The N-terminal Domain of Tomato 3-Hydroxy-3-methylglutaryl-CoA Reductases

SEQUENCE, MICROsomAL TARGETING, AND GLYCOSYLATION*

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The enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) catalyzes the conversion of 3-hydroxy-3-methylglutaryl-CoA to mevalonic acid, considered the rate-limiting step in isoprenoid biosynthesis. In plants, isoprenoid compounds play important roles in mediating plant growth and development, electron transport, photosynthesis, and disease resistance. Sequence comparisons of plant HMGR proteins with those from yeast and mammalian systems reveal high levels of sequence identity within the catalytic domain but significant divergence in the membrane domain. Mammalian HMGRs are integral membrane proteins of the endoplasmic reticulum with eight membrane-spanning regions. In contrast, the membrane domain of plant HMGRs is predicted to contain only one to two transmembrane spans. We have isolated and sequenced a clone (pCD4) encoding exon 1 of tomato hmg1. The membrane domain structures of two differentially regulated tomato HMGR isoforms, HMG1 and HMG2, were analyzed using in vitro transcription and translation systems. Microsomal membrane insertion of the tomato HMGRs is co-translational and does not involve cleavage of an N-terminal targeting peptide. HMGR membrane topography was established by protease protection studies of the HMG1 membrane domain and an analogous region of HMG2 engineered to contain a c-myc epitope tag. The data indicate that both tomato HMGRs span the membrane two times with both the C and N termini located in the cytosol. Luminal localization of the short peptide predicted to lie within the endoplasmic reticulum was further confirmed by in vitro glycosylation of an asparagine-linked glycosylation site present in HMG2.

The enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR, EC 1.1.1.34) catalyzes the synthesis of mevalonic acid, a major rate-limiting step in the isoprenoid biosynthetic pathway (1). This enzyme has been extensively studied in mammalian systems due to its critical role in mediating cholesterol biosynthesis. In mammals, HMGR is a 97-kDa transmembrane glycoprotein of the endoplasmic reticulum (ER), encoded by a single gene, and functions in the production of sterols, dolichol, prenyl modifications of proteins, steroid hormones, and several other non-sterol isoprenoid compounds (2). As shown in Fig. 1, plants synthesize a significantly greater and more diverse array of isoprenoids (in excess of 22,000 different compounds), which function in many aspects of plant growth, development, reproduction, and disease resistance (1, 3). Consistent with this complexity, plant HMGRs exist as multiple isoforms and are encoded by small gene families. Specific members of the HMGR gene families are differentially expressed during development or in response to environmental factors, and distinct HMGR isoforms may be critical in directing the flux of pathway intermediates into specific isoprenoid compounds (Refs. 3–6 and references therein). Although synthesis of many of these compounds occurs in the cytosol, several classes of isoprenoids are synthesized in plastids (e.g. carotenoids, plastoquinone, phylloquinone), mitochondria (e.g. ubiquinone), or specialized vesicles (e.g. rubber). HMGR activity has been associated with mitochondria and chloroplasts in addition to microsomes although the organellar location of plant HMGRs remains controversial (1).

The domain structure of plant, animal, and fungal HMGRs can be roughly divided into three regions: the C-terminal catalytic domain, the N-terminal transmembrane domain, and a divergent linker or hinge region. The catalytic domain, consisting of about 400 amino acids at the C terminus of the protein, is highly conserved between organisms (7–10). The N-terminal membrane domain shows greater divergence between organisms and is significantly smaller in plant species compared with yeast or animal HMGRs (10, 11). In mammalian systems, the N-terminal domain of HMGR contains a highly hydrophobic region of about 340 amino acids, which lacks a typical N-terminal signal peptide or signal peptide cleavage site. This domain is necessary and sufficient to confer localization to ER (microsomal) membranes (12–14) and has been implicated in the rapid degradation of HMGR triggered by increases in serum cholesterol or low density lipoproteins (15, 16). Initial models proposed for mammalian HMGR secondary structure and membrane orientation, based on limited proteolysis and hydrophathy plot analyses, suggested a transmembrane domain of seven membrane-spanning regions (9). More recent evidence using specific antibodies raised against synthetic peptides from the HMGR membrane domain supports a model with eight membrane-spanning regions and both the C and N termini localized on the cytosolic face (13, 17). The HMGR membrane domains of yeast, Drosophila, sea urchin, and Xenopus show a similar transmembrane organization of 7–8 membrane spans based on hydrophathy and sequence comparisons (7, 8, 18, 19). In contrast, the N-terminal hydrophobic domain of plant HMGRs is less than 120 amino acids and has been predicted to...
Fig. 1. A simplified biosynthetic pathway for plant isoprenoids highlighting important end products. PP, pyrophosphate; PGR, plant growth regulator.

contain one (20, 21) or two (11, 22) transmembrane spans. The significance of this reduced membrane domain is unknown although plants presumably lack the requirement for rapid down-regulation of sterol biosynthesis since no dietary source is utilized.

Because the membrane domain of plant HMGRs differs so markedly from that of animal and yeast HMGRs, we are interested in experimentally determining the transmembrane structure of plant HMGRs and the role of this simplified domain in subcellular localization. As a first step toward this goal, we initiated experiments to analyze microsomal targeting and transmembrane orientation of two distinct tomato HMGR isoforms. Tomato contains at least 4 genes encoding HMGR (20, 22). The current study uses HMG1 and HMG2 N-terminal peptides generated from the differentially regulated tomato HMGR isoforms, hmg1 and hmg2. Tomato hmg1 expression is elevated in rapidly growing cells consistent with a role in sterol biosynthesis (23, 24). In contrast, hmg2 is induced in response to pathogens and wounding associated with host defense responses (4, 22, 25). Defense-related induction is correlated with increases in microsomal HMGR activity and production of iso-prenoid phytoalexin antibiotics (6, 26–28).

EXPERIMENTAL PROCEDURES

cDNA Amplification and Cloning—Tomato hmg1 sequences encoding the putative membrane domain were generated by reverse transcription PCR using DNA synthesized from poly(A)- RNA (29, 30) from immature tomato fruit (2–5 mm, Lycopersicon esculentum cultivar cv.PT) using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) and oligo(dT) primers (Promega Corp., Madison, WI). A partial hmg1 cDNA was amplified via the polymerase chain reaction (PCR; 40 cycles: 95°C, 1 min; 58°C, 2 min; 72°C, 3 min) using hmg1-specific primers 14 (5'-GGGCTGGCTGTTCCATCCATTGCA-3') and 15 (5'-GAGAAGAATACTCATGTGTAAAC-3') that spanned the translation start site in intron I, and part of exon II, was inserted into the Smal site of pBluescript SK- to yield plasmid pXY12. A c-myc epitope (31) was incorporated into a BglII site of pXY12 just downstream of the translation start site. The resulting plasmid, pXY12(c-myc), encodes a polypeptide (HMG2(myc)) that begins MDVRRSE-OKLIEEDGLSEE... (c-myc epitope is underlined).

In vitro Transcription—RNA was transcribed in vitro from plasmids pCD4A and pXY12(c-myc) following the manufacturer’s protocols (Promega). Plasmid pCD4A (5 µg) was linearized with EcoRI and transcribed using SP6 RNA polymerase in the presence of the RNA cap analog P-5'-[7-(7-methyl)-guanosine-P-5'-guanosine triphosphate (Boehringer Mannheim). The plasmid pXY12(c-myc) (5 µg) was linearized with SstI and transcribed using T7 RNA polymerase. The resulting RNAs were resuspended in 20 µl of water, and 1-µl aliquots were used for in vitro translation.

In vitro Translation, Microsomal Insertion, and Protease Protection Analyses—Translation of in vitro synthesized RNA was carried out using rabbit reticulocyte lysates or a wheat germ translation system in the presence or absence of dog pancreatic microsomal membranes according to the manufacturer’s specifications (Promega). Proteins were synthesized in the presence of [35S]methionine (32) or non-radioactively by substituting L-methionine. Proteins translated in the presence of microsomes were fractionated into membrane-bound and soluble fractions; the microsomes from five translations were combined, pelleted by ultracentrifugation for 1 h at 100,000 × g at 4°C (Beckman SW50), and resuspended in 20 µl of buffer (50 mM HEPES, 1 mM dithiothreitol, 20% glycerol, 1 mM EDTA, 2 mM CaCl2). To confirm membrane insertion, translation mix (10 µl) was treated with a high salt wash (50 mM HEPES, pH 7.6, 500 mM potassium acetate, 10 mM EDTA, 8% sucrose) on ice for 10 min andrecentrifuged (32). For protease protection studies, 10 µl of translation mix were incubated with 1 µl of proteinase K (10 µg/ml); reactions were stopped by adding 5 µl of 0.5 M phenylmethylsulfonyl fluoride (Sigma) in ethanolo (32). For experiments designed to assess the presence of N-linked glycans, 10 µl of the protease-protected microsomal fragments were treated with 0.1 µl of Endo H (Boehringer Mannheim) and 1% Triton X-100 (Sigma) overnight at 37°C.

Translation products, including those subsequently fractionated or protease-treated, were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (33). Ten µl of the original volume of the translation mixture (25 µl) were added to 20 µl of gel loading buffer (10% v/v glycerol, 0.01% bromphenol blue, 1% SDS, 1% [γ-32P]ATP (34). The HMG1 sequences show 74.8% nucleic acid sequence identity and 79.6% amino acid identity (87.4% similar-
ity (35)) with the analogous region of tomato HMG2 previously isolated in this laboratory (22). The N-terminal sequences of tomato HMG1 and HMG2 are compared with other plant HMGRs in Fig. 2A. Although the N-terminal 200 amino acids are relatively divergent compared with the C-terminal catalytic domain (4), several regions of high sequence conservation are evident. The first six amino acids are quite conserved and are high in basic amino acids that may represent an ER retention signal (41). The most highly conserved region (that which aligns with tomato HMG2 residues 44–120) contains two hydrophobic segments (Fig. 2B), each of which extends over 20 amino acids, a distance sufficient to span a membrane bilayer. The regions preceding the first hydrophobic sequences and those linking these second hydrophobic sequences to the catalytic domains are variable both in amino acid sequence and in length. In contrast, the hydrophilic region between the two putative transmembrane spans is highly conserved among plant HMGRs. Interestingly, this conserved region, which is predicted to lie within the ER lumen, varies with respect to a potential asparagine-linked glycosylation site (N-X-S/T; Fig. 2A).

Insertion of HMG1 and HMG2 into Microsomal Membranes—In order to demonstrate microsomal targeting of tomato HMG1 and HMG2, labeled peptides were synthesized in vitro. For both HMGRs, regions encoded by exon I and utilizing in-frame stop codons provided by intron I sequences yielded N-terminal peptides comprising about 60% of full-length HMGR. Microsomal targeting, integral membrane association, and N-terminal signal peptide cleavage were assessed by comparing HMGR peptides in the presence or absence of microsomes and/or following proteinase treatment. The hydropathy data (Fig. 2) were used to predict the sizes of proteinase K-insensitive peptides of HMG1 and HMG2 based on one versus two transmembrane spans (Fig. 3).

The sizes of HMG1 and HMG2 N-terminal peptides resulting from rabbit reticulocyte lysate-mediated translation of in vitro synthesized mRNAs (Fig. 4) were consistent with those predicted from the derived amino acid sequences (37.5 kDa for HMG1- and 39.3 kDa for HMG2-exon I-encoded products). In vitro translation was enhanced in the presence of microsomes (Fig. 4, lanes 3). The peptides synthesized in the presence of microsomes were the same size as those synthesized without microsomes (Fig. 4, lanes 2 and 3), suggesting either that microsomal insertion was unsuccessful or that no signal peptide was cleaved during synthesis and insertion. That HMGR peptides were in fact integrally associated with microsomal membranes was demonstrated by co-sedimentation of labeled HMGR peptides with microsomal membranes upon re-isolation by centrifugation (Fig. 4, lanes 4). Membrane insertion was confirmed by the inability of a high salt wash to remove the protein from the microsomal pellet (data not shown) and the detection of protease-insensitive fragments (Fig. 4, lanes 6). Proteinase K treatment of peptides translated in the absence of microsomes resulted in complete loss of the protein (Fig. 4, lanes 5), whereas small protected fragments were evident fol-
and HMG2 was estimated at 6–8 kDa (Fig. 4, model B). These data are consistent with co-translational, signal recognition particle-dependent insertion (data not shown). These data are consistent with predictions based on two transmembrane spans linked by a short lumenal domain as shown in model A of Fig. 3.

In vitro synthesized mRNA encoding HMG1 and HMG2 peptides were translated in the presence of [35S]methionine. Translation was carried out in the presence of dog pancreatic microsomes (M) and/or followed by treatment with protease K (P) as indicated prior to separation by SDS-PAGE. The positions of molecular mass markers are indicated. Bands corresponding to HMGR products and protected fragments (PF) are indicated by arrows. Lane 1, no RNA control; lane 2, translation of mRNA in absence of microsomes; lane 3, translation of mRNA in the presence of microsomes; lane 4, microsomal pellet; lane 5, translation in absence of microsomes and followed by protease K treatment; lane 6, translation in the presence of microsomes and followed by protease K treatment.

Following digestion of peptides synthesized in the presence of microsomes (Fig. 4, lanes 6). The protease-insensitive fragments were not detected if protease treatment was carried out in the presence of 1% Triton X-100, which should liberate the protease-sensitive fragments. The protease-insensitive fragments had not run off the gel. Additional gels (not shown) were run to ensure that protease-insensitive bands had not run off the gel.

Fig. 3. Sizes of transmembrane and lumenal peptides predicted to be protected from proteinase K digestion, comparing models having one versus two membrane-spanning regions and maintaining the location of the C terminus (catalytic domain in the full-length HMGR) in the cytosol.

Microsomal Targeting of Plant HMGR

In vitro synthesized mRNA encoding HMG1 and HMG2-myc peptides were translated in the presence of dog pancreatic microsomes. Microsomal translation products were separated by centrifugation, treated with protease K as indicated, and detected by Western immunoblotting using c-myc monoclonal antibody. Protein standards (Amersham Rainbow protein markers) are indicated. Lanes 1 and 2 contained untagged HMG1 microsomal pellets, plus or minus protease K, as controls. Lane 3 contained the HMG2-myc microsomal pellet (cross-reactive band of expected size denoted by arrow). Lane 4 contained the HMG2-myc microsomal pellet treated with protease K showing loss of the cross-reacting material. Additional gels (not shown) were run to ensure that protease-protected fragments had not run off the gel.

HMG2 were reproducibly smaller than that predicted for a single transmembrane span (Fig. 3, model B). These data are consistent with predictions based on two transmembrane spans linked by a short lumenal domain as shown in model A of Fig. 3.

Localization of the N Terminus to the Cytosol—To further confirm that the N terminus is cytosolic, the sequence for a c-myc epitope was engineered into the hmg2 sequence immediately after the region encoding the first six amino acids. Western blot analysis of the in vitro translated HMG2-c-myc construct using an anti-c-myc monoclonal antibody showed detection of a full-length product (39 kDa) (Fig. 5, lane 3). All cross-reactivity was lost upon protease K treatment of the microsomal pellet (Fig. 5, lane 4), indicating that the c-myc epitope is exposed on the cytosolic face.

Tomato HMG2 is Glycosylated in Vitro—The membrane orientation of tomato HMGR1 and HMGR2 suggested by the experiments above positions a small stretch of 14–22 amino acids within the ER lumen. This region of HMGR2 contains a potential asparagine-linked glycosylation site that is absent in HMGR1 (see Fig. 2A). Since addition of N-linked glycans is an ER-localized event, evidence for glycosylation of the microsomal fragment of HMG2 would further support this model. The protease K-protected fragments of tomato HMGR1 and HMGR2 were treated with Endo H, an enzyme that cleaves N-linked glycans of the high-mannose type (42). Treatment of the HMG2 protease-protected fragment with Endo H resulted in a mobility shift as seen on SDS-PAGE (Fig. 6). As a control, the analogous HMG1 fragment, which lacks a glycosylation site, showed no mobility shift following Endo H treatment. This indicated that the glycosylation site of tomato HMG2 is utilized in our in vitro systems and suggests that this region is indeed localized within the ER lumen.

DISCUSSION

In mammals, HMGR is an integral membrane glycoprotein localized to the endoplasmic reticulum and is encoded by a single gene (2, 9, 14). In contrast, plant HMGRs are encoded by small gene families, and HMGR enzyme activity has been associated with plastid, mitochondrial, and ER membranes. We have shown that two tomato HMGR isoforms that are differentially regulated during development and in response to stress are both targeted to microsomal membranes in vitro.
Attempts to demonstrate post-translational insertion of tomato HMGR peptides into intact chloroplasts were unsuccessful (data not shown). Our results are in agreement with those of Enjuto et al. (5) in which they reported that both Arabidopsis HMGR isoforms are co-translationally targeted to microsomal membranes. These findings do not preclude the possibility that other plant HMGR isoforms may be targeted to plastids or mitochondria, although it is unlikely that those HMGRs that are highly conserved in the membrane-spanning domain (e.g., those listed in Fig. 2) are targeted to these organelles.

Mechanisms mediating ER targeting and insertion of integral membrane proteins of the endomembrane system have been reviewed (42, 43). For mammalian HMGR, the N-terminal 39 residues, which includes the first transmembrane domain, function as a permanent uncleaved signal for targeting mammalian HMGR to the ER membrane (12, 13). Orientation of the protein within the ER membrane is determined by a total of eight transmembrane spans such that both N and C termini are cytosolic (13, 17). Based on our results we propose a similar model for the membrane topology of plant HMGRs localized to the endomembrane system. Tomato HMG1 and HMG2 appear to be targeted to and co-translationally inserted into the ER by a permanent, uncleaved signal. The proteins span the membrane twice such that both the N and C termini are located in the cytoplasm and thus are classified as group III transmembrane proteins (transmembrane protein classifications as reviewed in Refs. 43 and 44). Three direct experimental approaches were used to assess orientation within the membrane.

First, tomato HMGR peptides, synthesized and inserted into microsomes in vitro, were treated with protease K. Those amino acids embedded in the membrane or within the lumen are protected from proteolysis. The sizes of the protected fragments for both HMG1 and HMG2 are consistent with digestion of the C termini of the N termini and protection of peptides consisting of two hydrophobic helices and the hydrophilic residues in between (e.g. residues 48–117 of tomato HMG2; see Fig. 4).

Second, cytosolic localization of the N terminus of tomato HMG2 was demonstrated by proteinase K digestion of a c-myc epitope engineered between residues 6 and 7. In addition, immunodetection of the c-myc epitope in the full-length product further confirms that no signal peptide is cleaved during microsome-associated translation. Finally, our experiments indicate that an asparagine within the predicted lumenal domain of tomato HMG2 is glycosylated. The HMG1 sequence used for these experiments contains no N-linked glycosylation sites. The tomato HMG2 peptide contains two potential sites, one within the stretch of 14–22 amino acids predicted to be within the ER lumen and one just downstream of the second transmembrane span and predicted to be cytosolic. Our proteinase K digestion experiments indicate that this second site is cytosolic and thus should not be glycosylated and would be absent from the peptides used for Endo H treatment. As predicted by our model, treatment of protease-protected fragments of in vitro synthesized and microsomal targeted HMGRs with Endo H resulted in a shift of molecular weight of HMG2 but not HMG1.

We find it intriguing that only a subset of plant HMGRs has an N-linked glycosylation site within the lumenal segment (see Fig. 2A). Of plant HMGRs currently in the data bases, those HMGRs associated with constitutive expression, rapid growth involving membrane biogenesis, or showing activity levels coordinated with squalene synthase activity (sterol branch (3, 6)) (e.g. tomato HMG1, potato HMG1, Arabidopsis HMG1, Hevea HMG3) lack this glycosylation site. All HMGRs in dicots that have expression patterns associated with production of defense compounds or other secondary products such as rubber or coordinated with expression of sesquiterpene cyclase (key branch pathway enzyme for phytoalexin synthesis in the Solanaceae) contain the glycosylation site (e.g. tomato HMG2, Hevea HMG1, the Nicotiana sylvestris and Catharanthus roseus HMGRs). The one exception is an elicitor-inducible rice HMGR gene, the first HMGR membrane domain sequence reported for a monocot species, which is highly divergent in the transmembrane and lumenal region and lacks the lumenal glycosylation site (45). In the dicot systems, both defense responses and rubber biosynthesis are associated with specific endomembrane differentiation (39, 46). Although N-linked glycans have not been shown to function as sorting signals in plants (42, 47), the conservation of the glycosylation site in a specific and highly specialized subset of plant HMGRs suggests that the glycans may play an important biological role. We are currently designing experiments to differentially tag tomato HMG1 and HMG2 to address whether these two isoforms localize to distinct compartments within the plant endomembrane system. Future experiments will address the importance of HMGR isoform-specific expression, subcellular localization, and protein–protein interaction in directing the production of specific isoprenoid compounds and thus mediating key physiological functions involving plant isoprenoids.

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Note Added in Proof—During review of this article, related work analyzing the membrane topology of Arabidopsis HMGR was reported (Campos, H., and Boronat, A. (1995) Plant Cell, 7, 2163–2174).

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