cDNA Cloning of MEV, a Mutant Protein That Facilitates Cellular Uptake of Mevalonate, and Identification of the Point Mutation Responsible for Its Gain of Function*

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Christine M. Kim†, Joseph L. Goldstein, and Michael S. Brown
From the Department of Molecular Genetics, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235

We report the expression cloning of pMev, a cDNA that facilitates cellular uptake of mevalonate. pMev was isolated from the met-18b-2 clone of Chinese hamster ovary (CHO) cells, which were selected for growth in low concentrations of mevalonate when synthesis is blocked by compactin (Faust, J. R., and Krieger, M. (1997) J. Biol. Chem. 262, 1996–2004). pMev encodes a 494-residue protein, Mev, that is predicted to have 12 membrane-spanning regions, consistent with a membrane transporter. Surprisingly, levels of Mev mRNA and protein are similar in CHO and met-18b-2 cells. The Mev gene differs from the wild-type gene by a single base change that substitutes a cysteine for phenylalanine in the 10th membrane-spanning region. met-18b-2 cells are heterozygous for this dominant gain-of-function mutation. Transfection of a cDNA encoding pMev, but not the wild-type cDNA, elicited a marked increase in [3H]mevalonate uptake and incorporation into cellular lipids in stably and transiently transfected cells. The availability of pMev will facilitate studies of [3H]mevalonate incorporation into trace products, including p21 proteins and other prenylated proteins.

Mevalonate is a key intermediate in the synthesis of sterols and isoprenoids. In animals the bulk product of mevalonate metabolism is cholesterol, some of which is converted to steroid hormones and bile acids. Mevalonate is also converted into dolichols, which act as carriers in the assembly of carbohydrate chains of glycoproteins; ubiquinones, which participate in electron transport; and isopentenylated transfer RNAs, which play specific roles in protein synthesis (Goldstein and Brown, 1990). The most recently discovered products of mevalonate metabolism are a family of proteins whose adherence to membranes is facilitated by covalent attachment of mevalonate-derived prenyl groups, namely, farnesyl and geranylgeranyl. Such prenylated proteins include p21 proteins, which regulate cell growth; lamins, which help to form the nuclear envelope; membrane-bound subunits of signal-transducing G proteins; and a large family of Ras-related 21–25-kDa proteins that bind GTP and regulate the budding and fusion of membranous vesicles (Glomsset et al., 1991).

Mevalonate is produced by a membrane-bound enzyme of the endoplasmic reticulum, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reducetase. Complex regulatory mechanisms adjust the activity of this enzyme over a range of several 100-fold, assuring the production of sufficient mevalonate for conversion into essential sterol and nonsterol products while avoiding toxic overaccumulation (Goldstein and Brown, 1990).

Interest in the quantitatively minor products of mevalonate metabolism has been heightened by the discovery that the growth-promoting activities of wild-type or oncogenic p21 proteins are dependent on the presence of a farnesyl group attached in thioether linkage to a cysteine residue (Hancock et al., 1989; Casey et al., 1989; Schafer et al., 1990). This attachment is catalyzed by a cytosolic enzyme that uses farnesyl pyrophosphate as a donor (Reiss et al., 1990). If a Ras protein is altered so that it can no longer accept a farnesyl group, the protein loses its ability to stimulate the growth of cells. This finding raises the possibility that growth of Ras-dependent tumors may be slowed or arrested by agents that inhibit the synthesis of farnesyl groups or their attachment to Ras proteins (Hancock et al., 1989; Schafer et al., 1989). The search for such inhibitors would be facilitated if assays of protein prenylation could be performed efficiently in living cells.

The study of prenylated proteins in living cells is most conveniently carried out by incubating the cells with radioactive mevalonate and following its incorporation into prenylated proteins by polyacrylamide gel electrophoresis and autoradiography. Although such studies have produced important results, they have been hampered by inefficient entry of mevalonate into cells and the consequent difficulty in incorporating detectable amounts of radioactivity into low abundance products. This problem can be circumvented in part by incubating the cells with inhibitors of HMG-CoA reductase such as compactin or lovastatin, which prevent dilution of the exogenous labeled mevalonate by endogenously synthesized unlabeled mevalonate (Brown and Goldstein, 1990). However, even when these inhibitors are used, only small amounts of radioactivity are incorporated into prenylated proteins. Typically, autoradiographic detection of such proteins requires exposures of several days to weeks (Schmidt et al., 1984; Maltese, 1990).

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1 The abbreviations used are: HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; LDL, low density lipoprotein; CHO, Chinese hamster ovary; MeLoCo medium, selection medium containing mevalonate, LDL, and compactin; PCR, polymerase chain reaction; kb, kilobase(s).
To overcome the problem of inefficient uptake of mevalonate, in the present study we have isolated a complementary DNA copy (cDNA) of a messenger RNA that produces a protein that facilitates mevalonate entry into cells. This cDNA was derived from a line of Chinese hamster ovary (CHO) cells that was isolated by Faust and Krieger (1987) and shown to have high mevalonate uptake. These cells were discovered as a by-product of a complex strategy that was designed to isolate the gene for the low density lipoprotein (LDL) receptor. This strategy took advantage of the fact that mammalian cells in tissue culture require cholesterol for growth. They can obtain cholesterol from two sources: endogenous synthesis from acetyl CoA through a multi-enzyme pathway that includes HMG-CoA reductase; and uptake from exogenous lipoproteins by means of the cell surface receptor for plasma LDL (Brown and Goldstein, 1980). Krieger et al. (1981) described a method to produce cells with mutations in the gene for the LDL receptor. Kingsley and Krieger (1984) used this method to isolate a clone of CHO cells, designated ldlA-7, that fail to produce active LDL receptors as a result of a mutation in the LDL receptor gene. Krieger and coworkers then tried to determine whether the defect in these cells could be corrected by introducing genomic DNA from normal CHO cells into the ldlA-7 cells by calcium phosphate-mediated transfection. The DNA was introduced together with the bacterial neo gene that renders mammalian cells resistant to the antibiotic G418 (reviewed in Faust and Krieger (1987)).

In order to select for cells that had acquired the gene for the LDL receptor, Faust and Krieger (1987) grew the transfected cells in a medium called MeLoCo. This medium contains ordinary culture medium supplemented with serum from which the cholesterol-carrying lipoproteins have been removed; compactin, an inhibitor of HMG-CoA reductase; and a low concentration of LDL. Importantly, the MeLoCo medium also contains a low concentration of mevalonate (250 μM) that provides sufficient substrate for the synthesis of nonsterol products, but is ordinarily not sufficient to meet the demands for the bulk product, cholesterol (Brown and Goldstein, 1980). Under these conditions the cells should only grow if they produce LDL receptors and thus satisfy their cholesterol requirement.

After transfection of CHO cell DNA into the ldlA-7 cells, Faust and Krieger (1987) isolated a single clone of cells, designated met-18b-2, that was able to grow in the MeLoCo medium. They expected these cells to have acquired the gene for the LDL receptor, but surprisingly the cells did not produce LDL receptors. Instead, the cells had acquired the ability to efficiently utilize the mevalonate in the culture medium so that low concentration of mevalonate was sufficient to satisfy their demands for cholesterol synthesis.

Faust and Krieger (1987) went on to show that the met-18b-2 cells take up and use [3H]mevalonate from the culture medium at a rate that was ca. 10-40 fold faster than the rate in CHO cells. Uptake appeared to be mediated by a saturatable transporter that showed half-maximal rates at about 0.3 mM (R,S)-mevalonate. Indirect evidence suggested that the transporter was specific for the physiologic R form of mevalonate. It did not appear to take up other organic anions such as acetocetate, β-hydroxybutyrate, pyruvate, or octanoate.

In the current study, we have used an expression cloning strategy to isolate a cDNA that encodes the protein responsible for rapid mevalonate uptake in met-18b-2 cells. The cDNA, designated pMev, encodes Mev, a hydrophobic protein of 494 amino acids that is predicted to contain 12 membrane-spanning regions. When pMev is inserted into human, hamster, monkey, or mouse cells by calcium phosphate-mediated transfection, the cells take up [3H]mevalonate with high efficiency, thereby facilitating the study of mevalonate metabolism in these cells. The nucleotide sequence of pMev differs from the corresponding cDNA in parental CHO cells at one nucleotide position that changes a codon for phenylalanine in the wild-type protein to cysteine in Mev. We conclude that Mev arose from a cellular gene by a point mutation that has endowed the protein with the ability to enhance mevalonate uptake. Mev may be the mevalonate transporter itself or a protein that stimulates the transporter. The point mutation that gave rise to Mev is an example of a gain-of-function mutation in animal cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Mevalonolactone (purchased from Fluka) and compactin (provided by Akira Endo, Tokyo Noko University, Tokyo, Japan) were converted to the sodium salts (Brown et al., 1978). [3-14C]Pyruvic acid (14.5 mCi/mmol) and ([R,S]-[5-3H]mevalonolactone (27.8–35 Ci/mmol) were obtained from Du Pont-New England Nuclear. The radiolabeled mevalonolactone was evaporated to dryness, mixed with varying amounts (10–140 μl) of unlabeled 0.2 mM sodium mevalonate, incubated with 0.35 ml of 0.1 N NaOH at 37 °C for 1 h, neutralized with 70 μl of 0.5 N HCl, and used immediately for experiments. 6-Fluoromevalonate (provided by Gary Quistad, Sanzor Crop Protection, Palo Alto, CA) was stored at −80 °C as a stock solution (0.27 M) in ethanol. ldlA-7 cells (Kingsley and Krieger, 1984), met-18b-2 cells (Faust and Krieger, 1987), human embryonic kidney 293 cells, and CHO-K1 cells were obtained, respectively, from Monty Kreiger (MFR), Jerry Faust (Tufts University School of Medicine), Arnold Berk (University of California at Los Angeles), and the American Type Culture Collection. cDNA probes for rat cyclophilin and rat glyceraldehyde-3-phosphate dehydrogenase were obtained from Karl Normington of this department.

**General Methods**—Standard molecular biology techniques were used (Sambrook et al., 1989). Total cellular RNA was isolated from solid organs or from cell lines by the guanidinium thiocyanate/TrisCl centrifugation procedure (Chirgwin et al., 1979). Poly(A)⁺ RNA was isolated by oligo(dT)-cellulose chromatography using a Pharmacia LKB Biotechnology Inc. mRNA purification kit. Blot hybridization of RNA was carried out (Lehrman et al., 1987) with either single-stranded 32P-labeled M13 DNA probes (Church and Gilbert, 1984) or with double-stranded 32P-labeled probes primed with random hexanucleotides (Feinberg and Vogelstein, 1983).

**Cell Culture**—All cells were grown in monolayer at 37 °C in an atmosphere of 5–9% CO₂. Human embryonic kidney 293 cells were maintained in medium A (Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin). CHO-K1 cells and ldlA-7 cells were grown in medium B (a 1:1 mixture of Ham's F-12 medium and Dulbecco's modified Eagle's medium containing 5% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin). Met-18b-2 cells were grown in medium C (medium B supplemented with 20 μM compactin and 0.2 mM sodium mevalonate). Experiments were carried out with either medium D (medium A in which the fetal calf serum was dialyzed against 0.9% (w/v) NaCl prior to use) or medium E (medium B with dialyzed fetal calf serum).

**Construction of cDNA Expression Library—**Poly(A)⁺ RNA prepared from met-18b-2 cells was used to construct a size-selected, directional cDNA library with a kit purchased from Gibco-BRL (catalog number 82585A) with minor modifications. Poly(A)⁺ RNA (5 μg) was denatured with methanol/mericyclic hydroxide at room temperature prior to first strand synthesis. NotI-(dT)₅-primed cDNA was synthesized and ligated to SalI adapters according to the manufacturer's protocol. cDNAs greater than ~800 base pairs in length were isolated from an 0.8% (w/v) agarose gel by electroelution and purified by DNA-Blue Jolla (Bio101, Inc., Vista, CA) and Elutip minicolumns (Schleicher & Schuell) prior to ligation into the SalI-NotI sites of a plasmid expression vector pRc/CMV78 (see below). The ligation mixture was electroporated into Escherichia coli HB101 cells, yielding a cDNA library with greater than 1 × 10⁷ independent recombinants. Approximately 560 pools of 10 cells each (1 × 10⁹ independent plasmids per pool) were grown overnight in 50-ml cultures of LB medium. Plasmid DNA was purified using Qiagen-tip 100 columns (Qiagen, Inc., Chatsworth, CA) prior to transfection.
Plasmid pRC/CMV7S was constructed from pRC/CMVSeCAT (kindly provided by Mark Evans of this department) by excising a 1-kb DNA stuffer fragment (designated cCAT) with SalI and NotI in order to create the cloning site for the cDNA library. pRC/CMV7S contains the CMV promoter-regulatory region, bovine growth hormone signal (bGH S/G) and bovine papilloma virus E1 early region and SV40 origin of replication (neo) (see Fig. 1). The parent plasmid pRC/CMV7S-cCAT was created by ligating a 3-kb MluI-Sall fragment from Rc/CMV (Invitrogen) into the MluI-XhoI sites of pGEM7Zf+ (Promega) and then converting the sole HindIII site to a unique SalI site. A 1-kb stuffer fragment, designated cCAT, was inserted between the SalI and NotI sites. This fragment was inserted before insertion of the library, as described above.

Expression Cloning of pMev from Met-18b-2-derived mRNA—Pools of cDNAs in pRC/CMV7S were transfected into 293 cells, which were subsequently analyzed for uptake and metabolism of [3H]mevalonate in a transient expression assay. On day 0, replicate dishes of 2 × 10⁶ cells per 60-mm dish were plated in medium A. On day 1, duplicate dishes of cells were each transfected by the calcium phosphate method (Sambrook et al., 1989) with 5 μg of the cDNA plasmid pool plus 0.5 μg of pRNA, a plasmid encoding adenovirus VA RNA, which is believed to enhance translation (Akusjarvi et al., 1987). On days 2 and 4, the cells received fresh medium A and D, respectively. On day 5, the cells were assayed for [3H]mevalonate uptake as described above. Plasmid DNA from one positive pool of -100 independent transformants. Plasmids were isolated and sequenced by automated methods using an Applied Biosystems 373A DNA sequencer and the M13 universal and specific internal primers. The two other positive pools identified by Southern blotting were designated 370-64-26d and 370-64-31a. All subsequent studies were carried out with clone 370-64-26d, hereafter designated as pMev.

For monolayers transfected with the vast majority of plasmid cDNAs, the [H]/[C] ratio was 2 to 3. In a screen of ~10⁷ cDNAs each, three pools gave a [H]/[C] ratio of >7 and were considered to be positive. The DNA from one positive pool, number 370, was electroeluted into E. coli HB101 to generate multiple pools of ~10⁰ independent transformants. Plasmids were isolated and transfected into 293 cells, which were assayed for [3H]mevalonate uptake as described above. Plasmid DNA from one positive pool of 100 cDNAs was retransformed, and 144 colonies from this transformation were randomly picked and plated onto a 12 × 12 matrix. Bacterial cultures were prepared from pooled samples from each row and column of the matrix. Plasmids were isolated from these cultures and transfected into 293 cells, which were assayed for [3H]mevalonate incorporation into lipids. Two positive rows and columns were identified. Plasmids isolated from bacterial colonies at the intersection of the positive rows and columns of the original matrix were assayed individually for [3H]mevalonate incorporation into lipids. As expected, two of the four plasmids identified as positives were subsequently shown to have identical restriction maps. These cDNA clones were designated 370-64-26d and 370-64-31a. All subsequent studies were carried out with clone 370-64-26d, hereafter designated as pMev.

Stable Transfection of CHO Cells with pMev—CHO cells were seeded on day 0 at 3 × 10⁵ cells/100-mm dish in medium B. On the following day, they were transfected with 10 μg/dish of pMev that had been precipitated with calcium phosphate (van der Eb, 1980). The cells were incubated with DNA for 5 h in a 5% CO₂ incubator at 37°C and then reseeded with medium B. On day 2, the cells were fed medium D supplemented with 700 μM NaCl, 0.1 mM Hepes at pH 7.4, and 10 μM NaF and were maintained for 8 days after which G418-resistant colonies were isolated and analyzed for their ability to take up and metabolize [3H]mevalonate. One strongly positive colony was cloned by dilution plating, and one of its clones (designated as the TR-1875-1 cell line) was used for further studies.

cDNA Cloning of pMev-wt from CHO-derived mRNA—A double-stranded bacteriophage cDNA library was constructed from CHO cell poly(A)⁺ RNA as described above. cDNAs greater than 1 kb in length were ligated into the NotI-Sall arms of Agt22A. After in vitro packaging (GIBCO-BRL λ Packaging Kit), the phage were plated out on host strain E. coli C600 hisF. Approximately 4 × 10⁶ plaques were picked and the plaques were picked and the plaques were plated onto nitrocellulose filters and probed with uniformly ²⁵P-labeled random hexanucleotide-primed HindIII-BglII DNA fragment from pMev. One positive clone, designated pMev-wt, with an insert of ~3.5 kb was plaque-purified. Phage DNA was subcloned into pBlueScript SK± (Stratagene) and M13 for restriction mapping and sequencing, respectively. The entire coding region of pMev-wt was sequenced on one strand; the cDNA corresponding to amino acids 286-419 was sequenced on both strands. Another positive clone with an insert of ~2.2 kb was plaque-purified, subcloned into pBlueScript SK±, and determined to have the entire coding region by restriction mapping.

PCR Sequencing of Genomic DNA—A 338-base pair PCR product was obtained by amplifying genomic DNA from CHO cells, met-18b-2 cells, and Syrian hamster white blood cells with the following primers: oligo A, 5-AGTAATTTGTGAGCTCAGATT-3 and oligo B, 5-CAATGTTGACCAACCCACAGCA-3 corresponding to base pairs 1043-1067 in pMev; and oligo B, 5-CAATGTTGACCAACCCACAGCA-3 corresponding to base pairs 1357-1381 in pMev. The thermal profile used was 94°C for 1 min, 65°C for 1 min, then 72°C for 1 min for 35 cycles. The PCR-amplified DNA products were either used without gel purification (Syrian hamster DNA) or isolated from a 1.5% agarose gel (CHO and met-18b-2 cell DNA). All three samples were denatured and sonicated on a Cem100 microconicer (Microcon-100, Amersham). PCR products were sequenced on one strand. The sequencing conditions included 20 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min. The thermal PCR sequencing conditions included 20 cycles of 95°C for 30 s, 60°C for 30 s, 70°C for 30 s, followed by 10 cycles of 95°C for 1 min, 70°C for 1 min. An aliquot of each sequencing reaction was loaded on a 6% acrylamide denaturing gel and electrophoresed at 60 watts for 2 h at room temperature. The gels were dried and exposed to XAR-5 film for 2 h (Syrian hamster) or 10 h (CHO and met-18b-2).

Immunoblots Analysis—A polyclonal anti-peptide antibody directed against the COOH terminus of Mev (lgG-C811) was produced by immunizing rabbits with a synthetic peptide (EQISSGDPAAEESV (Peninsula Laboratories) corresponding to amino acids 481-494 (Fig. 3). The peptide was coupled to keyhole limpet hemocyanin using m-maleimidoxybenzoic acid N-hydroxysuccinimide ester (Sigma) (Harlow and Lane, 1988). IgG fractions were prepared from rabbit serum (preimmune or immune) by protein A agarose chromatography (Biesele et al., 1981). Proteins were transferred from 8% SDS-polyacrylamide gels to nitrocellulose membranes. The membranes were incubated in buffer A (35 mM Tris-HCl (pH 7.4), 0.5 mM sodium chloride, 10% (v/v) nonfat dried milk, and 0.2% (v/v) Tween 20) for 2 h at room temperature, after which the following sequential steps were undertaken: incubation with either preimmune or immune polyclonal antibody at 20 μg/ml in buffer A for 1 h at room temperature; two 15-min washes with vigorous shaking with buffer B (35 mM Tris-HCl (pH 7.4), 0.5 mM sodium chloride, 1% SDS, 1% Nonidet P-40, and 0.5% (v/v) sodium deoxycholate); incubation at room temperature with donkey anti-rabbit IgG-horseradish peroxidase conjugate secondary antibody (1:2000 dilution; Amersham. Catalog number RPN 2108) in buffer A supplemented with 1% Nonidet P-40; and two 15-min washes in buffer B. The membranes were then subjected to enhanced chemiluminescence detection according to the manufacturer’s protocol (Amersham) and exposed to Kodak XAR-5 film at room temperature for 75 s.

RESULTS

The strategy for the expression cloning of the cDNA responsible for mevalonate uptake in met-18b-2 cells is outlined in Fig. 1 and described in detail under “Experimental Procedures.” A size-fractionated cDNA library was prepared from met-18b-2 cells in an expression vector that uses the CMV promoter. Plasmids prepared from pooled cultures of 1000 bacterial transformants each were introduced into human kidney 293 cells by means of calcium phosphate-mediated transfection. After 4 days the 293 cells were assayed for mevalonate transport by incubation with a mixture of [³H]mevalonate and [¹⁴C]pyruvate. The cells were incubated with compactin to block endogenous mevalonate synthesis. After 60 min, an organic extract of the cells was prepared with

M. Evans, unpublished data.
brane-spanning regions are
plated 144 individual plasmids from subpool 370-64 on 12
Doolittle (1982) with a window of 9 residues showed that the 
preceded by three stop codons within
protein data banks failed to reveal any proteins with signifi-
cant sequence identities. We also found no evidence for the
consensus sequences for nucleotide binding, which are present
the 12th membrane-spanning region.

A total of 560 pools of 1000 transformants each were screened, and three pools gave a positive result as evidenced by a \(^3\)H/\(^4\)C ratio that was clearly above that produced by the other pools. Fig. 2A shows the results of one screening experi-
ment, conducted in duplicate, which showed a positive result for plasmid pool 370. For comparison, in each screening experiment we included dishes of 293 cells that had not been transfectioned or were transfected with control (salmon sperm) DNA. We also included dishes of met-18b-2 cells as a positive control for the assay.

From pool 370, we prepared 27 subpools of approximately 100 plasmids each, and two of these subpools gave positive results in the mevalonate uptake assay (Fig. 2B). We then plated 144 individual plasmids from subpool 370-64 on a 12 \( \times \) 12 matrix and assayed pools of plasmids from each row and column. Two of the rows gave positive results (Fig. 2C), as did two of the columns (not shown). The four plasmids at the intersections of the positive rows and columns were assayed individually, and two of these were positive, as expected (Fig. 2D). Restriction maps of these two plasmids were identical, and one of them (26d) was designated pMev and used for further study.

The sequence of the cDNA insert in pMev shows one long open reading frame that encodes a protein of 494 amino acids plus 5- and 3-untranslated regions of 194 and 1624 base pairs, respectively (Fig. 3). The putative initiator methionine is preceded by three stop codons within 50 base pairs, and it is placed within a good consensus for initiation of translation as defined by Kozak (1984).

Hydropathicity plots calculated by the method of Kyte and Doolittle (1982) with a window of 9 residues showed that the Mev protein has 12 relatively distinct hydrophobic segments of 19 to 28 residues each (Fig. 4). These 12 putative membrane-spanning regions are underlined in Fig. 3. The two longest stretches of hydrophilic residues are located between the 6th and 7th membrane-spanning regions and following the 12th membrane-spanning region. A search of several protein data banks failed to reveal any proteins with significant sequence identities. We also found no evidence for the consensus sequences for nucleotide binding, which are present in the cytoplasmic sequences of several transporters (Walker et al., 1982). The protein has two potential sites of \(N\)-linked glycosylation, which are boxed in Fig. 3.

To confirm that expression of pMev can lead to enhanced mevalonate uptake, we prepared a permanent line of CHO cells that were transfected with pMev, which is under control of the CMV promoter and contains in the same plasmid the neo gene that confers resistance to G418. Clones of cells that grew in G418 were analyzed for mevalonate uptake. To study mevalonate uptake in the absence of extensive metabolism, we performed the uptake studies in the presence of 6-fluoro-
mevalonate, which blocks the decarboxylation of mevalonate pyrophosphate and thus prevents the conversion of \[^3\]H\]merv-
uptake of [3H]mevalonate permits ready labeling of mevalon- 

calculated potential transmembrane regions are 

rate that was similar to the uptake rate in the met-

of the cDNA library are denoted by the 

18b-2 cells and much faster than the rate in CHO cells. At 

(3H]mevalonate in one of these clones as compared with CHO 

In cells expressing the Mev gene product, the efficient 

FIG. 3. Nucleotide and predicted amino acid sequences of pMev. Nucleotide residues are numbered on the right; amino acid residues are numbered on the left. Residue 1 is the putative initiator methionine. Two sites of potential N-linked glycosylation are boxed. Twelve potential transmembrane regions are underlined. Sequences of the 5'-SS adaptor and the 3'-Nol primer-adaptor used in the construction of the cDNA library are denoted by the double underline.

alinate into all products beyond mevalonate pyrophosphate (Nave et al., 1985). Fig. 5A shows a time course of uptake of [3H]mevalonate in one of these clones as compared with CHO cells and with the original met-18b-2 cells. Uptake was linear for 30 min. The transfected CHO cells had a mevalonate uptake rate that was similar to the rate in the met-18b-2 cells and much faster than the rate in CHO cells. At the 30-min time point, the uptake process in the transfected cells and the met-18b-2 cells was saturable (Fig. 5B), and the calculated \( K_m \) value for (R,S)-mevalonate was 1.5–2 mm in both cell types.

In cells expressing the Mev gene product, the efficient uptake of [3H]mevalonate permits ready labeling of mevalonate-derived proteins. Fig. 6 shows an experiment in which

parental CHO cells, met-18b-2 cells, and CHO cells transfected with pmMev were incubated with [3H]mevalonate, and detergent extracts were subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography for 48 h. In the detergent-soluble fraction of the met-18b-2 cells and the pmMev-transfected CHO cells, intense bands of radioactivity were seen in the position of 20–25 kDa, corresponding to Ras and p21. In parental CHO cells, met-18b-2 cells, and CHO cells transfected with pmMev, intense bands of radioactivity were seen in the position of 20-25 kDa, corresponding to Ras and related small GTP-binding proteins. In contrast, no radioactivity was seen in the extracts of parental CHO cells at 48 h (Fig. 6, lanes 1 and 4) and even after 7 days (not shown). On Northern blots of mRNA from met-18b-2 cells, radio-
the method of Kyte and Doolittle (1982) using the Genetics Computer Group Sequence Analysis Software Package, Version 7.1 (Devereux et al., 1984).

Fig. 4. Hydropathy plot of the amino acid sequence of Mev. Positive values represent increased hydrophobicity. The residue specific hydropathy index was calculated over a window of 9 residues by the method of Kyte and Doolittle (1982) using the Genetics Computer Group Software Package, Version 7.1 (Devereux et al., 1984).

Fig. 5. Uptake of [3H]mevalonate by monolayers of CHO cells (○), CHO cells transfected with pMev (□), and met-18b-2 cells (▲). On day 0, replicate dishes of 4×10^5 cells per 60-mm dish were plated in medium B and refed on day 2 with identical medium. On day 3, each monolayer was preincubated for 30 min in medium E containing 10 mM Hepes at pH 7.4, 50 mM compactin, and 0.5 mM fluoromevalonate, after which [3H]mevalonate (0.1 mM at 880 dpm/pmol; B) was added. After incubation at 37 °C for the indicated time (A) or 30 min (B), each monolayer was washed at 4 °C three times (5 min/wash) with 2 ml of buffer (50 mM Tris-HCl [pH 7.4], 155 mM NaCl, and 2 mg/ml bovine serum albumin), followed by three 5-min washes with 2 ml of buffer without albumin. Each monolayer was then incubated at room temperature for 30 min in 1 ml of 0.1 N NaOH, after which one aliquot of the cell suspension (0.1 ml) was counted in 10 ml of scintillation fluid, and another aliquot (50 μl) was used to measure the content of cellular protein (Lowry et al., 1951). Each value represents the average of duplicate incubations.

Fig. 6. Incorporation of [3H]mevalonate into cellular proteins in CHO cells (lanes 1 and 4), met-18b-2 cells (lanes 2 and 5), and CHO cells transfected with pMev (lanes 3 and 6). On day 0, 2.5×10^5 cells for each type were plated into a 60-mm dish in medium B and refed on day 2 with identical medium. On day 3, each monolayer was incubated at 37 °C with 1 ml of medium E containing 100 μM compactin for 1 h, followed by 1 ml of fresh medium E containing 100 μM compactin and 110 μCi/ml [3H]mevalonate (specific activity, 35 Ci/mmol). After incubation for 6 h, each cell monolayer was washed twice with 2 ml of phosphate-buffered saline, scraped into buffer containing 50 mM Tris-HCl (pH 8), 0.15 M NaCl, 0.5% (v/v) Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 10 μg/ml leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride. The cellular extract was centrifuged at 14,000 × g for 5 min at 4 °C. Aliquots of the detergent-soluble fraction (80 μg of protein) and insoluble fraction (80 μg of protein) were subjected to electrophoresis on a 14% gel and blotted onto a nylon membrane as described under "Experimental Procedures." The gel was impregnated with Intensify (Du Pont-New England Nuclear), dried, and exposed to Kodak XAR-5 film with an intensifying screen at -80 °C for 48 h. No radiolabeled bands were seen in lanes 1 and 4 after a 7-day exposure. [3H]Labeled molecular weight standards (Amersham) were run in an adjacent lane.

Fig. 7. Blot hybridization of Mev mRNA in CHO cells and met-18b-2 cells. An aliquot of poly(A)+ RNA (5 μg) from the indicated cell line was subject to electrophoresis on a 1.5% agarose gel and blotted onto a nylon membrane as described under "Experimental Procedures." The RNA was probed at 42 °C with a single-stranded, uniformly 32P-labeled M13 cDNA probe (3.5×10^6 cpm/ml) corresponding approximately to nucleotides 550–750 of pMev (Fig. 3). The filter was washed as described in Fig. 8 and exposed to Kodak XAR-5 film with an intensifying screen for 5 h at -80 °C. The same filter was reprobed with a 32P-labeled M13 probe to rat cyclophilin cDNA (5×10^4 cpm/ml), washed in 1× SSC with 0.1% SDS at 37 °C for 2 h, and exposed to Kodak XAR-5 film for 15 min at -80 °C. The blot is confirmed by SDS gel analysis of the translation product of Mev mRNA produced in a reticulocyte lysate system (data not shown). Furthermore, transfection of pMev into 293 cells (see below, Fig. 11) and simian COSm6a cells (data not shown) produced a 43-kDa protein by immunoblot analysis. The above data suggested that the protein produced by pMev in met-18b-2 cells had acquired a new property, namely,
the ability to cause enhanced uptake of mevalonate. To determine whether this new property was attributable to a mutation in the Mev gene, we isolated a plasmid containing the entire Mev sequence from a cDNA library that was constructed from RNA isolated from parental CHO cells. We designate this cDNA pMev-wt (for wild-type). The nucleotide sequence of the entire coding region of pMev-wt was identical to that of pMev from met-18b-2 cells except for one base pair position: an adenosine at position 1290 instead of the cytosine encoding the mutant protein.

Two independent clones from the CHO cDNA library. It was also confirmed by direct sequencing of an amplified copy of this region after reverse transcription of mRNA from CHO and met-18b-2 cells followed by PCR (data not shown).

To further confirm the existence of the mutation in the met-18b-2 cells, we determined the sequence of this region after PCR amplification of genomic DNA from parental CHO cells, fresh Syrian hamster leukocytes, and met-18b-2 cells (Fig. 10). The CHO and Syrian hamster DNA had a single nucleotide (adenosine) at nucleotide position 1290. In contrast, the met-18b-2 cell DNA was heterozygous at this position, showing roughly equal proportions of adenosine (encoding the wild-type protein) and cytosine (encoding the mutant protein).

Fig. 11 compares the incorporation of [3H]mevalonate into cellular lipids in 293 cells transfected transiently with pMev-wt and pMev. Both cDNAs produced comparable amounts of protein as detected by immunoblot analysis (Fig. 11, inset).
**DISCUSSION**

This paper reports the isolation of pMev, a cDNA whose expression leads to enhanced uptake of mevalonate in mammalian tissue culture cells. Mev, the protein encoded by pMev, contains 494 amino acids and is extremely hydrophobic. A hydropathy plot suggests that Mev is a membrane protein that contains 12 hydrophobic membrane-spanning regions separated by short hydrophilic loops ranging from 3 to 22 residues. The only exception is a 60-residue hydrophilic segment separating membrane spans 6 and 7, which divides the molecule in half. There is also a segment of 56 hydrophilic amino acids at the COOH terminus.

The structure of Mev, as predicted from the cDNA sequence, suggests that the protein functions directly as a mevalonate transporter. A similar membrane topology with 12 putative transmembrane segments has been modeled for other transport proteins, such as the glucose transporter (Mueckler et al., 1985); several members of the ATP-binding cassette (ABC) superfamily of transporters, such as the multidrug resistance protein (P-glycoprotein) (Gottesman and Pastan, 1988); and a family of transporters for neurotransmitters including dopamine, serotonin, norepinephrine, and γ-aminobutyric acid (Snyder, 1991). Like Mev, the glucose transporter and the P-glycoprotein each contain a large cytoplasmic hydrophilic loop that divides the multiple membrane-spanning domains into two symmetric halves. The hydrophilic loops in some transporters, such as the P-glycoprotein, contain ATP binding sites that are recognizable by certain amino acid motifs. Such motifs are absent in Mev, as they are in the glucose transporters and the neurotransmitter transporters. We cannot rule out the possibility that Mev is a regulator of some other protein that transports mevalonate, but this seems unlikely based on its predicted transporter-like structure.

When pMev is introduced into CHO cells by transfection, the cells acquire the characteristic properties of the met-18b-2 cells from which pMev was derived. They take up mevalonate rapidly by a saturable process, which suggests a carrier-mediated event (Fig. 5). The affinity for mevalonate is relatively low with $K_m$ values for (R,S)-mevalonate of 2 mM (for met-18b-2 cells) and 1.5 mM (CHO transfected with pMev) as measured in the current study. If the carrier is specific for the physiologic R form of mevalonate as previously suggested (Faust and Krieger, 1987), the affinity would be 2-fold higher than these measured values. The concentration of mevalonate in cells is much lower than this value, suggesting that the uptake mechanism is carrier-mediated facilitated transport rather than energy-dependent transport against an electrochemical gradient. It should be noted that the apparent $K_m$ value observed in the current study for met-18b-2 cells (2 mM) is several-fold higher than that reported by Faust and Krieger (1987). The explanation for this difference is unknown.

The linearity of mevalonate uptake with time up to 30 min is likely attributable to rapid phosphorylation by mevalonate kinase, which prevents back-diffusion (Fig. 5). We cannot rule out the possibility that the actual rate of mevalonate uptake is the same in control and Mev-expressing cells, but that the Mev-expressing cells retain more mevalonate owing to more rapid phosphorylation. Mev does not resemble mevalonate kinase structurally (Tanaka et al., 1990), but it might be a protein that regulates mevalonate kinase, thus allowing mevalonate to be retained. We have ruled out the possibility that Mev acts by enhancing any of the reactions distal to mevalonate kinase, because it enhances mevalonate uptake in cells in which the metabolism of mevalonate pyrophosphate is blocked with 6-fluoromevalonate (Fig. 5). Definitive demonstration that Mev is a mevalonate carrier will require studies of the ability of the isolated Mev protein to directly mediate transport in vitro.

A most interesting aspect of these studies is the observation that the gene encoding Mev arose from a pre-existing gene through a point mutation that allowed the encoded protein to gain a new function, namely, the enhancement of mevalonate uptake. Although such gain-of-function mutations are observed in invertebrates, they have been observed less frequently in mammalian cells. Perhaps the clearest parallel to the current results is the finding of Owen et al. (1983), who described a variant form of α₁-antitrypsin in which a single amino acid substitution converted the protein from its normal function as an inhibitor of elastase to that of an inhibitor of thrombin. The mutation occurred in a patient who had a bleeding disorder, owing to the altered protein. The notion that amino acid substitutions in the membrane-spanning regions of membrane transporters can alter their specificity for ligands is exemplified by studies of mutations in the P-glycoprotein (Gros et al., 1991; Devine et al., 1992).

Presumably the mutation in met-18b-2 cells occurred spontaneously, and this allowed the cells to survive the intense selection pressure of growth in MeLoCo medium (see the Introduction). Although Faust and Krieger (1987) transfected...
these cells with CHO DNA, there is no evidence that this procedure had any influence on the occurrence of the Mev mutation. The met-18b-2 cells show no signs of having taken up the cotransfected neo resistance gene marker (Faust and Krieger, 1987), and there is no overexpression of the Mev mRNA (Fig. 7). Moreover, the genomic sequencing experiment of Fig. 10 indicates that the met-18b-2 cells have one copy of the wild-type gene and one copy of the mutated Mev gene, which would be expected for a dominant gain-of-function mutation.

An important question relates to the normal function of the protein encoded by the wild-type Mev gene. As discussed above, the sequence suggests that it is a membrane transporter. It may be a transporter for mevalonate that is kept under regulation so that it is nonfunctional. In this case the Phe to Cys mutation at codon 360 may have allowed it to escape regulation. This explanation seems unlikely for several reasons: 1) we have been unable to observe rapid mevalonate uptake into CHO cells under any condition, even when the cells are starved for mevalonate by exposure to compactin, which would be expected to activate any regulated form of mevalonate uptake; 2) the affinity of Mev for mevalonate is much lower than the physiologic concentration of mevalonate in serum, which is about 50-100 nM (Parker et al., 1984), indicating that it would be an ineffective transporter in vivo; and 3) intravenously administered mevalonate is cleared primarily by the kidney (Hellstrom et al., 1973), whereas Mev mRNA is expressed most abundantly in heart as well as kidney (Fig. 8). It is possible that wild-type Mev is a high affinity, low capacity transporter for mevalonate and that the Phe to Cys substitution endows it with a lower affinity, but higher capacity. Further detailed studies of the properties of Mev-wt in vitro transport systems will be necessary to answer this question.

In view of these considerations, we favor the hypothesis that Mev-wt is normally a transporter for some other substance and that the substitution at codon 360 changed the specificity so that it can now bind and transport mevalonate. We do not know the putative normal substrate for Mev-wt, but it seems likely that it is an organic anion that is structurally related to mevalonate. It is possible that the mutation that allows mevalonate transport simultaneously abolishes transport of the normal substrate. Alternatively, Mev-wt could transport both substances. Many organic anions bear some superficial similarity to mevalonate. Krieger and Faust (1987) showed previously that the enhanced uptake of mevalonate in the met-18b-2 cells was relatively specific.

A clue to the normal function of Mev-wt may be provided by the tissue distribution of the mRNA. The mRNA is expressed at low levels in most tissues of the body, but in both the Syrian hamster and the rat the expression was highest in the heart. If Mev-wt is normally a transporter, it may carry a substance that is required for cardiac function. Further studies will be necessary to determine the nature of this substance.

The final aspect of these studies that merits discussion is the utility of pMev for studies of mevalonate metabolism in eukaryotic cells. So far, pMev has elicited enhanced mevalonate uptake in every cell type into which it has been introduced (CHO, simian COS cells, human embryonic kidney 293 cells, and mouse 3T3 cells). If this protein can function in other mammalian cells, especially in malignantly transformed cells, and if it can function in yeast, it should facilitate the further study of mevalonate metabolism, and particularly the role of prenylated proteins in cell division and cell biology.

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