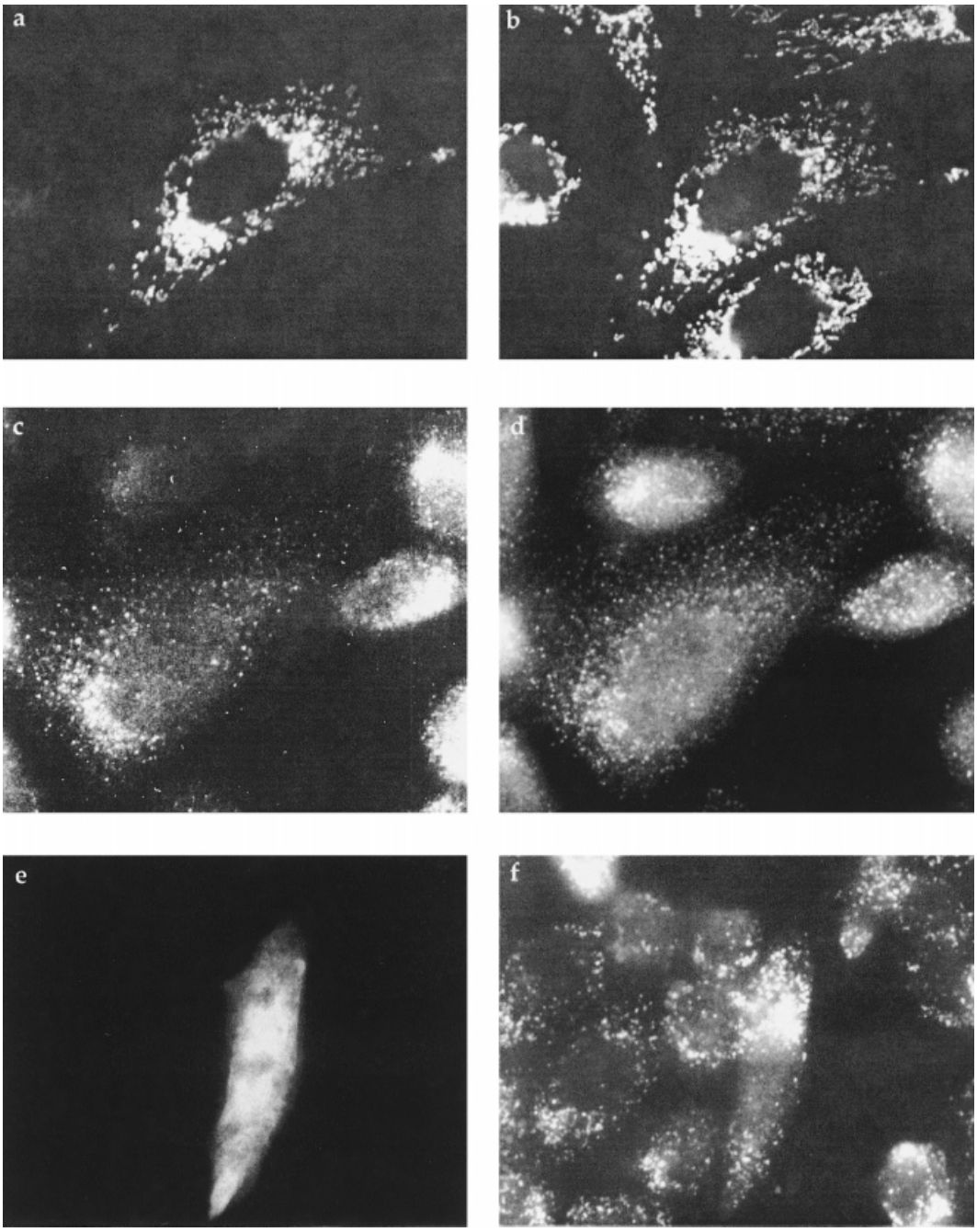

Fig. 2. The mitochondrial AA-CoA thiolase contains both an amino-terminal mitochondrial targeting signal and a carboxy-terminal PTS-1. Expression vectors for AA-CoA thiolase containing a Myc epitope tag were transiently transfected into CHO cells. Full-length AA-CoA thiolase (a and b), AA-CoA thiolase T1 (c and d), and AA-CoA thiolase T2 (e and f). Transfected cells were processed for double-label immunofluorescence using anti-Myc (a, c, and e), anti-cytochrome oxidase (b), and anti-catalase (d and f) antibodies. Expression of the full-length mitochondrial AA-CoA thiolase construct resulted in strong mitochondrial labeling that is completely superimposable onto the pattern of the mitochondrial marker protein, cytochrome oxidase (a and b). However, expression of the mitochondrial AA-CoA thiolase T1 construct revealed a punctate distribution with the Myc antibody that was similar to that for catalase (c and d). Expression of the mitochondrial AA-CoA thiolase T2 construct (which has both the mitochondrial and peroxisomal targeting sequences removed) resulted in cytosolic labeling for the Myc antibody (e) and as expected, a punctate pattern with catalase (f).

while the other encodes an enzyme believed to be localized to the cytosol (32) and required for cholesterol biosynthesis. Furthermore, these genes are differentially regulated through their respective promoter elements (33). Moreover, the promoter for cytosolic HMG-CoA synthase contains a sterol regulatory element (SRE) that activates transcription through the binding of SRE-binding proteins in response to low cellular sterol levels (34).

HMG-CoA synthase activity has also been reported in rat liver peroxisomes (13). In efforts to verify this initial observation suggesting the presence of peroxisomal HMG-CoA synthase activity, subcellular fractionation studies were done. Data from these experiments demonstrate that HMG-CoA synthase activity is detected in three fractions of rat liver: in the mitochondria (5.2 U/min per mg), in the cytosol (3.7 U/min per mg), and in the peroxisomal fraction (3.2 U/min per mg). However, because of the ease of rupture of peroxisomes during fractionation, and the release of matrix enzymes in the cytosol, these types of experiments are not accurate in determining subcellular localization. Therefore, to examine further the peroxisomal localization of HMG-CoA synthase, cryosections of normal rat liver were immunolabeled with affinity-purified antibodies made against cytosolic HMG-CoA synthase and processed for immunoelectron microscopy. Western blots utilizing this antibody demonstrated that this antibody specifically recognized only one band of the correct molecular size for cytosolic HMG-CoA synthase (data not shown). In addition, this antibody does not cross-react with the mitochondrial protein. As shown in **Fig. 3**, significant labeling is observed in the peroxisomal matrix with only a small amount of labeling found in the cytosol. In addition, we also investigated the subcellular localization of endogenous HMG-CoA synthase by double-label indirect immunofluorescence. **Figure 4a** and **b** illustrates the immunofluorescence pattern obtained with the HMG-CoA synthase and catalase antibodies in JD15 cells (JD15 cells are CHO cells transfected with a plasmid expressing

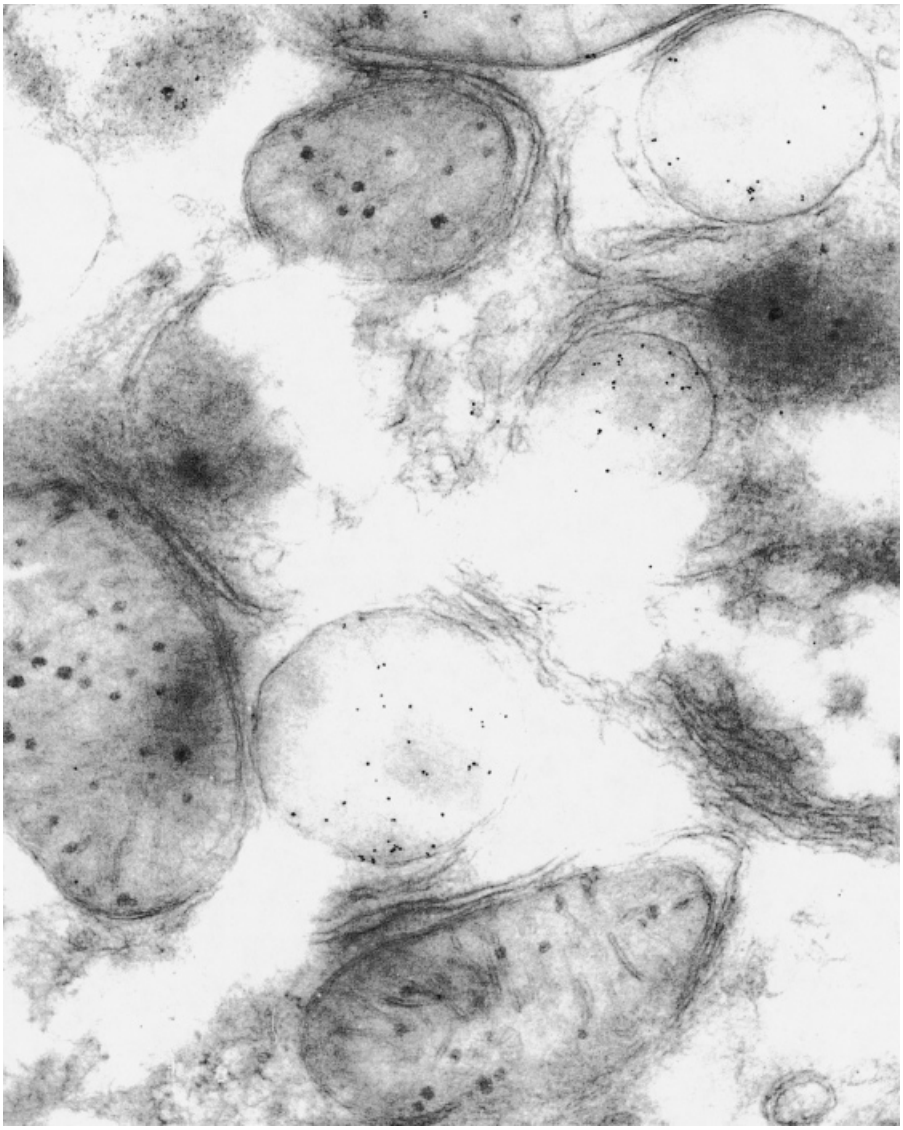

Fig. 3. Demonstration of a peroxisomal localization of HMG-CoA synthase in rat liver by immunoelectron microscopy. Shown is a representative micrograph of a normal rat liver cryosection that was immunolabeled with an affinity-purified antibody against the cytosolic HMG-CoA synthase, followed by a colloidal gold adduct of goat anti-rabbit IgG. The peroxisomes are uniformly immunolabeled for HMG-CoA synthase. In addition to the immunolabeling of the peroxisomal matrix, some gold particles can be detected in the cytosol surrounding the peroxisomes.

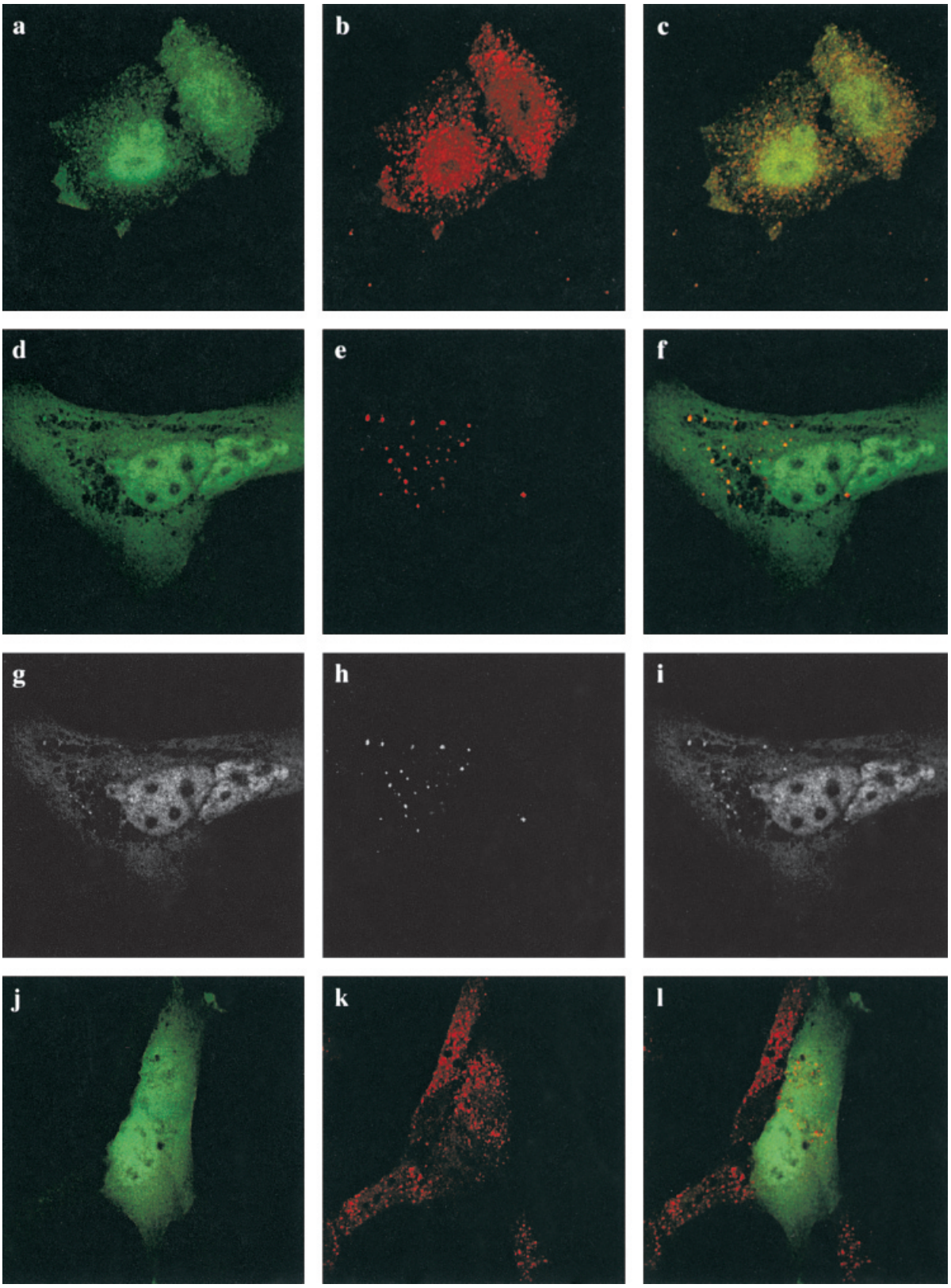

Fig. 4. Demonstration that endogenous HMG-CoA synthase is localized in peroxisomes in JD15 cells and that in transiently transfected cells it is targeted to peroxisomes by a PTS-2. JD15 cells were processed for indirect immunofluorescence, using antibodies specific for cytosolic HMG-CoA synthase (a) and catalase (b). Human fibroblasts deficient in PTS-1 (d–i) or PTS-2 (j–l) protein import were transiently transfected with expression vectors containing HMG-CoA synthase plus the PTS-2. Cells were processed for double-label indirect immunofluorescence, using antibodies specific for the Myc tag (d, g, and j) and PMP70 (e, h, and k). (c, f, i, and l) Overlay images. The grayscale images shown in (g–i) are identical to the color images in (d–f), respectively, and are included for clarity of the punctate distribution of fluorescence. A significant degree of the immunofluorescence pattern obtained with the HMG-CoA synthase antibody is superimposable over that obtained for the catalase antibody (c). Immunofluorescence for the Myc epitope in cells deficient in the PTS-1 import pathway revealed a punctate labeling pattern (d and g), which completely superimposes the pattern of PMP70 (f and i). On the other hand, cells deficient in PTS-2 protein import displayed cytosolic labeling for the Myc epitope (j) and a punctate pattern for PMP70 (l).

rat cholesterol-7α-hydroxylase gene, which results in increased levels of cholesterol biosynthesis proteins). A significant degree of the immunofluorescence pattern obtained with the HMG-CoA synthase antibody is superimposable over that obtained for the catalase antibody (Fig. 4c), indicating that these two enzymes are colocalized. Taken together, these biochemical and immunological data indicate that a significant amount of HMG-CoA synthase is found in the peroxisomes.

HMG-CoA synthase is targeted to peroxisomes by a PTS-2

On analysis of the protein sequences of the two HMG-CoA synthase proteins, neither a consensus PTS-1 nor PTS-2 was evident in either sequence. However, the HMG-CoA synthase, believed to be located in the cytosol, does contain a sequence similar to a PTS-2. This PTS-2-like sequence diverges from the consensus amino acids, with the basic amino acids (either arginine or lysine) being replaced with a serine (SVX_5QL). Therefore, in experiments aimed to determine if this sequence is responsible for the peroxisomal localization, two constructs were generated. These vectors contain a Myc epitope and the HMG-CoA synthase coding sequence, which either includes (HMG-CoA synthase plus PTS-2) or does not include (HMG-CoA synthase minus PTS-2) the putative PTS-2. The HMG-CoA synthase expression construct containing the putative PTS-2 was transfected into two peroxisomal import-deficient cell lines and processed for double-label indirect immunofluorescence, using antibodies to the Myc epitope (Fig. 4d, g, and j) and to PMP70 (Fig. 4e, h, and k). Immunofluorescence for the Myc epitope in cells deficient in the PTS-1 import pathway revealed a punctate labeling pattern (Fig. 4d and g), which is the predicted result if the protein does not depend on the PTS-1 receptor, whereas cells deficient in PTS-2 protein import displayed cytosolic labeling (Fig. 4j). This is the expected result if the protein depends on the PTS-2 receptor. The immunofluorescent pattern for the Myc epitope tag in PTS-1 import-deficient cells completely superimposes the pattern of PMP70 (Fig. 4f and i). As can be seen in Fig. 4d–j, the PTS-1 import-deficient cell lines contain a significantly reduced number of peroxisomes (35).

To analyze further the targeting requirements of the protein, the HMG-CoA synthase plus PTS-2 and minus PTS-2 constructs were transiently transfected into CHO cells and processed for double-label immunofluorescence, using antibodies to the Myc epitope and to the peroxisomal marker catalase. The HMG-CoA synthase expression construct containing the putative PTS-2 displayed a punctate distribution of fluorescence (**Fig. 5a**). However, the expression of the HMG-CoA synthase construct minus the putative PTS-2 resulted in complete cytosolic labeling (Fig. 5c), in contrast to the punctate labeling of catalase (Fig. 5d). These data indicate that the putative PTS-2 found in cytosolic HMG-CoA synthase is required for peroxisomal localization of the enzyme and depends for import on the PTS-2 receptor.

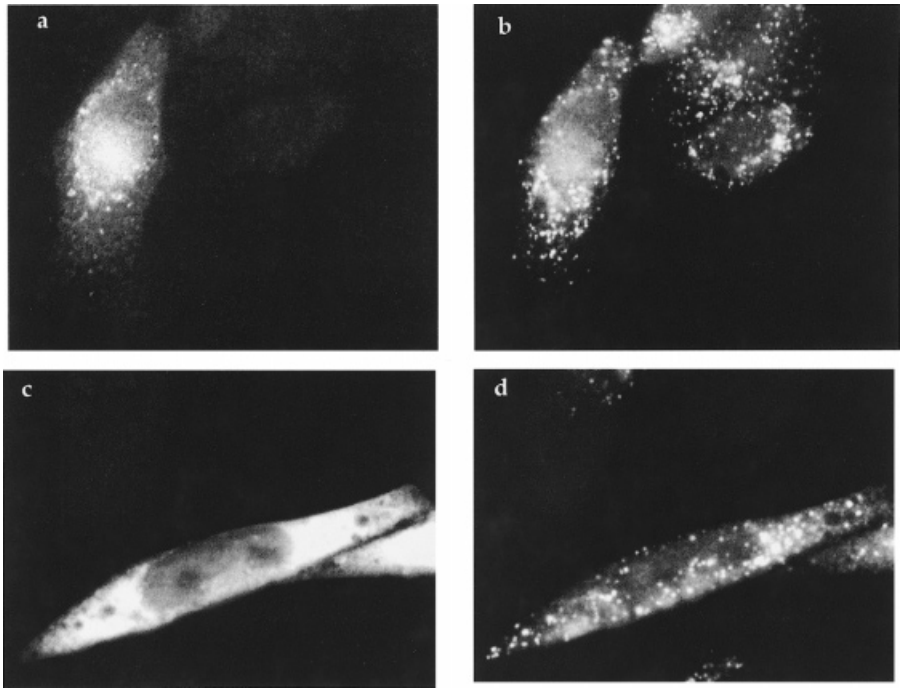

Fig. 5. HMG-CoA synthase is targeted to peroxisomes by a PTS-2. CHO cells were transiently transfected with expression vectors either containing HMG-CoA synthase plus the PTS-2 (a and b) or minus the PTS-2 (c and d). Transfected cells were processed for double-label indirect immunofluorescence with anti-Myc (a and c) and with anti-catalase (b and d) antibodies. The expression of HMG-CoA synthase plus the PTS-2 (a) resulted in punctate labeling that was similar to that for catalase (b) whereas the expression of the HMG-CoA synthase construct minus the putative PTS-2 resulted in complete cytosolic labeling (c).

MPPD is targeted to peroxisomes by a PTS-2

MPPD catalyzes the sixth reaction of the cholesterol biosynthetic pathway, in which the six-carbon mevalonate diphosphate is dehydrated and decarboxylated to form IPP. As previously mentioned, the localization of MPPD to peroxisomes has been inferred indirectly by studies based on utilizing permeabilized cells in which the cytosol has been removed (22), and by analysis of tissues obtained from PD patients (20). However, how this enzyme is targeted to peroxisomes is unknown. On analysis of the MPPD amino acid sequence a putative PTS-2 was identified, 39 amino acids from the amino terminus. While this putative PTS-2 did not fit the consensus sequence, it has the same bipartite sequence found in HMG-CoA synthase (SVX_5QL). Therefore, two MPPD constructs containing only the amino-terminal region of the protein tagged with the HA epitope were developed; one coding through the putative PTS-2 (pCR3.1 MPPD HA) and the other terminating immediately prior to the putative PTS-2 (pCR3.1 MPPD HA w/o PTS). These constructs were transiently transfected into CHO cells and visualized by double-label indirect immunofluorescence, using antibodies to HA and to the peroxisomal marker catalase. Analysis of the cells transfected with pCR3.1 MPPD HA, containing the putative PTS-2, revealed a punctate pattern that was similar to the distribution of catalase (**Fig. 6a** and **b**). Cells transfected with pCR3.1 MPPD HA, without the PTS-2 sequence, revealed cytosolic fluorescence labeling (Fig. 6c) whereas catalase labeling was punctate (Fig. 6d). Subsequently, pCR3.1 MPPD HA was transiently transfected into the two peroxisomal import-deficient cell lines and subcellular localization was again visualized by indirect immunofluorescence. In the PTS-1 import-deficient cell line we observed punctate labeling whereas in the PTS-2 import-deficient cells the fluorescence was cytosolic (data not shown). Furthermore, this PTS-2 is the same as found in HMG-CoA synthase. Therefore, these data suggest that MPPD is a peroxisomal protein that utilizes the newly identified PTS-2 sequence (SVX_5QL) as a peroxisomal targeting signal and requires a functional PTS-2 receptor for import into the organelle. Further studies utilizing the full-length protein will be required to conclude unequivocally that the identified PTS-2 is functional in the intact protein.

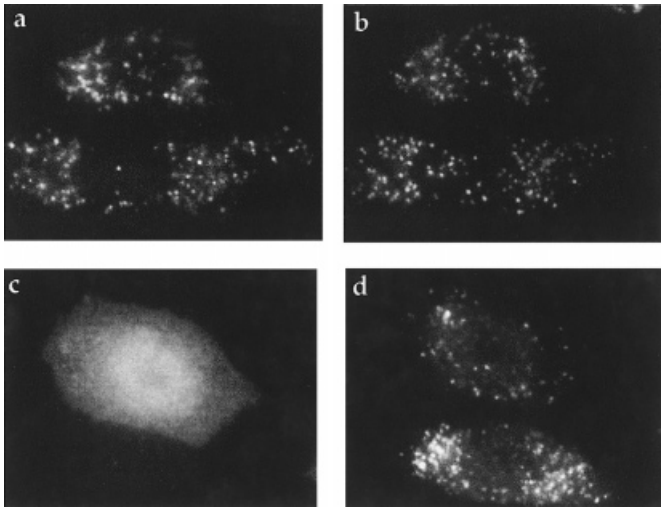

Fig. 6. Demonstration in transiently transfected cells that MPPD is targeted to peroxisomes by a PTS-2. CHO cells were transiently transfected with pCR3.1 MPD-HA (a and b) or with pCR3.1 MPD-HA without PTS-2 (c and d) and processed for double-label immunofluorescence with anti-HA (a and c) and with anti-catalase (b and d) antibodies. Analysis of the cells transfected with pCR3.1 MPD HA, containing the putative PTS-2, revealed a punctate pattern that was similar to the distribution of catalase (a and b). On the other hand, cells transfected with pCR3.1 MPD HA, without the PTS-2, revealed a cytosolic labeling for the Myc epitope (c) and a punctate pattern with catalase (d).

The signal required for peroxisomal targeting of FPP synthase is contained within the first 20 amino acids of the protein

FPP synthase catalyzes two sequential 1′-4 condensation reactions of IPP with the allylic diphosphates dimethylallyl diphosphate and geranyl diphosphate (36). The final product, FPP, is utilized in the synthesis of squalene, cholesterol, farnesylated and geranylgeranylated proteins, dolichols, coenzyme Q, and the isoprenoid moiety of heme a (37). FPP synthase has been well characterized as being predominantly a peroxisomal protein based on immunofluorescence and immunoelectron microscopy studies (20, 21). These studies have clearly demonstrated that the majority, if not all, of the endogenous FPP synthase protein is colocalized with catalase in hepatic H35 cells as well as in CHO cells (21). Moreover, in peroxisomal-deficient CHO cells, FPP synthase is cytosolic (21). However, analysis of the amino acid sequence does not reveal any sequence related to either of the two known peroxisomal targeting signals. Therefore, studies were designed to identify the peroxisomal targeting signal contained in FPP synthase. First, a full-length FPP synthase fusion construct was produced that contained the Myc epitope tag at the carboxy-terminal end. This full-length FPP synthase (pcDNA3-Myc FPS) expression vector was transfected into three peroxisomal import-deficient cell lines: a cell line deficient in both PTS-1 and PTS-2 protein import, a cell line deficient in PTS-1 protein import only, and a cell line deficient in PTS-2 protein import only. Immunofluorescence for the Myc epitope in cells deficient in both import pathways revealed cytosolic labeling (data not shown). However, transfection of this construct into cells deficient in PTS-1 protein import only resulted in a punctate distribution consistent with a peroxisomal localization (**Fig. 7a–c**). This is illustrated by the use of the peroxisomal membrane marker protein PMP70, for which peroxisomal import is unaffected by the import deficiency of these cells. Figure 7c illustrates the colocalization of this construct with PMP70. In addition, when this construct was transfected into cells deficient in PTS-2 protein import only, cytosolic labeling was observed (Fig. 7f), whereas PMP70 remained punctate (Fig. 7g). Therefore, these data suggest that FPP synthase requires the PTS-2 import pathway for peroxisomal localization.

Two different cell lines are presented in Fig. 7, PTS-1

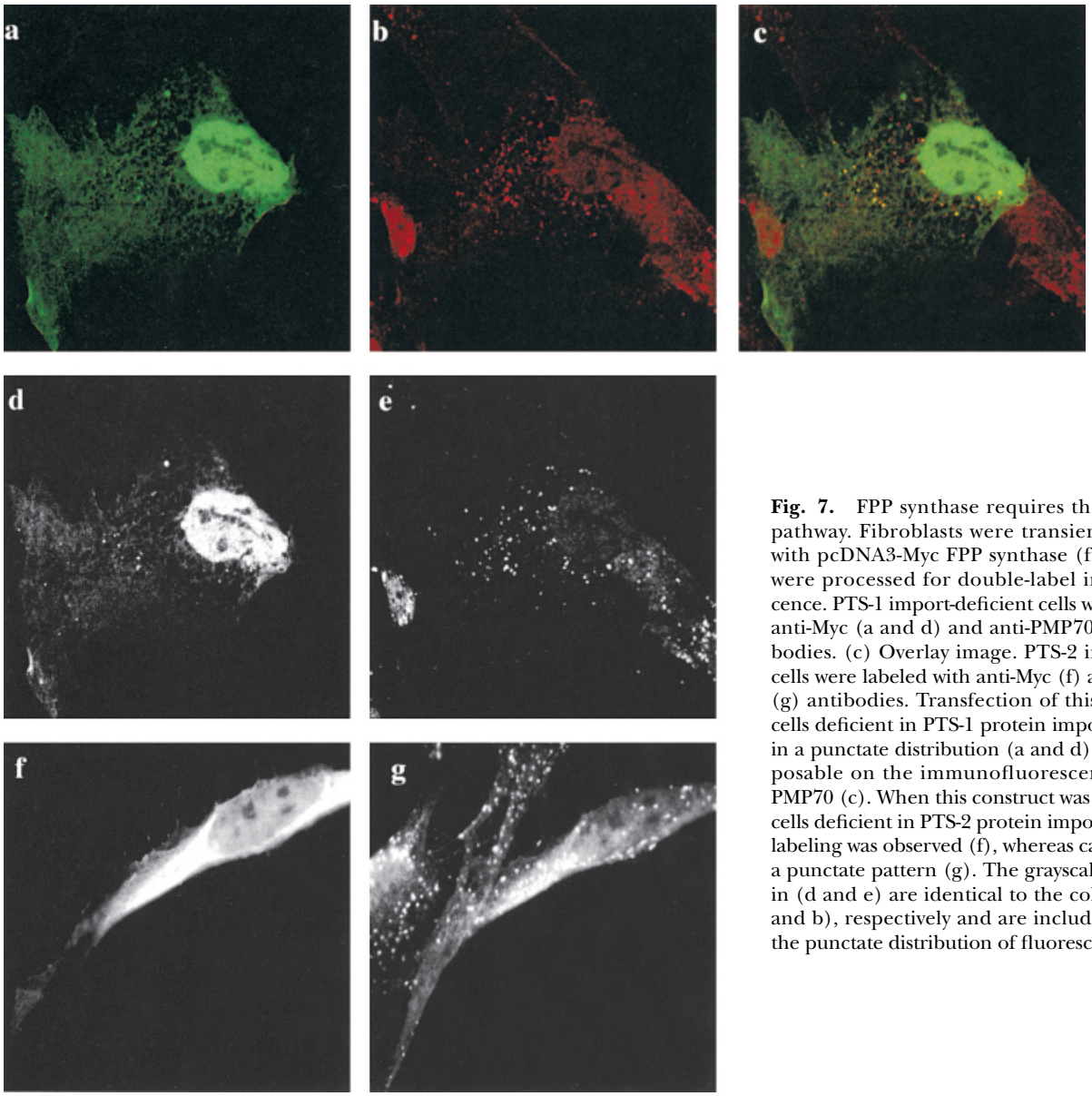

Fig. 7. FPP synthase requires the PTS-2 import pathway. Fibroblasts were transiently transfected with pcDNA3-Myc FPP synthase (full length) and were processed for double-label immunofluorescence. PTS-1 import-deficient cells were labeled with anti-Myc (a and d) and anti-PMP70 (b and e) antibodies. (c) Overlay image. PTS-2 import-deficient cells were labeled with anti-Myc (f) and anti-catalase (g) antibodies. Transfection of this construct into cells deficient in PTS-1 protein import only resulted in a punctate distribution (a and d) that is superimposable on the immunofluorescence pattern for PMP70 (c). When this construct was transfected into cells deficient in PTS-2 protein import only, cytosolic labeling was observed (f), whereas catalase displayed a punctate pattern (g). The grayscale images shown in (d and e) are identical to the color images in (a and b), respectively and are included for clarity of the punctate distribution of fluorescence.

import-deficient cell lines in Fig. 7a–e and PTS-2 import-deficient cell lines in Fig. 7f and g. These cell lines transfect differently, with the PTS-2 import-deficient cell lines showing a higher transfection efficiency. Therefore, one possible difference in the fluorescence seen in Fig. 7a, d, and f could be due to a difference in copy number being expressed. We again included grayscale images (Fig. 7d and e) for clearer visualization of the pattern. Although in Fig. 7a and d the peroxisomal labeling is faint, the main goal is the demonstration of colocalization, which can be clearly seen in Fig. 7c of the FPPS construct with PMP70.

To determine the region of FPP synthase required for peroxisomal import a number of fusion expression vectors were generated. The first expression vector contained 20 amino acids of the amino terminus of FPP synthase attached to the Myc epitope (pcDNA3-Myc FPS-N20). After transfection of this construct into a cell line deficient in PTS-1 protein import only, immunofluorescence patterns for the Myc epitope (**Fig. 8a**) and PMP70 (Fig. 8b) were similar. That the labeling of the two proteins is colocalized is shown by the superimposed image (Fig. 8c), indicating that the amino-terminal 20 amino acids of FPP synthase are sufficient for targeting FPP synthase to peroxisomes. Immunofluorescence for the Myc epitope in cells deficient in the PTS-2 protein import pathway revealed cytosolic labeling (Fig. 8g), which is the predicted result if the protein depends on the PTS-2 receptor. Transfection of pcDNA3-Myc FPS-N20 in CHO cells also resulted in a punctate distribution of labeling that consistently colocalized with catalase, indicating a peroxisomal localization (**Fig. 9a** and **b**). The second Myc fusion expression vector contained the carboxy-terminal 266 amino acids of FPP synthase (pcDNA3-Myc FPS C-terminal). The transfection of this vector in CHO cells produced cytosolic labeling (Fig. 9c), in contrast to the punctate labeling of catalase (Fig. 9d). Therefore, these data suggest that the signal re-

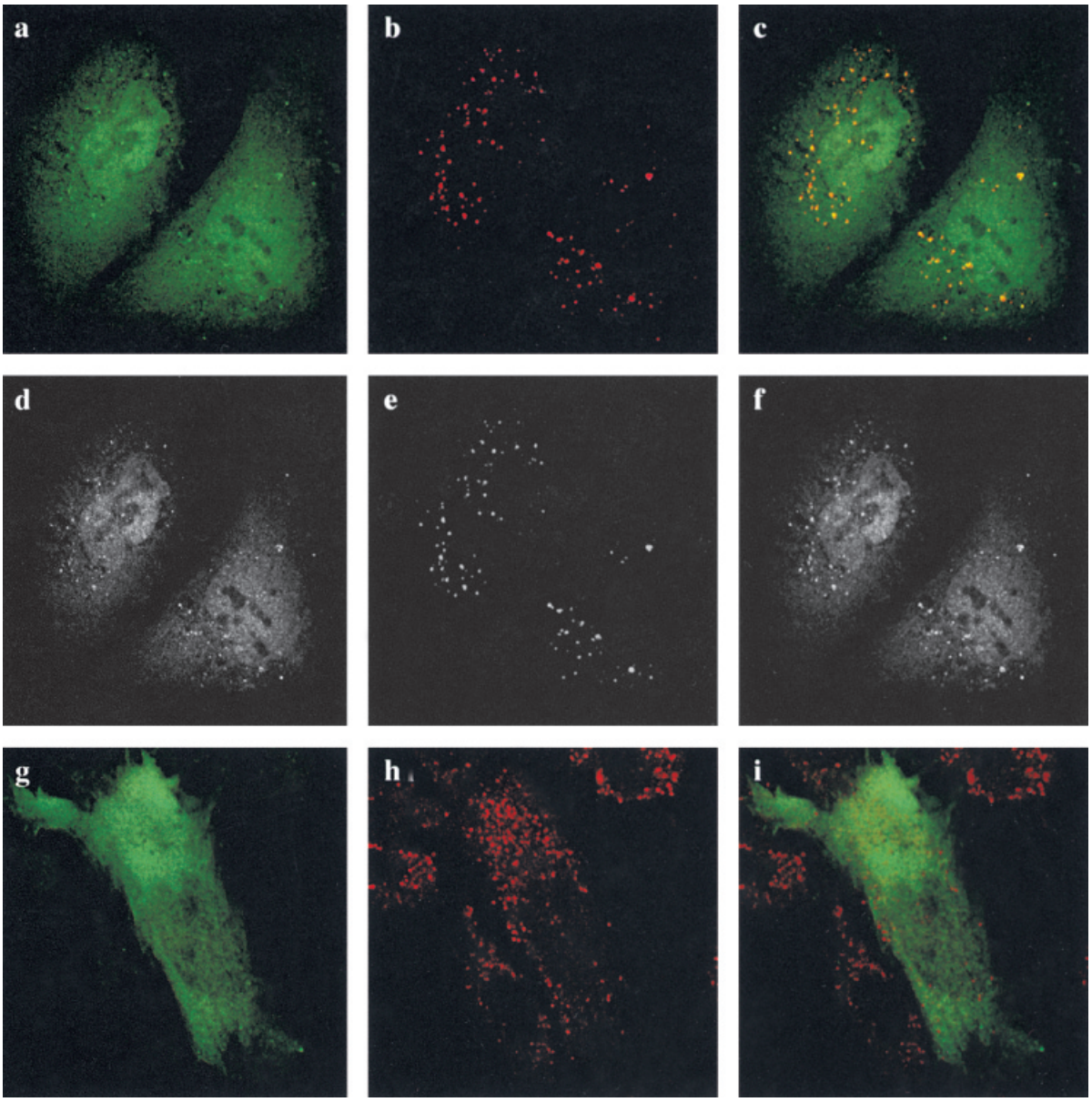

Fig. 8. The amino-terminal 20 amino acids of FPP synthase contains a peroxisomal targeting signal and requires the PTS-2 import pathway. Human fibroblasts deficient in PTS-1 (a–f) or PTS-2 (g–i) protein import were transiently transfected with pcDNA3-Myc FPS-N20 and were processed for double-label immunofluorescence, using antibodies specific for the Myc tag (a, d, and g) and PMP70 (b, e, and h). (c, f, and i) Overlay images. Transfection of this construct into cells deficient in PTS-1 protein import only resulted in a punctate distribution (a and d) that is completely superimposable onto the immunofluorescence pattern of PMP70 (c and f). When this construct was transfected into cells deficient in PTS-2 protein import only, cytosolic labeling was observed (g), whereas PMP70 displayed a punctate pattern (h). The grayscale images shown in (d–f) are identical to the color images in (a–c), respectively, and are included for clarity of the punctate distribution of fluorescence.

quired for peroxisomal targeting of FPP synthase is contained within the first 20 amino acids of the protein and that the protein lacks a peroxisomal targeting signal within the carboxy-terminal portion.

In efforts to identify more clearly the sequence responsible for the peroxisomal targeting of FPP synthase, additional Myc epitope-FPP synthase expression fusion constructs containing truncated regions of FPP synthase were generated. Three constructs were generated, one in which the first four amino acids of FPP synthase were removed from the N-20 sequence (pcDNA3-Myc FPP synthase N-16), one in which the first six amino acids were removed (pcDNA3-Myc FPP synthase N-14), and one in which amino acids 11 and 12 were changed from QE to GA (pcDNA3-Myc FPP synthase QEΔGA). Truncation of FPP synthase by four amino acids did not alter its peroxisomal import; however, removing the first six amino acids relocated the protein to the cytosol (**Table 2**). These results indicate that the fifth and sixth amino acids are a necessary part of the targeting signal contained in FPP synthase. Furthermore, when amino acids 11 and 12 were changed from QE to GA, the immunofluorescence pattern again

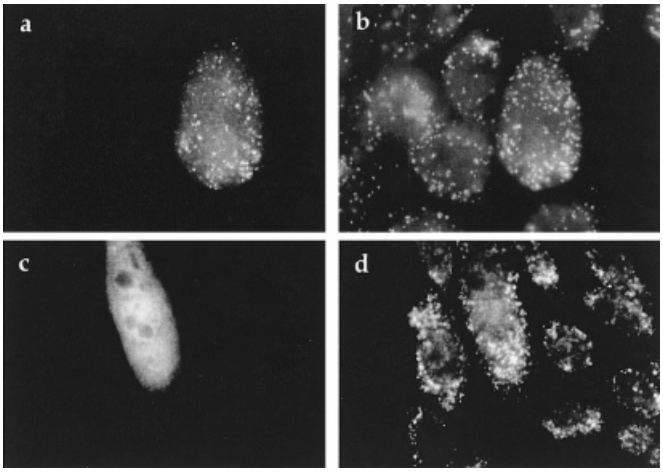

Fig. 9. The amino-terminal 20 amino acids of FPP synthase contains a peroxisomal targeting signal. CHO cells were transiently transfected with pcDNA3-Myc FPP synthase N20 (a and b) and pcDNA3-Myc FPP synthase C-terminal (c and d) and were processed for double-label immunofluorescence. Transfected cells were processed for double-label immunofluorescence with anti-Myc (a and c) and with anti-catalase (b and d) antibodies. Transfection of pcDNA3-Myc FPS-N20 in CHO cells resulted in a punctate distribution of labeling that consistently colocalized with catalase (a and b). The transfection of the C-terminal 266 amino acids of FPP synthase pcDNA3-Myc FPS C-terminal vector produced cytosolic labeling (c) in contrast to the punctate labeling of catalase (d). The CHO cells used in this figure have high transfection efficiency and survive the transfection procedure well, whereas the peroxisome-deficient cells used in Fig. 7 do not transfect well and only a fraction survive the transfection procedure. Therefore, the different transfection efficiencies may explain the different signal intensities between the two figures

revealed only cytosolic distribution, indicating that QE is also a requirement in the targeting sequence.

DISCUSSION

Cholesterol biosynthesis occurs through more than 30 discrete reactions. As mentioned previously, studies have indicated that a number of the enzymes involved in these reactions are localized to the peroxisomes. However, mechanisms for targeting to peroxisomes have been demonstrated only for PMvK and IPP isomerase (26, 27). In the current study we identify the peroxisomal targeting signals required for four other enzymes of the cholesterol biosynthetic pathway: AA-CoA thiolase, HMG-CoA synthase, MPPD, and FPP synthase.

We present evidence here that FPP synthase is targeted to peroxisomes through a sequence contained in the first 20 amino acids of the amino-terminal portion of the protein. Furthermore, the fifth and sixth amino acids as well as amino acids 11 and 12 are essential for retaining the peroxisomal localization (Table 2). Although the FPP synthase targeting sequence KL(DVHN)QE is reminiscent of a PTS-2, it clearly does not fit the consensus sequence. Furthermore, when the amino acids of human FPP synthase (38) are compared with rat FPP synthase (39) the sequences in this region are not conserved. The human sequence for these amino acids is NS(DVYA)QE. In contrast, a comparison of human and rodent sequences for AA-CoA thiolase (28, 40), HMG-CoA synthase (41, 42), MvK (43, 44), PMvK (26, 45), MPPD (46, 47), and IPP isomerase (27, 48) reveals that the peroxisomal targeting signals are conserved. Therefore, this apparent lack of sequence conservation of FPP synthase between mammalian species might indicate that the sequence is not a specific receptor-binding site. However, it is clear that FPP synthase is a peroxisomal protein in a variety of mammalian cells. FPP synthase has been shown to colocalize with catalase by use of confocal immunofluorescent microscopy, in a human hepatoma cell line (Hep G2), in a rat hepatoma cell line (H35), and in CHO cells (21). Previous studies have also demonstrated the peroxisomal location of FPP synthase in a human carcinoma cell line (SKB-R3) and in monkey kidney fibroblasts (CV-1) (20). Several studies have shown that peroxisomal proteins without targeting signals can gain access to the matrix by piggybacking, a process in which the enzyme lacking a PTS multimerizes with other proteins that do contain peroxisomal targeting signals and the complex as a whole is imported into the matrix (5, 6). Other groups have reported data that indicate some proteins such as acyl-CoA oxidase are imported into the peroxisomal matrix independent of either import pathway, suggesting the existence of a third import pathway (49). Therefore, while it is clear from the studies presented here that FPP synthase utilizes the PTS-2 import pathway, it is as yet unclear if the sequence identified as being required for import is a degenerate PTS-2 like sequence or a sequence required for the piggybacking of FPP synthase with another PTS-2-containing protein.

Another unexpected result from these studies is the identification of both a mitochondrial targeting signal at the amino terminus and a peroxisomal targeting signal

TABLE 2. Analysis of FPP synthase amino-terminal peroxisomal targeting signal

Construct	Sequence	Location
pcDNA3-Myc FPS N-20	M N G D Q K L D V H N Q E K Q N F I Q N	P
pcDNA3-Myc FPS N-16	M K L D V H N Q E K Q N F I Q N	P
pcDNA3-Myc FPS N-14	M D V H N Q E K Q N F I Q N	C
pcDNA3-Myc FPS QEΔGA	M N G D Q K L D V H N *GA* K Q N F I Q N	C

FPP synthase-Myc fusion constructs were transiently transfected into CHO cells. The sequence of FPP synthase contained in each construct is shown along with the observed localization. Flourescent location is indicated as peroxisomal (P) or cytosolic (C).

(PTS-1) (QKL) at the carboxy terminus in mitochondrial AA-CoA thiolase. In our studies using full-length AA-CoA thiolase we were able to visualize only mitochondrial labeling; however, these results do not exclude the possibility that peroxisomal labeling was present but not detectable by our methods. Interestingly, another enzyme, HMG-CoA lyase, has been shown to have a unique dual localization in both peroxisomes and mitochondria (30). The HMG-CoA lyase contains a 27-amino acid amino-terminal mitochondrial targeting sequence and a carboxy-terminal consensus PTS-1 (CKL). Although the subcellular targeting ability of these sequences has not been directly tested, the enzyme has been localized to both peroxisomes and mitochondria through subcellular fractionation studies (31). Furthermore, after entry into mitochondria the amino-terminal sequence is cleaved, causing this form of the enzyme to be 2.5 kDa smaller than the peroxisomal form, which does not undergo any cleavage. In addition, other studies indicate that the peroxisomal activity is lacking in fibroblasts from patients with Zellweger syndrome (31). While mitochondrial HMG-CoA lyase is known to be required for ketogenesis, the function of the enzyme in peroxisomes is unknown. Another example of an enzyme dually distributed in both peroxisomes and mitochondria is the mammalian $\Delta^{3,5}$-$\Delta^{2,4}$- dienoyl-CoA isomerase (50). The protein contains both a 40-amino acid amino-terminal mitochondrial targeting sequence and a carboxy-terminal PTS-1 (SKL) (50). The amino-terminal amino acid sequence is cleaved on mitochondrial import and as a consequence the mitochondrial polypeptide is 4 kDa smaller than the peroxisomal isoform. Interestingly, Kotti et al. (51) reported that mouse α-methylacyl-CoA racemase can be alternatively targeted to peroxisomes or mitochondria without modifications. They concluded that the non-cleavable amino-terminal sequence of this enzyme acts as a weak mitochondrial targeting signal and the carboxy-terminal sequence -KANL acts as a peroxisomal targeting signal. The sequence -KANL has previously been shown to act as a functional PTS-1 in catalase (52). The α-methylacyl-CoA racemase is exceptional in general, because, unlike other enzymes, which are modified when present in different compartments, here the same gene product can be targeted to two subcellular compartments.

In the studies presented, we also analyzed a new variation of PTS-2 sequences required to target HMG-CoA synthase and MPPD to peroxisomes. These data indicate that both HMG-CoA synthase and MPPD are peroxisomal proteins that utilize the newly identified PTS-2 sequence (SVX_5QL) and that requires a functional PTS-2 receptor for import into the peroxisomes. However, the functionality of the PTS-2 sequence in the intact MPPD has yet to be determined.

The peroxisomal localization of MvK, which phosphorylates mevalonate in the fourth reaction of the cholesterol biosynthetic pathway, has been conclusively demonstrated (18, 19). On analysis of the amino acid sequence for MvK a putative PTS-2 has been identified that fits the consensus sequence (KVX_5HA). Therefore, in additional experiments we attempted to demonstrate that the putative PTS-2 contained in MvK targets the protein to peroxisomes. Fusion constructs were generated linking either the full-length MvK (100% of amino acids) or the amino-terminal region containing the putative PTS-2 (from 13 to 88% of amino acids) to either Myc or HA epitope tags, or to reporter constructs (chloramphenicol acetyltransferase and green fluorescent protein). However, all MvK-containing expression constructs that were developed, and all conditions tested, resulted in cytosolic distribution of the fusion proteins in CHO, CV-1, and human fibroblast cells (data not shown). We conclude from these data that the targeting of MvK fusion proteins to peroxisomes is either irreversibly obstructed because of interference from the reporter or epitope tag, or that the targeting signal is no longer accessible to the receptor, or that the protein has significantly changed conformation because of the additional sequences making it unrecognizable to the receptor. It is clear, however, that full-length expression constructs of MvK, without epitope tags, are targeted to peroxisomes, as previously shown (19). **Table 3** summarizes the peroxisomal targeting signals identified to date in the cholesterol biosynthetic enzymes.

TABLE 3. Peroxisomal targeting signals in cholesterol biosynthetic enzymes

Enzyme	Signal	Sequence	Ref.
AA-CoA thiolase (mito)	PTS-1	QKL	
HMG-CoA synthase	PTS-2	SV (X5) QL	
HMG-CoA reductase	Unknown		
Mevalonate kinase	PTS-2	KV (X5) HA	
Phosphomevalonate kinase	PTS-1	SRL	26
Mevalonate-PP decarboxylase	PTS-2	SV (X5) QL	
Isopentenyl-PP isomerase	PTS-1	HRM	27
Farnesyl-PP synthase	PTS-2	KL (X4) QE	

The only enzyme in the cholesterol biosynthetic pathway required for the conversion of acetyl-CoA to FPP for which we as yet have no peroxisomal targeting information is HMG-CoA reductase. HMG-CoA reductase, the rate-limiting step of the cholesterol biosynthetic pathway, catalyzes the conversion of HMG-CoA into mevalonate. As mentioned previously, a number of studies indicate that HMG-CoA reductase is located not only in the ER but also in peroxisomes (15–17). In addition, one study suggests that the peroxisomal reductase is functionally and structurally different from the ER HMG-CoA reductase (53). Analysis of the ER HMG-CoA reductase amino acid sequence does not reveal a peroxisomal targeting signal.

The biosynthesis of cholesterol is a lengthy and complex process in which the required enzymes are distributed amongst several subcellular compartments (Fig. 1). Currently, expression and stability of pathway enzymes have been shown to be major sites for the regulation of this pathway (54). However, it is attractive to speculate that further research into the compartmentalization of the cholesterol biosynthesis pathway may reveal another level by which this complex pathway is regulated.

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