Communication

Transfection of the Ketogenic Mitochondrial 3-Hydroxy-3-methylglutaryl-coenzyme A Synthase cDNA into MeV-1 Cells Corrects Their Auxotrophy for Mevalonate*

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A somatic cell mutant of the Chinese hamster ovary (CHO)-K1 (called Mev-1), auxotrophic for mevalonate by virtue of a complete lack of detectable cytosolic 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase activity, was transfected with a plasmid containing the cDNA for ketogenic mitochondrial HMG-CoA synthase under the control of SV40 early promoter. The resulting stable cell line (Mev-SM) was able to grow in the absence of mevalonate. Analysis by Western blot showed that the new cell line strongly expressed mitochondrial HMG-CoA synthase protein. Immunocytochemical studies using specific antibodies against mitochondrial HMG-CoA synthase showed that the protein was located exclusively inside the mitochondria. The prototroph cell line Mev-SM can incorporate labeled acetate into cholesterol in the absence of mevalonate. These results show that the new cell line may circumvent the lack of cytosolic HMG-CoA synthase activity by producing cholesterol-convertible HMG-CoA inside the mitochondria.

3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase activity catalyzes the condensation of acetocetyl-CoA and acetyl-CoA into HMG-CoA plus free CoA. HMG-CoA synthase activity is located in two separate compartments: cytosol and mitochondria. The HMG-CoA produced by cytosolic HMG-CoA synthase is converted into mevalonate by the action of HMG-CoA reductase. This is the beginning of the isoprenoid pathway, which, in addition to cholesterol as the main end product, also produces a vast number of isoprenoid derivatives such as ubiquinone, dolichol, isopentenyl tRNA, and farnesylated proteins. In contrast the HMG-CoA produced by mitochondrial HMG-CoA synthase is converted into acetoacetate by the action of HMG-CoA lyase; then acetoacetate is transformed into β-hydroxybutyrate and acetone, all of which are usually called ketone bodies.

We have demonstrated previously that rat mitochondrial and cytosolic HMG-CoA synthases are encoded by two different genes (1). Further studies showed that mitochondrial HMG-CoA synthase is expressed vigorously not only in liver, testis, and ovary from adult rats (2) but also in liver and intestine from suckling rats (3, 4). Immunocytochemical studies using specific antibodies performed by our group clearly showed that mitochondrial HMG-CoA synthase protein is present inside the mitochondria of some specialized steroidogenic cells: Leydig cells from testis, theca interna cells from the ovarian follicle, some corpus luteum cells of the rabbit, and the epi-thelial cells lining the proximal portion of the epididymis. This particular pattern of expression suggests that, in addition to its role as an important rate-limiting step in ketogenesis, mitochondrial HMG-CoA synthase is expressed in these specialized cells, probably to produce cholesterol for steroidogenesis (2).

In this paper we report that transfection of the mevalonate auxotroph Mev-1 cells (5) with a plasmid containing the cDNA for ketogenic mitochondrial HMG-CoA synthase under the control of the promoter of the SV40 virus reverses the mevalonate auxotrophy. The resulting cell line, Mev-SM, does not depend on the presence of mevalonate for growth and division. Western blot analyses show considerable expression of mitochondrial HMG-CoA synthase in Mev-SM but not in Mev-1 cells. Immunocytochemical studies using specific antibodies clearly show the occurrence of mitochondrial HMG-CoA synthase inside the mitochondria. These results demonstrate that such cells may produce cholesterol-convertible HMG-CoA inside the mitochondria via the activity of mitochondrial HMG-CoA synthase.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Plasmid pSG5SM was constructed by insertion of a 1725-base pair BamHI fragment excised from lcmMS1, which contains the cDNA for mitochondrial HMG-CoA synthase (1), into the BamHI site in the polylinker of the eukaryotic expression vector pSG5 (6).

Cell Culture and Transfection Experiments—Mev-1, a somatic cell mutant of the Chinese hamster ovary (CHO)-K1 cell devoid of detectable cytosolic HMG-CoA synthase and auxotrophic for mevalonate, was kindly provided by Dr. Sinensky. Cells were cultured as described by Schnitzer-Polokoff et al. (5). Briefly, cells were maintained in Ham’s F-12 medium supplemented with 10% fetal calf serum, 0.4 mM mevalonate, and antibiotics. DNA transfections were carried out by the standard calcium phosphate method (7). 48 h after transfection, the trypsinized cells were cultured in medium supplemented with 10% fetal calf serum, without mevalonate, and the plates were maintained for 8–10 days. By this time colonies of revertant cells were distinguishable and resistant colonies were pooled (150–300 colonies) and expanded in mass culture in the absence of mevalonate. These revertants were named Mev-SM.

Antibodies against Mitochondrial HMG-CoA Synthase—Polyclonal antibodies against mitochondrial HMG-CoA synthase were elicited against peptides corresponding either to the N terminus of the protein (sequence 37–49, STIPPAFLAKTD) or to the C terminus (sequence 469–484, QYHVKVFSPFGDTSN) of the rat mitochondrial HMG-CoA synthase protein as reported previously (2). The antibodies were spe-
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Table 1
HMG-CoA synthase activity in extracts prepared from mutant and wild type cells

| Cell type | Specific activity | -MgCl₂ | +MgCl₂ |
|-----------|-------------------|--------|--------|
| CHO-K1    | 0.87              | 0.86   |        |
| MeV-1     | ND                | ND     |        |
| MeV-SM    | 1.9               | ND     | 0.02   |

Fig. 1. Mevalonate auxotrophy reversion by mitochondrial HMG-CoA synthase. 2.5 x 10⁵ cells were inoculated on 35-mm plates and incubated in media supplemented with 10% fetal calf serum or 2% lipoprotein-deficient serum, in the presence or absence of 0.4 mM mevalonate as indicated. 48 h after plating, cells were stained with 0.1% methylene blue in 50% methanol.

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For 45 min at 37 °C in darkness. After the last incubation, nuclear staining was carried out with 1 μg bis-benzimide (Sigma). Finally the coverslips were mounted with immunofluorescence mounting medium (ICN Biomedicals Inc., Costa Mesa, CA). Negative controls were performed by using preimmune immunoglobulins and by omission of the primary antibody. Immunostaining was visualized with an epifluorescence microscope (Polyvar II, Reichert-Jung, Wien, Austria).

For electron immunocytochemistry, cells grown on 60-mm plates were fixed in 2% paraformaldehyde, 2% sucrose and 0.5% glutaraldehyde, for at least 1 h at room temperature. Cell pellets were embedded in 10% gelatin blocks and post-fixed overnight in 2% paraformaldehyde at 4 °C. Blocks were then cryoprotected in polyvinylpyrrolidone (Sigma) and 2% sucrose solution for 24 h, mounted on a metal stub and rapidly frozen in liquid nitrogen. Ultrathin sections (60-85 nm) were obtained by cryoultramicrotomy (Ultracut FC4D, Reichert-Jung) and placed on formvar-coated gold grids (200-mesh) for transmission electron microscopy. The grids were treated in 150 mM ammonium chloride and then blocked in 0.5% ovalbumin in 10 mM PBS, 20 μM glycine solution at room temperature. Antisera were incubated for 30 min at 1:800 dilution and secondary protein A-gold 15 nm (Dr. J. Slot, University of Utrecht, Utrecht, The Netherlands) was then applied for 20 min. After successive
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RESULTS AND DISCUSSION

Mevalonate Auxotrophy Reversion by Mitochondrial HMG-CoA Synthase—To determine whether the cDNA for mitochondrial HMG-CoA synthase could revert the mevalonate auxotrophy of the MeV-1 cells, a BamHI fragment corresponding to such cDNA, containing the 5'-untranslated region, the whole coding sequence, and 200 base pairs of the 3'-untranslated region, was subcloned into an eukaryotic expression vector (pSG5). The introduction of this cDNA into MeV-1 cells, which are defective mutants for cytosolic HMG-CoA synthase activity, produced (as a result of a stable transfection) colonies able to grow in the absence of mevalonate. When MeV-1 cells were transfected with the empty vector (pSG5) and transferred to a medium without mevalonate, all the cells died in about 30 h. Fig. 1 shows that MeV-SM (the result of the stable transfection) but not MeV-1 (the parental cell line) are able to grow in the absence of added mevalonate in medium supplemented with either fetal bovine serum or lipoprotein-deficient serum. This experiment suggests that the expression of the mitochondrial HMG-CoA synthase gene, which is not expressed in mutant MeV-1 cells, confers on the cells the capacity to synthesize inside the mitochondria HMG-CoA (supposedly ketogenic), which is susceptible to transformation into cholesterol, and also in the mevalone-derived isoprenoid compounds necessary for cells growth and division.

Since these results were unexpected, we investigated whether: i) MeV-SM cells lack cytosolic HMG-CoA synthase activity, ii) the protein produced after transfection was indeed mitochondrial HMG-CoA synthase, iii) it was located inside mitochondria, and iv) MeV-SM cells were able to synthesize cholesterol from [14C]acetate.

Sensitivity to MgCl2 of HMG-CoA Synthase Activity in MeV-SM Cells—To confirm that MeV-SM cells lack cytosolic HMG-CoA synthase activity, we measured the HMG-CoA activity in the presence or absence of MgCl2 in order to distinguish between the two forms of the enzyme (11). As can be seen in Table I, the activity present in MeV-SM cells is completely inhibited by 20 mM MgCl2, as it corresponds to the mitochondrial enzyme.

Immunolocalization of Mitochondrial HMG-CoA Synthase in Mutant or Revertant Cells—To test whether the enzyme responsible for the reversion of the auxotrophy was indeed mitochondrial HMG-CoA synthase, we assessed the presence of mitochondrial HMG-CoA synthase protein in MeV-SM by Western blot analysis (Fig. 2). Results show that neither CHO nor MeV-1 cells express mitochondrial HMG-CoA synthase. In contrast, MeV-SM cells vigorously expressed the mitochondrial HMG-CoA synthase protein. We also performed immunocytochemical studies using the antibodies described under "Experimental Procedures." Immunofluorescence microscopy of MeV-SM cells revealed a very intense dotted fluorescence pattern that suggested intramitochondrial localization, which was not present in the MeV-1 cells (Fig. 3). Immunoelectron microscopy on ultrathin cryosections confirmed that HMG-CoA synthase was located in the mitochondrial matrix of MeV-SM cells (Fig. 4).

MeV-SM Cells Synthesize Cholesterol from Acetate—The capacity of mevalonate to support the growth of MeV-1 cells im-

FIG. 4. Immunoelectron localization of mitochondrial HMG-CoA synthase in Mev-SM cells. Cells were processed for immunoelectron microscopy as indicated under "Experimental Procedures." Immunolabeling was performed using anti-HMG-CoA synthase immunoglobulins and protein A-gold (14 nm). Inset shows high magnification of the matrix localization of mitochondrial (m) label. pm, plasma membrane, n, nucleus. Bar in A, 2 μm; bar in inset, 0.5 μm.

washes, grids were contrasted in 0.03% uranyl acetate solution and a thin surface membrane of methyl-cellulose (Sigma) was applied. Negative control sections were prepared by omission of the primary antibody. Sections were observed by conventional transmission electron microscopy (Hitachi 600 AB, Hitachi Inc., Tokyo, Japan).

Incorporation of Labeled Acetate into Cholesterol—CHO-K1, MeV-1, or MeV-SM cells (0.5 × 106) were seeded into 35-mm plates and incubated overnight in Ham's F-12 (0.1% fetal calf serum) supplemented with 200 units/ml aprotinin. After 24 h of incubation, cells were harvested by scraping into PBS, 0.1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 200 units/ml aprotinin. After 24 h of dialysis at 4°C in 0.2 M potassium phosphate buffer, pH 7.5, 0.1 mM EDTA, HMG-CoA synthase was assayed by the radiometric procedure of Clinkenbeard et al. (10). The standard assay mixture contained, in a final volume of 0.2 ml, 0.25 M Tris, pH 8.0, 50 μM acetoacetyl-CoA, 200 μM [1-14C]acetyl-CoA (2 μCi/ mmol), and 30 μg of protein.
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FIG. 5. Incorporation of [14C]acetate into cholesterol. CHO, MeV-1, or MeV-SM cells were incubated overnight in F-12 + 10% fetal calf serum. The medium was then changed to F-12 + 10% lipoprotein-deficient serum, and 2 μCi of [14C]acetate was added. After incubation for 24 h, cells were harvested and the non-saponifiable fraction was analyzed in two different separation systems, petroleum ether/diethyl ether/acetic acid (A) or chloroform/methanol (B). Arrows on the left indicate the position of cholesterol and the origin of the chromatography. The migration of pure [14C]cholesterol is shown on the right. After localization of the radioactive bands by autoradiography, the silica gel was cut out of the plate and the radioactivity of samples was measured on a liquid scintillation counter. The mean of the incorporation of radioactivity to cholesterol in two different experiments was: 310.5 cpm in CHO-K1 cells, 33.95 cpm in MeV-1, and 450 cpm in MeV-SM in A; and 472.15 cpm in CHO-K1 cells, 33.25 cpm in MeV-1, and 368.3 in MeV-SM in B.

Mitochondrial HMG-CoA synthase activity in liver cells of adult mammals is located inside the mitochondria, and it constitutes a regulatory site in the HMG-CoA pathway to synthesize ketone bodies (1, 12–14). It has also been localized in large amounts in liver and intestine mitochondria from suckling rats (3), although smaller quantities have also been observed in kidney from neonatal rats (4). In all these tissues, the HMG-CoA produced by the enzyme reaction could be converted into acetoacetate by the action of HMG-CoA lyase.

The expression of this gene in testis and ovary, not only as mRNA but as immunodetectable mitochondrial HMG-CoA synthase protein, and the fact that this expression is restricted to specialized steroidogenic cells led us to propose the hypothesis that, in gonads, HMG-CoA synthase has an important role in cholesterol synthesis as a precursor of steroid hormones (2). Our present results effectively show that HMG-CoA produced in the mitochondria by the action of mitochondrial HMG-CoA synthase could be further transformed into cholesterol, although the mechanism of such transformation is unknown. Based on the presence of HMG-CoA reductase in mitochondria from Leydig cells and the incorporation of labeled acetyl-CoA into digitonin-precipitable sterols in purified mitochondria, Pignauro et al. (15) postulated mitochondrial biosynthesis of cholesterol in Leydig cells from rat testis. The entire pathway from acetyl-CoA to cholesterol may be located inside the mitochondria, as has been predicted for peroxisomes from the presence of several enzymes of the mevalonate pathway in this organelle (for a review, see Ref. 16). Moreover, it has been shown recently that mitochondria utilize endogenously-produced farnesyl pyrophosphate for isoprenoid biosynthesis and that the biosynthetic steps in mitochondria are regulated independently from those occurring in other subcellular compartments (17). Alternatively, a metabolite of HMG-CoA could traverse the mitochondrial membrane and be incorporated in the cytosolic pathway.

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