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Targeting and Topology in the Membrane of Plant 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase

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The enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) catalyzes the synthesis of mevalonate. This is the first committed step of isoprenoid biosynthesis. A common feature of all known plant HMGR isoforms is the presence of two highly conserved hydrophobic sequences in the N-terminal quarter of the protein. Using an in vitro system, we showed that the two hydrophobic sequences of Arabidopsis HMGR1S function as internal signal sequences. Specific recognition of these sequences by the signal recognition particle mediates the targeting of the protein to microsomes derived from the endoplasmic reticulum. Arabidopsis HMGR is inserted into the microsomal membrane, and the two hydrophobic sequences become membrane-spanning segments. The N-terminal end and the C-terminal catalytic domain of Arabidopsis HMGR are positioned on the cytosolic side of the membrane, whereas only a short hydrophilic sequence is exposed to the lumen. Our results suggest that the plant HMGR isoforms known to date are primarily targeted to the endoplasmic reticulum and have the same topology in the membrane. This reinforces the hypothesis that mevalonate is synthesized only in the cytosol. The possibility that plant HMGRs might be located in different regions of the endomembrane system is discussed.

INTRODUCTION

Higher plants have developed a complex multibranched metabolic pathway for isoprenoid biosynthesis (Gray, 1987; Chappell, 1995b; McGarvey and Croteau, 1995). The first committed step of the pathway is the synthesis of mevalonic acid, which is catalyzed by the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR; EC 1.1.1.34). The subsequent reactions allow the synthesis of isopentenyl diphosphate (IPP). This metabolite may be viewed as a basic building block in the biosynthetic pathway. A series of prenyl transfer reactions, involving IPP and other prenyl diphosphates, mediates the synthesis of prenyl diphosphates of increasing size, which are the starting points for the multiple ramifications that lead to the final isoprenoid products. This biosynthetic strategy allows the synthesis of an astonishing variety of plant isoprenoids that play many different roles (Conolly and Hill, 1992; Chappell, 1995b; McGarvey and Croteau, 1995). The isoprenoid pathway is fundamental in plants not only because it provides compounds that are essential for particular functions (for example, carotenoids and the side chain of chlorophylls for photosynthesis, the side chain of ubiquinone for respiration, sterols for membrane architecture, and phytoalexins for defense), but also because some of the products synthesized (gibberellins, abscisic acid, and the side chain of cytokinins) play a role in the control of various physiological processes and contribute to the establishment of the developmental pattern of the plant.

Despite the importance of isoprenoid biosynthesis for plant growth and development, some essential aspects, mainly concerning the subcellular location and the control of the pathway, still await clarification. A major point of controversy is the subcellular location of IPP biosynthesis. At least three subcellular compartments, the cytosol, plastids, and mitochondria, are involved in the synthesis of isoprenoid compounds. T.W. Goodwin's group proposed that the synthesis of IPP occurs in these three compartments (Rogers et al., 1966). This proposal is known as the segregation hypothesis. On the other hand, H. Kleinig's group suggested that IPP is synthesized exclusively in the cytosol and that this compound is subsequently translocated to the organelles to allow the synthesis of the specific isoprenoid end products (Kreuz and Kleinig, 1984; Lutkebrinkhaus et al., 1984). Evidence in favor of or against these two hypotheses has been discussed in recent reviews (Gray, 1987; Kleinig, 1989; Bach et al., 1990). The observation that the competence for IPP biosynthesis changes during chloroplast development in barley is interesting and might be a clue to reconciling the two opposite theories (Heintze et al., 1990).

The study of plant HMGR is particularly valuable in this context and has received considerable attention. After subcellular fractionation, HMGR activity has been detected in membrane fractions derived from the endoplasmic reticulum (ER), in plastids, and in mitochondria (for review, see Bach et al., 1990). In addition, it has been observed in different plant systems

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that treatment with mevinolin, a specific inhibitor of HMGR activity, prevents sterol biosynthesis but has very little effect on the synthesis of plastidic isoprenoids (Bach and Lichtenthaler, 1983). These results have been considered as evidence in favor of the segregation hypothesis (Bach et al., 1990). However, it has been argued that the cell fractionation studies cited above are not conclusive and that the results of inhibition with mevinolin cannot be interpreted properly until precise knowledge of the enzymatic activities and the flow of metabolites through the pathway is available (Gray, 1987).

In all plant species studied so far, HMGR is encoded by a multigene family. The number of genes composing each multigene family varies in different plants (Stermer et al., 1994; Chappell, 1995b). The HMGR mRNAs of a given species differ in their expression pattern, indicating that the encoded enzymes might be involved in the synthesis of specific isoprenoids (Dean et al., 1991; Narita et al., 1991; Yang et al., 1991; Choi et al., 1992, 1994; Chye et al., 1992; Cramer et al., 1993; Enjuto et al., 1994, 1995; Lumbreras et al., 1995). It has been suggested that the functional diversity of these enzymes could correlate with differences in subcellular location (Choi et al., 1992; Chappell, 1995a, 1995b). Of particular interest are the studies carried out with Arabidopsis. In this plant, two differentially expressed genes (*HMG1* and *HMG2*) encode three different HMGR isoforms: HMGR1S, HMGR1L, and HMGR2. The HMGR1S and HMGR1L isoforms derive from the *HMG1* gene (Lumbreras et al., 1995). These proteins are identical in sequence, but HMGR1L is extended at its N terminus by an additional region of 50 amino acids. Arabidopsis HMGR2 shows a remarkable divergence in sequence with regard to Arabidopsis HMGR1S and to the other plant HMGRs characterized so far (Enjuto et al., 1994). In spite of the differences observed, the three Arabidopsis HMGR isoforms insert into ER-derived membranes (Enjuto et al., 1994; Lumbreras et al. 1995).

Comparative studies have shown that all plant HMGR isoforms known to date have a similar structural organization, reflecting a common evolutionary origin. Four regions have been defined in the primary structure of this protein: the N-terminal region, the membrane domain, the linker region, and the catalytic domain (Figure 1; Monfar et al., 1990). The membrane and the catalytic domains are well conserved among plant HMGRs, whereas the N-terminal and the linker regions are highly divergent both in length and in amino acid sequence.

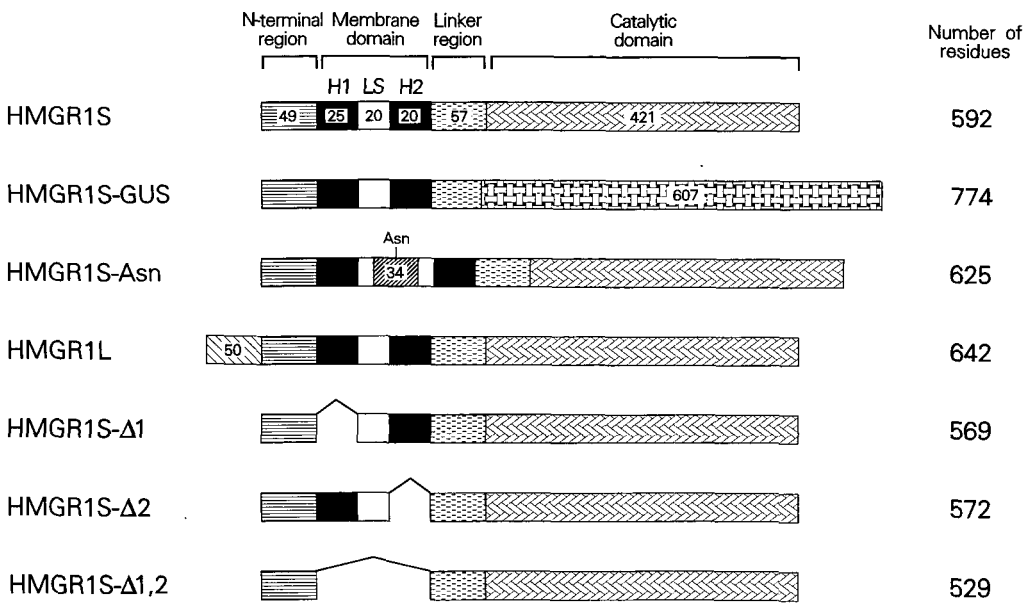

Figure 1. Schematic Representation of the Proteins Synthesized in the in Vitro Translation Experiments.

The four regions defined in the primary structure of plant HMGR are indicated on the top. The H1, H2, and LS sequences of the membrane domain are also depicted. The numbers inside the boxes representing the proteins correspond to the number of amino acid residues of the different sequences. HMGR1S is the short Arabidopsis HMGR isoform encoded by the *HMG1* gene (Caelles et al., 1989); HMGR1S-GUS is the chimeric protein resulting from the fusion of the N-terminal part of HMGR1S (residues 1 to 167) and GUS (Jefferson et al., 1987); HMGR1S-Asn is the derivative of HMGR1S carrying an N-glycosylation site in the LS sequence; HMGR1L is the long Arabidopsis HMGR isoform encoded by the *HMG1* gene (Lumbreras et al., 1995). HMGR1L is identical to HMGR1S, but it is extended at the N terminus by an additional region of 50 amino acids. HMGR1S-Δ1 is the mutated variant of HMGR1S in which the first hydrophobic sequence (H1, residues 52 to 74) has been deleted; HMGR1S-Δ2 is the mutated variant of HMGR1S in which the second hydrophobic sequence (H2, residues 95 to 114) has been deleted; and HMGR1S-Δ1,2 is the mutated variant of HMGR1S in which the whole membrane domain (H1, LS, and H2 sequences, residues 52 to 114) has been deleted.

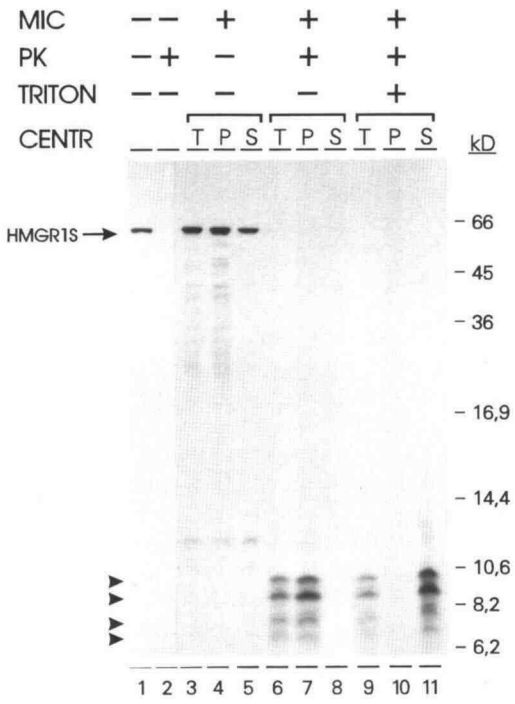

Figure 2. Analysis of Arabidopsis HMGR1S Inserted in Microsomal Membranes.

In vitro–synthesized mRNA coding for HMGR1S was translated using L-3,4,5(n)-^{3}H-leucine as labeled precursor. Translations were performed in the presence (+) or absence (–) of ER-derived microsomes from dog pancreas (MIC) as indicated on the top. Post-translational treatments of the samples were performed in the following order: (1) digestion with proteinase K (PK); (2) disruption of the microsomes with 1% Triton X-100 (TRITON); and (3) fractionation by centrifugation at 51,700*g* (CENTR). Before the addition of Triton X-100, proteinase K was inactivated by adding phenylmethylsulfonyl fluoride. Samples were separated in an SDS–16% polyacrylamide gel (Schaegger and von Jagow, 1987). The fluorographed gel was exposed for 14 hr. Bands corresponding to the protein fragments generated by digestion with proteinase K are indicated by arrowheads. The positions of the molecular mass markers are indicated at right. +, sample subjected to the indicated treatment; –, sample not subjected to the indicated treatment; T, total translation products; P, pellet; and S, supernatant.

The membrane domain is characterized by the presence of two hydrophobic sequences separated by a short hydrophilic one. It has been suggested that this hydrophobic domain might be involved in anchoring the protein to cellular membranes (Caelles et al., 1989; Learned and Fink, 1989). Accordingly, this region was named the membrane domain (Monfar et al., 1990). In particular, it was proposed that Arabidopsis HMGR1S might span cellular membranes once (Learned and Fink, 1989) or twice (Caelles et al., 1989). In spite of these considerations, no direct demonstration of a structural or functional involvement of the membrane domain in the insertion of the protein into membranes has been reported so far. In this work, we define the topology of plant HMGR in the membrane. Our data show that the two hydrophobic sequences of Arabidopsis HMGR1S are membrane-spanning segments. In addition, we demonstrate that these sequences function as internal signal sequences. They interact specifically with the signal recognition particle (SRP) and mediate the targeting of the protein to ER-derived membranes. Because these topogenic sequences are well conserved in all plant HMGR isoforms known to date, our results suggest that these proteins are primarily targeted to the ER and have the same topology in the membrane.

RESULTS

Catalytic Domain of Arabidopsis HMGR1S Is Positioned on the Cytosolic Side of ER-Derived Membranes

To study the topology of plant HMGR in the membrane, we used an in vitro system in which the appropriate transcripts were translated in the presence of ER-derived microsomes from dog pancreas. As a first approach, translation products derived from the Arabidopsis HMGR1S transcript were digested with proteinase K and/or fractionated by centrifugation. The results are shown in Figure 2. As expected from previous results (Enjuto et al., 1994), Arabidopsis HMRG1S was inserted into microsomal membranes. This protein cosedimented with the vesicles and was found in the pellet after centrifugation (lane 4). However, no processing of HMGR1S occurred upon insertion, as indicated by the coincidence in electrophoretic mobility of the protein synthesized in the absence of microsomes (lane 1) and the protein inserted in the microsomal membrane (lane 4). Post-translational digestion with proteinase K yielded no protected fragment if translation had been performed in the absence of microsomes (lane 2) and two major fragments of 9 to 10 kD and a few smaller fragments if translation had been performed in the presence of microsomes (lane 6). This result indicates that one or several small regions of the inserted protein were buried in the lipid bilayer or exposed to the lumen of the vesicles. To confirm that the protected fragments were bound to the membranes, we fractionated the digested sample by centrifugation. As expected, the protected fragments sedimented with the microsomes (compare lanes 7 and 8), but if the microsomal membranes were disrupted with Triton X-100 before centrifugation, they were found in the supernatant (compare lanes 10 and 11).

The appearance of several protected fragments in the digestion assays shown in Figure 2 might be due to the insertion of separate regions of HMGR1S in the membrane. Alternatively, the protected fragments might derive from a single inserted sequence if this region had been digested at several preferential sites by proteinase K. To confirm that none of the protected fragments was derived from the C-terminal catalytic domain of HMGR1S, we prepared an expression plasmid encoding the chimeric protein HMGR1S-GUS, in which the catalytic domain

of HMGR1S (residues 168 to 592) was replaced by β-glucuronidase (GUS; Figure 1). The results shown in Figure 3A demonstrate that the HMGR1S-GUS protein synthesized in vitro inserts into the microsomal membrane and behaves as an integral membrane protein. Post-translational isolation of the microsomes, followed by extraction of the lumenal content with Na_2CO_3, pH 11.0, and subsequent centrifugation, showed that this protein was tightly associated with the pelleted open membrane fragments (lane 4). In the same conditions, the processed IgG light chain, which is known to translocate into the lumen of the microsomes, was released and recovered as a soluble protein (lane 3). It can be concluded that the N-terminal part of Arabidopsis HMGR1S (residues 1 to 167) is sufficient for insertion into the microsomal membrane. When the HMGR1S and the HMGR1S-GUS proteins synthesized in the presence of microsomes were digested with proteinase K, the same pattern of protected fragments was obtained (compare lanes 2 and 4 of Figure 3B). This indicates that the protected peptides derived exclusively from sequences located in the N-terminal part of the proteins and that the corresponding C-terminal catalytic domains were fully exposed to the cytosolic side of the membrane.

Arabidopsis HMGR1S Spans the Microsomal Membrane Twice

Three different arrangements of HMGR1S in the membrane could be proposed, considering the presence of two hydrophobic segments in the N-terminal part of the sequence (Figure 1) and the cytosolic location of the catalytic domain. Arabidopsis HMGR1S might span the lipid bilayer once or twice. If the protein spans the membrane once, either the first (H1) or the second (H2) hydrophobic sequence might be the membrane-spanning segment.

To discern whether the hydrophilic region between H1 and H2 is translocated to the lumen or remains on the cytosolic side of the membrane, we engineered a high-mannose–type glycosylation site in this region. A sequence coding for the glycosylation site of the maize Zm-ERabp1 protein, together with 30 surrounding amino acids, was inserted in the HMGR1S cDNA (see Methods for details). The resulting protein was named HMGR1S-Asn (Figure 1). As shown in Figure 4A, a single translation product (Hm) was generated from the HMGR1S-Asn transcript in the absence of microsomes (lane 1). In the presence of microsomes, an additional protein with

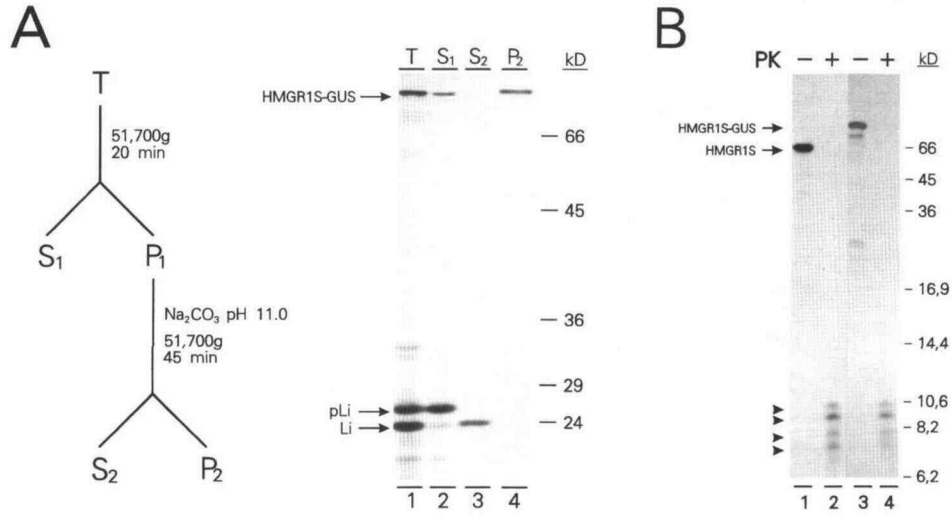

Figure 3. Cytosolic Location of the Catalytic Domain of Arabidopsis HMGR1S.

(A) In vitro–synthesized mRNA coding for HMGR1S-GUS was translated in the presence of ER-derived microsomes from dog pancreas, using L-^{35}S-methionine as the labeled precursor. The system was also supplemented with control mRNA coding for the IgG light chain. Translation products were fractionated by two sequential centrifugations as indicated in the diagram (see Methods for details). Samples were separated in an SDS–10% polyacrylamide gel (Laemmli, 1970). The fluorographed gel was exposed for 8 hr. T, total translation products; S_1, supernatant of the first centrifugation; P_1, pellet of the first centrifugation; S_2, supernatant of the second centrifugation; P_2, pellet of the second centrifugation.
(B) In vitro–synthesized mRNAs coding for HMGR1S (lanes 1 and 2) and HMGR1S-GUS (lanes 3 and 4) were translated in the presence of ER-derived microsomes from dog pancreas using L-3,4,5(n)-^{3}H-leucine as labeled precursor. Post-translational treatment with proteinase K (PK) was performed as indicated: +, sample treated; −, sample not treated. Samples were separated in an SDS–15% polyacrylamide gel (Schaegger and von Jagow, 1987). The fluorographed gel was exposed for 18 hr. The positions of molecular mass markers are indicated on the right. Bands corresponding to HMGR1S, HMGR1S-GUS, the processed IgG light chain (Li), and the unprocessed IgG light chain (pLi) are indicated. Bands corresponding to the protein fragments generated by digestion with proteinase K are indicated by arrowheads.

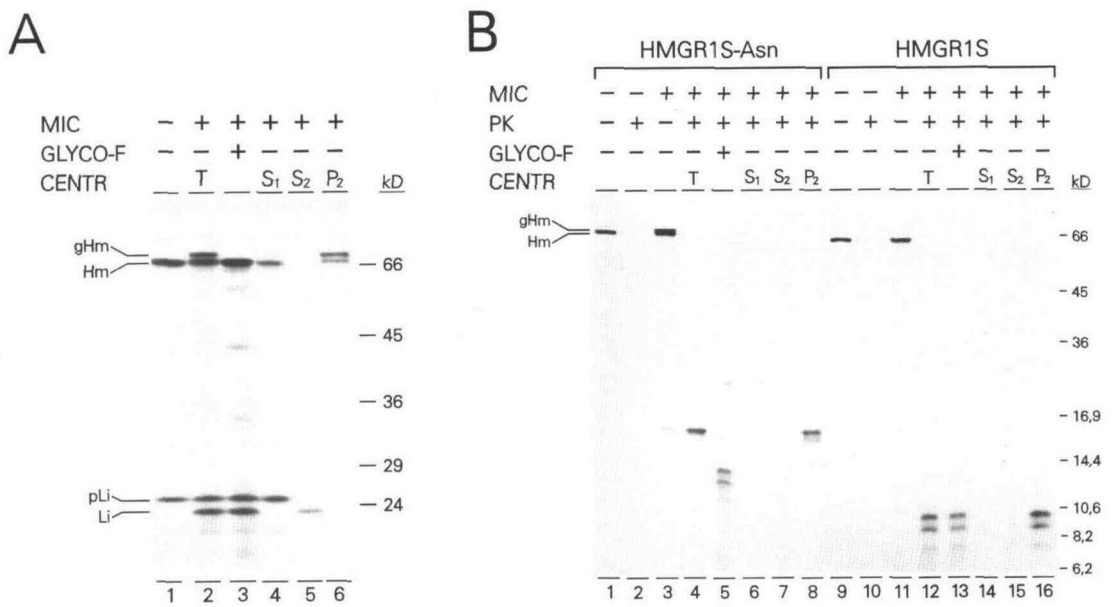

Figure 4. Lumenal Location of the LS Sequence of Arabidopsis HMGR1S.

(A) In vitro–synthesized mRNA coding for HMGR1S-Asn was translated in the presence (+) or absence (–) of ER-derived microsomes from dog pancreas (MIC), using L-^{35}S-methionine as the labeled precursor. The translation mixture was also supplemented with mRNA coding for the IgG light chain. Post-translational treatments were performed in the following order: (1) digestion with glycosidase F (GLYCO-F), and (2) fractionation by two sequential centrifugations (CENTR) as depicted in Figure 3A. Samples were separated in an SDS–10 to 15% gradient polyacrylamide gel (Laemmli, 1970). The fluorographed gel was exposed for 7 hr.

(B) In vitro–synthesized mRNAs coding for HMGR1S and HMGR1S-Asn were translated in the presence (+) or absence (–) of ER-derived microsomes from dog pancreas (MIC), using L-3,4,5(n)-^{3}H-leucine as labeled precursor. Post-translational treatments were performed in the following order: (1) digestion with proteinase K (PK); (2) digestion with glycosidase F (GLYCO-F); and (3) fractionation by two sequential centrifugations (CENTR) as depicted in Figure 3A. Samples were separated in an SDS–14.4% polyacrylamide gel (Schaegger and von Jagow, 1987). The fluorographed gel was exposed for 22 hr.

The abbreviations used to refer to the post-translational treatments are as follows: +, sample subjected to the indicated treatment; –, sample not subjected to the indicated treatment. The T, S_1, S_2, and P_2 abbreviations are explained in the legend to Figure 3A. The positions of the molecular mass markers are indicated on the right of **(A)** and **(B)**. Bands corresponding to the unglycosylated HMGR1S-Asn (Hm), the glycosylated HMGR1S-Asn (gHm), the processed IgG light chain (Li), and the unprocessed IgG light chain (pLi) are indicated.

slightly lower electrophoretic mobility (gHm) was synthesized (lane 2). The gHm protein was converted to the Hm protein by glycosidase F treatment (compare lanes 2 and 3). This demonstrates that gHm was the glycosylated form of HMGR1S-Asn. When a sample equivalent to the one loaded on lane 2 was subjected to fractionation by centrifugation and Na_2CO_3 treatment, the glycosylated protein (gHm) was found in the P_2 fraction (lane 6). Neither form of IgG light chain (processed, Li, or unprocessed, pLi) was affected during glycosidase F treatment, and both fractionated correctly upon centrifugation (lanes 2 to 5). It can be concluded that the HMGR1S-Asn protein was inserted into the microsomal membrane and glycosylated.

To confirm that the region between the two hydrophobic sequences of the inserted HMGR1S-Asn protein was located in the lumenal side of the membrane, we performed post-translational assays of digestion with proteinase K. The results are shown in Figure 4B. When translation of the HMGR1S-Asn transcript was performed in the absence of microsomes, no peptide was protected against proteinase K digestion (lane 2). When translation of the HMGR1S-Asn transcript was performed in the presence of microsomes, the treatment with proteinase K yielded a protected fragment of ~16 kD (lane 4). Treatment with glycosidase F after digestion with proteinase K yielded two fragments of ~12 and 13 kD (lane 5). The removal of one oligosaccharide side chain from a protein backbone causes a decrease of ~3 kD in the apparent molecular mass of the peptide (Lipp et al., 1989). This is consistent with the appearance of a 13-kD peptide in the sample of lane 5. The additional 12-kD fragment might have been generated by partial proteolysis occurring during glycosidase F treatment. The deglycosylated fragments derived from the HMGR1S-Asn protein (lane 5) were ~3.5 kD larger than the protected fragments derived from the HMGR1S protein (lane 12), as might be expected, considering the differences between the corresponding

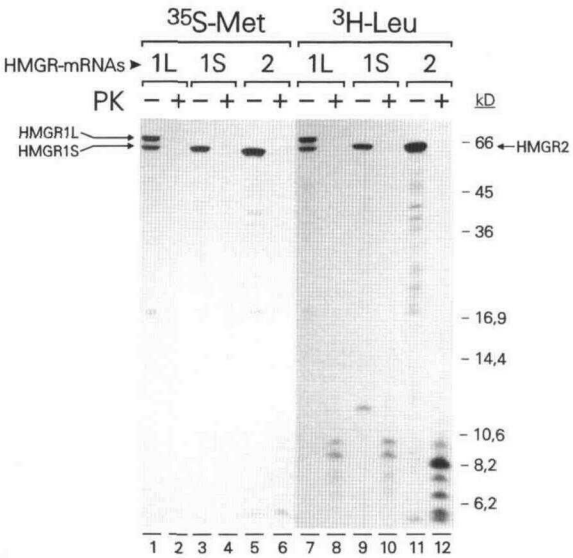

Figure 5. Cytosolic Location of the N-Terminal Region of Arabidopsis HMGR Isoforms.

In vitro–synthesized mRNAs coding for Arabidopsis HMGR1S, HMGR1L, and HMGR2 were translated in the presence of ER-derived microsomes from dog pancreas, using L-^{35}S-methionine (lanes 1 to 6) or L-3,4,5(n)-^{3}H-leucine (lanes 7 to 12) as the labeled precursor. As expected from previous results (Lumbreras et al., 1995), two different translation products, corresponding to HMGR1S and HMGR1L, were generated from the HMGR1L transcript in the in vitro system. Digestion with proteinase K (PK) was performed post-translationally as indicated: +, sample treated; −, sample not treated. Samples were separated in an SDS–15% polyacrylamide gel (Schaegger and von Jagow, 1987). Time of exposure of the fluorographed gel was 4 hr for lanes 1 to 6 and 13 hr for lanes 7 to 12. The positions of the molecular mass markers are indicated at right. The transcripts used are indicated on the top: 1L, HMGR1L mRNA; 1S, HMGR1S mRNA; and 2, HMGR2 mRNA. Bands corresponding to the proteins synthesized are indicated at left and right.

sequences. The glycosylated protected fragment derived from the HMGR1S-Asn protein (lane 4) was tightly bound to the membrane, as shown by centrifugation and Na_2CO_3 treatment (lanes 6 to 8). It can be concluded that the hydrophilic region located between the two hydrophobic sequences was positioned on the lumenal side of the microsomal membrane, upon insertion of the protein. Thereafter, this hydrophilic region was named LS or lumenal sequence.

To determine whether the N-terminal region of plant HMGR was positioned on the lumenal or the cytosolic side of the membrane, we synthesized Arabidopsis HMGR1S, HMGR1L, and HMGR2 in vitro, in the presence of microsomes, and compared the electrophoretic patterns of the protected peptides obtained by digestion with proteinase K. The results are shown in Figure 5. When the translation assays were performed in the presence of L-^{35}S-methionine, the three Arabidopsis HMGR isoforms were labeled (lanes 1, 3, and 5), but no labeled fragment was protected from digestion (lanes 2, 4, and 6). The membrane domain of the Arabidopsis HMGR isoforms contains no methionine, and the N-terminal region of these proteins contains one (HMGR1S and HMGR2) or two (HMGR1L) methionines. The absence of labeling in the digestion products, even after long exposures, suggested that the N-terminal region of the HMGR proteins was positioned on the cytosolic side of the membrane. When in vitro translation was performed in the presence of L-3,4,5(n)-^{3}H-leucine as labeled precursor, detectable digestion products were derived from the three proteins (Figure 5, lanes 8, 10, and 12). This confirmed that the absence of digestion products labeled with L-^{35}S-methionine in the previous experimental conditions was not due to the absence of protected fragments but to the absence of labeling in the protected fragments.

The coincidence of the patterns of protected fragments derived from HMGR1S and HMGR1L (compare lanes 8 and 10) is additional evidence of the cytosolic location of the N-terminal region of these proteins. As mentioned above, HMGR1S and HMGR1L are identical in sequence, but the HMGR1L isoform is extended at the N terminus by an additional region of 50 amino acids. If the N-terminal regions of these proteins were positioned on the lumenal side of the membrane, clear differences in the size of the protected fragments (~5.7 kD) would have been observed. The pattern of protected fragments derived from HMGR2 is different from the pattern of protected fragments derived from HMGR1S or HMGR1L (compare lane 12 with lanes 8 and 10), as might be expected considering the differences between the protein sequences.

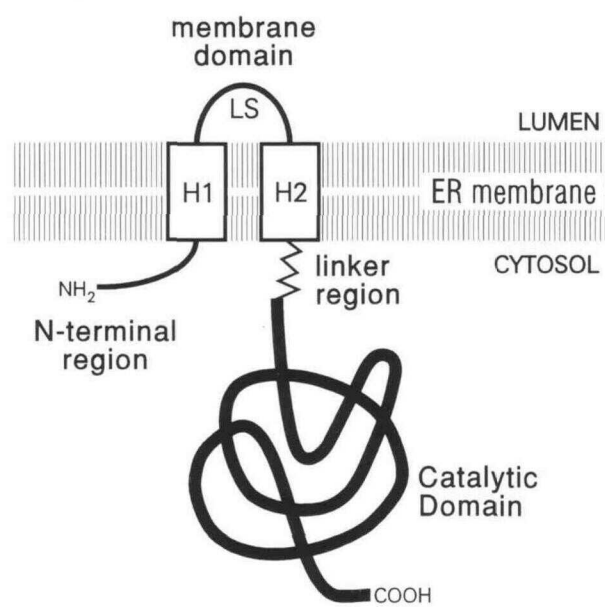

Figure 6. Topological Model Proposed for Plant HMGR in the ER Membrane.

For simplicity, the different regions of HMGR are not drawn to scale. H1 and H2, highly conserved membrane-spanning sequences; LS, highly conserved lumenal sequence.

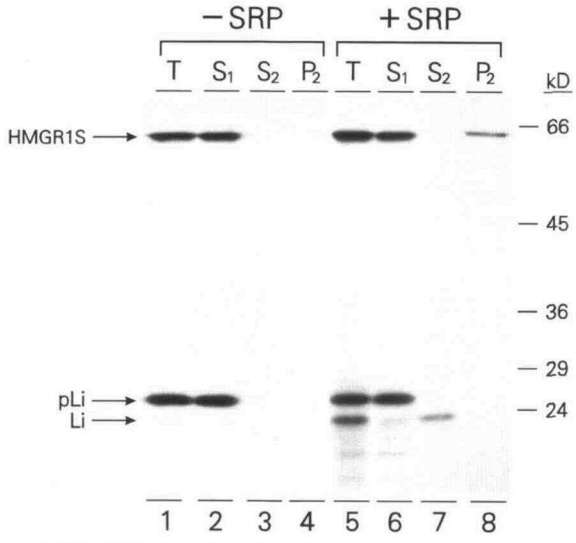

Figure 7. SRP-Mediated Insertion of Arabidopsis HMGR1S in the Membrane of ER-Derived Microsomes.

In vitro–synthesized mRNA coding for HMGR1S was translated in the presence of ER-derived microsomes from dog pancreas, using L-^{35}S-methionine as labeled precursor. Canine SRP was present (+SRP) or absent (−SRP) in the translation mixture, as indicated at the top. The system was also supplemented with control mRNA coding for the IgG light chain. Translation products were fractionated by two sequential centrifugations, as depicted in Figure 3A. The nomenclature of the fractions obtained, T, S_1, S_2, and P_2, is defined in the legend of Figure 3A. Samples were separated in an SDS–12.5% polyacrylamide gel (Laemmli, 1970). The fluorographed gel was exposed for 9 hr. The positions of molecular mass markers are indicated at right. Bands corresponding to HMGR1S, the processed IgG light (Li) chain, and the unprocessed IgG light chain (pLi) are indicated.

Only one of the above-mentioned topological models is consistent with the data presented. This model is shown in Figure 6. Plant HMGR spans the microsomal membrane twice. The N-terminal region and the catalytic domain are located on the cytosolic side of the membrane. The hydrophilic LS sequence is the only part of the protein exposed to the lumen.

Targeting of Arabidopsis HMGR1S to the ER-Derived Membranes Is Mediated by the Specific Interaction of SRP with the H1 and H2 Hydrophobic Sequences

According to the above-mentioned results, the H1 and H2 sequences of Arabidopsis HMGR are membrane-spanning segments. We asked whether the H1 and H2 sequences are not only structurally but functionally involved in the insertion process. In particular, we attempted to determine if these hydrophobic regions are signal sequences that mediate the insertion of the protein in the membrane. Targeting of proteins to the ER is mediated by the SRP (Walter and Johnson, 1994). The SRP recognizes the hydrophobic N-terminal or internal signal sequences at the time they emerge from the ribosome and transfers these sequences to the translocation machinery in the membrane, which initiates the translocation process (Gilmore, 1993). During this process, the internal signal sequences and the stop transfer signals are transferred to the lipid bilayer in a definite orientation and become membrane-spanning segments (Wickner and Lodish, 1985). In vitro, the specific interaction of SRP with signal sequences can be detected in two ways. In the presence of ER-derived membranes, SRP mediates translocation or insertion of the synthesized proteins. In the absence of ER-derived microsomes, SRP specifically inhibits the synthesis of proteins that contain one or several signal sequences (Walter and Johnson, 1994).

As a first step toward studying the role of H1 and H2 sequences in the insertion of Arabidopsis HMGR, we analyzed the insertion process to determine if it is SRP dependent. Transcripts coding for Arabidopsis HMGR1S and for murine IgG light chain, used as a control, were translated in the presence of SRP-depleted microsomes and either in the presence or absence of SRP. Post-translationally, the samples were fractionated by centrifugation, and the microsomes present in the sediment were extracted with Na_2CO_3, pH 11.0. The results are shown in Figure 7. Arabidopsis HMGR1S was recovered in the fraction containing the open microsomal membranes only when translation was performed in the presence of SRP (lanes 4 and 8). As expected, translocation and processing of the control protein occurred only in the presence of SRP (lanes 5 to 7). It can be concluded that SRP is required for insertion of Arabidopsis HMGR1S in the microsomal membrane.

To ascertain whether SRP initiates the insertion process upon interaction with H1 or H2, we prepared expression plasmids encoding deleted versions of the HMGR1S protein. The H1 sequence was missing in the HMGR1S-Δ1 protein, the H2 sequence was missing in the HMGR1S-Δ2 protein, and the whole membrane domain (H1, LS, and H2 sequences) was missing in the HMGR1S-Δ1,2 protein (Figure 1). In vitro translation experiments showed that none of the modified proteins was inserted into ER-derived microsomal membranes (data not shown). It is clear from these results that both hydrophobic sequences are required for the insertion process. However, the evidence obtained does not determine which hydrophobic sequence is recognized by SRP, as was originally intended. In an alternative approach, we performed in vitro translation experiments in the presence of several concentrations of SRP and in the absence of microsomal membranes. The results are shown in Figure 8. The presence of SRP inhibited the synthesis of HMGR1S, which was used as a reference in this experiment. This inhibitory effect was proportional to the SRP concentration. SRP also inhibited the synthesis of the HMGR1S-Δ1 protein, in which only the second hydrophobic sequence was present. The same effect was observed for the HMGR1S-Δ2 protein that contained only the first hydrophobic sequence. However, SRP had no effect on the synthesis of the HMGR1S-Δ1,2 protein, in which both hydrophobic sequences were missing. These results demonstrate that the two

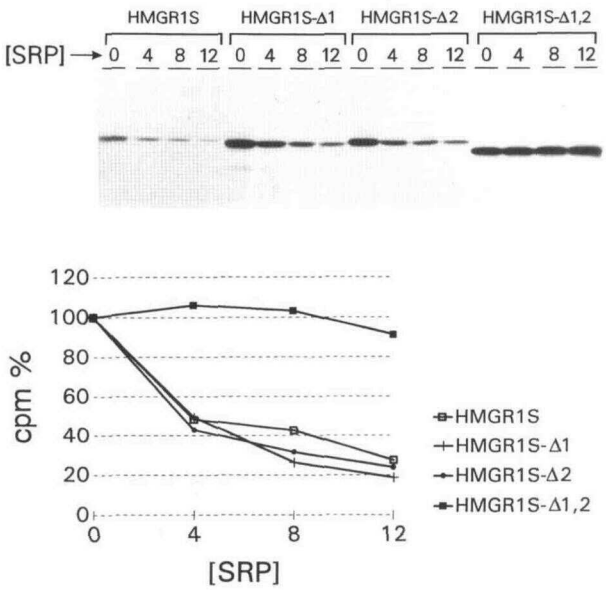

Figure 8. Effect of SRP on the Synthesis of the HMGR1S-Deleted Derivatives.

In vitro–synthesized mRNAs coding for HMGR1S, HMGR1S-Δ1, HMGR1S-Δ2, and HMGR1S-Δ1,2 were translated in the absence of microsomes, using L-^{35}S-methionine as the labeled precursor. The concentration of canine SRP present in the translation mixtures is indicated in arbitrary units above the gel. Samples were separated in an SDS–12.5% polyacrylamide gel (Laemmli, 1970). The fluorographed gel was exposed for 2 hr. Protein bands were excised from the gel, and the radioactivity incorporated was quantified as described in Methods. The radioactive incorporation corresponding to each protein is represented against the SRP concentration. The incorporation values are expressed as percentages of the incorporation obtained in the absence of SRP. The radioactive incorporations obtained in the absence of SRP were as follows: HMGR1S, 30,663 counts per min (cpm); HMGR1S-Δ1, 107,164 cpm; HMGR1S-Δ2, 79,081 cpm; and HMGR1S-Δ1,2, 87,751 cpm.

hydrophobic sequences, H1 and H2, can interact with SRP and indicate that no other functional signal sequence is present in this protein. We conclude that the H1 and H2 sequences of Arabidopsis HMGR1S play a key role both in targeting the protein to the ER-derived microsomes and in anchoring this polypeptide in the membrane.

DISCUSSION

One of the major open questions concerning isoprenoid biosynthesis in plants is the subcellular location of the enzymes involved in the synthesis of IPP. Unraveling this problem is fundamental if we are to understand the organization and control of the isoprenoid pathway. The subcellular location of HMGR is particularly significant in this context because this enzyme catalyzes the synthesis of mevalonic acid, the first committed precursor of isoprenoids. In the present study, we focused on two particular aspects of this issue: the targeting of plant HMGR and the topology of this protein in the membrane.

Our data show that the region of Arabidopsis HMGR1S extending from residues 1 to 167 is sufficient for insertion into microsomal membranes of dog pancreas. The targeting to the ER-derived membranes is mediated by the specific interaction of SRP with the two hydrophobic sequences present in this region (H1 and H2). No processing of the protein is produced upon insertion. After insertion, Arabidopsis HMGR1S spans the membrane twice. The catalytic domain and the N-terminal region are positioned on the cytosolic side of the membrane, whereas only the short hydrophilic region (LS) located between the two hydrophobic sequences is exposed to the lumen (Figure 6). It can be concluded that H1 and H2 of Arabidopsis HMGR1S function as internal signal sequences and topogenic sequences in the in vitro system. Because the insertion of proteins in the ER membrane requires the specific interaction of the targeting sequences with different components of the translocation machinery (Rapoport, 1992), and because this machinery is highly conserved in evolution (Prehn et al., 1987; Ng and Walter, 1994; Wolin, 1994), our results strongly suggest that Arabidopsis HMGR1S is primarily targeted to and inserted into the ER membrane in vivo.

A large number of full-length sequences corresponding to HMGR isoforms from different plant species have been reported: three from Arabidopsis (Enjuto et al., 1994; Lumbreras et al., 1995), two from radish (Vollack et al., 1994) and *Hevea brasiliensis* (Chye et al., 1991, 1992), and one from potato (Choi et al., 1992), tomato (Park et al., 1992), tobacco (Genschik et al., 1992), periwinkle (Maldonado-Mendoza et al., 1992), *Camptotheca acuminata* (Burnett et al., 1993), pea (Monfar, 1990), and rice (Nelson et al., 1994). All of these HMGR isoforms have a similar structural organization. In particular, all known plant HMGRs possess the structural motif formed by two highly conserved hydrophobic sequences (H1 and H2) separated by a highly conserved hydrophilic one (LS). As mentioned above, in Arabidopsis HMGR1S, this motif is sufficient for targeting to ER-derived microsomes and insertion into the membrane. Therefore, our results suggest that the plant HMGR isoforms known to date are competent for insertion into microsomal membranes and have the same topology in the membrane. This has been confirmed experimentally in the case of the three Arabidopsis HMGR isoforms (HMGR1S, HMGR1L, and HMGR2), tomato HMGR1 and HMGR2 (Denbow et al., 1995), and pea HMGR1 (N. Campos and A. Boronat, unpublished results). These HMGR isoforms insert into ER-derived microsomal membranes. In all cases, the results obtained after post-translational analysis of the inserted protein agree with the topological model shown in Figure 6. It is worth noting that pea HMGR1 has the most divergent hydrophobic domain among the known HMGR isoforms from dicotyledonous plants (M. Monfar, N. Campos, and A. Boronat, unpublished results). The finding that the catalytic domain of plant HMGR is positioned on the cytosolic side of ER-derived membranes is

consistent with the observation that the cytosol is the major subcellular location for plant HMGR activity (Gray, 1987; Bach et al., 1990).

No transit peptide typical of proteins targeted to chloroplasts or mitochondria (von Heijne, 1986; Baker and Schatz, 1987; von Heijne et al., 1989) has been identified by inspection of the plant HMGR sequences. Taking into account this observation and the above-mentioned results, targeting the known plant HMGR isoforms to plastids or mitochondria seems unlikely. The work carried out in Arabidopsis is particularly suggestive. The fact that the three Arabidopsis HMGR isoforms insert into ER-derived microsomal membranes reinforces the hypothesis that the ER is the only cell compartment for the primary targeting of Arabidopsis HMGR (Enjuto et al., 1994; Lumbreras et al., 1995). In principle, this kind of reasoning cannot be applied to other plant species, because not all the corresponding HMGR genes have been characterized. Nevertheless, the cumulative evidence obtained in different plant systems also points to the hypothesis that the ER is the major, or perhaps the only, primary targeting site for plant HMGR. Despite the large number of plant HMGR isoforms characterized so far, the diversity of physiological conditions in which mRNAs have been isolated for cDNA preparation, the variety of expression patterns of the corresponding genes, and the functional specialization indicated for the HMGRs of some species, all the known plant HMGR isoforms possess the highly conserved H1/LS/H2 motif, which suggests targeting to the ER. However, at present, the possibility that plant HMGR is targeted to plastids or mitochondria, either primarily or after insertion in the ER membrane, cannot be ruled out.

Another interesting possibility is the transport of plant HMGR down the endomembrane system. The inspection of the N-terminal sequence of plant HMGRs is very suggestive in this respect. Most of the known plant HMGR isoforms share the conserved motif MetAspXArgArgArg (X can be Val, Ile, Leu, or Ser) at their N-terminal end. The conserved sequence is located in an otherwise highly divergent region, suggesting that it has a definite function. A recent report shows that a motif consisting of a pair of arginine residues located at positions 2 and 3, 3 and 4, 4 and 5, 2 and 4, or 3 and 5 of the N-terminal end is sufficient for the retention of type II membrane proteins in the ER (Schutze et al., 1994). The conserved N-terminal sequence of plant HMGRs fits this consensus and is positioned on the cytosolic side of ER-derived membranes, which is the location expected for this kind of retention signal (Schutze et al., 1994). It is tempting to speculate whether the MetAspXArgArgArg N-terminal sequence might be involved in retaining plant HMGR in the ER. Other plant HMGR isoforms with different N-terminal sequences might have alternative locations in the endomembrane system. Physical segregation of different HMGR isoforms in the ER membrane and cytosolic subcompartmentalization of mevalonate production have been proposed in yeast (Wright et al., 1988; Casey et al., 1992). More recently, these have also been suggested in plants (Chappell, 1995a, 1995b). Subcompartmentalization of plant HMGR in the endomembrane system appears to be a very exciting hypothesis. Confirmation or refutation of this hypothesis would certainly provide important clues to understanding the organization of the isoprenoid biosynthetic pathway in plant cells.

METHODS

Enzymes and Chemicals

Restriction enzymes, the Klenow fragment of DNA polymerase I, T4 DNA ligase, and Taq DNA polymerase were obtained from Promega. Deoxynucleotides, dideoxynucleotides, proteinase K, and glycosidase F from *Flavobacterium meningosepticum* were supplied by Boehringer Mannheim. The *Escherichia coli* RNA polymerase and the T7 DNA polymerase were purchased from Pharmacia. All enzymes were used as indicated by the manufacturer. Primers used for DNA sequencing and for polymerase chain reaction (PCR) were synthesized by Oligos Etc Inc. (Wilsonville, OR). ^{35}S-dATPαS and L-^{35}S-methionine were provided by Amersham International. L-3,4,5(n)-^{3}H-leucine was obtained from DuPont–New England Nuclear.

DNA Constructs

The DNA coding sequences used in the transcription-translation experiments (Figure 1) were cloned in the expression plasmid pDS6 (Bujard et al., 1987). The pDS6-derived plasmids coding for the Arabidopsis HMGR1S, HMGR1L, and HMGR2 and for the chimeric protein HMGR1S-GUS were constructed by conventional subcloning procedures (Ausubel et al., 1989; Sambrook et al., 1989). The detailed description of the strategy and the steps involved in the generation of these plasmids are available on request. The methods used to prepare the other pDS6-derived plasmids are described below. Mutations generated in the DNA constructs were confirmed by sequencing using the dideoxy chain termination method (Sanger et al., 1977).

HMGR1S-Asn

To introduce a high-mannose–type glycosylation site in the Arabidopsis HMGR1S protein, the corresponding cDNA (Caelles et al., 1989), cloned in a pBluescript KS+ plasmid, was modified by site-directed mutagenesis (Kunkel et al., 1987). A unique MluI restriction site was generated at positions 253 to 258 of this cDNA using the primer 5′-TTACAATACGCGTCTTCACG-3′. The DNA fragment coding for the glycosylation site and a surrounding sequence of 30 amino acids was taken from the maize Zm-ERabp1 cDNA (Hesse et al., 1989). The Zm-ERabp1 protein was previously shown to be glycosylated in vivo and in vitro (Hesse et al., 1989; Campos et al., 1994). With this strategy, we intended to position the glycosylation site of the HMGR1S-Asn protein not only in an appropriate context for glycosylation but also at enough distance from the hydrophobic sequences to permit accessibility to the catalytic center of the glycosyl transferase (Wessels and Spiess, 1988). The 95-bp AluI-EcoRI restriction fragment of the Zm-ERabp1 cDNA was subcloned in the above-mentioned MluI site. Both the AluI-EcoRI fragment and the MluI-digested plasmid had been blunted with the Klenow enzyme to allow an in-frame fusion of the two coding sequences. The final inserted sequence was IRYNTRSLKYPGQPQEIPFFQ**NTT**FSIPVNDPHQVWNSR-LHVVT (the regions of the original HMGR1S protein flanking the inserted sequence are

underscored; the glycosylation site is shown in boldface). The chimeric construct, coding for the HMGR1S-Asn protein, was transferred to the pDS6 plasmid.

HMGR1S-Δ1, HMGR1S-Δ2, and HMGR1S-Δ1,2

DNA sequences coding for the HMGR1S-deleted derivatives (Figure 1) were obtained by PCR following modification of the protocols described by Imai et al. (1991) and Ponce and Micol (1992). In these assays, a pBluescript KS+ plasmid, containing the Arabidopsis HMGR1S cDNA (Caelles et al., 1989), was used as a template. Primers were designed in inverted tail-to-tail direction to amplify the cloning vector together with the cDNA sequence. The primers used were as follows: D1, 5′-AAGCGCGTCGGATGCTTTCGG-3′; D2, 5′-CACCGGTGGCGTGACAAGATC-3′; D3, 5′-TTCTGTGATAGTGACGACGTGAAG-3′; and D4, 5′-GACTTTGTTCAGTCATTTATCTCACG-3′. To obtain a DNA construct coding for HMGR1S-Δ1, primers D1 and D2 were used. Likewise, primers D3 and D4 were used to obtain the DNA construct coding for HMGR1S-Δ2, and primers D1 and D4 were used to obtain the DNA construct coding for HMGR1S-Δ1,2. Reaction mixtures for PCR were prepared in a final volume of 50 μL containing 100 ng of template, 200 μM of each deoxynucleotide triphosphate, 1 μM of each primer, 30 mM Tricine, pH 8.4, 2 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.01% gelatin, and 0.1% Triton X-100. The samples were covered with mineral oil, incubated at 94°C for 3 min, and cooled to 80°C. Taq DNA polymerase (2.5 units) was added, and the samples were incubated for 15 cycles consisting of 1 min at 94°C, 1 min at the appropriate annealing temperature, and 5 min at 72°C. The annealing temperature was 67°C for the reaction mixture containing primers D1 and D2 and 63°C for the other reaction mixtures. Amplified DNA fragments were purified by agarose gel electrophoresis, treated with the Klenow enzyme to remove possible 5′ overhanging nucleotides, and self-ligated. The samples of ligation were used to transform competent XL1 blue or DH5α *E. coli* cells. Mutations were confirmed by DNA sequencing. Appropriate restriction fragments containing the deletion sites were used to replace equivalent regions of a pDS6-HMGR1S plasmid not derived from PCR, to ensure the absence of additional mutations in the HMGR1S coding sequence.

In Vitro Transcription and Translation

The coding sequences of the pDS6-derived plasmids were transcribed in vitro using *E. coli* RNA polymerase, and the mRNAs generated were translated in a wheat-germ cell-free system, as described by Campos et al. (1988). The translation mixture contained SRPs and endoplasmic reticulum (ER)–derived microsomes from dog pancreas, unless stated otherwise. Wheat germ extract was prepared essentially according to Roberts and Patterson (1973). Microsomes were isolated, depleted of SRP, and treated with staphylococcal nuclease as described by Walter and Blobel (1983a, 1983b). SRP was purified according to Walter and Blobel (1983b). Control mRNA coding for IgG light chain was purified from murine MOCP-41 tumors (Blobel and Dobberstein, 1975).

Proteinase K Treatment

Aliquots of the translation reactions were incubated in the presence of 0.11 mg/mL of proteinase K at 4°C for 30 min. Digestion was stopped by addition of phenylmethylsulfonyl fluoride at a final concentration of 0.5 mg/mL (Figures 2 and 4B) or 2 mg/mL (Figures 3B and 5).

Post-Translational Fractionation

Samples were fractionated by two sequential centrifugations as described by Enjuto et al. (1994). Briefly, after the first centrifugation, the supernatant containing the proteins not associated to microsomes was recovered (supernatant S_1), and the pellet was resuspended in 0.1 M Na_2CO_3, pH 11.0, to release the lumenal content of the microsomes. A second centrifugation of the resuspended sample separated the former lumenal proteins (supernatant S_2) from the proteins integrated in the open microsomal membranes (pellet P_2). Proteins present in the supernatant fractions were precipitated with trichloroacetic acid, and the corresponding pellets were washed with deionized water. Pellet samples were analyzed by SDS-PAGE.

Glycosidase F Treatment

Aliquots of the translation samples (9 to 18 μL) were heated at 100°C for 3 min, in the presence of 1% SDS, to denature proteins. After cooling at room temperature, samples were diluted to 200 μL with the appropriate buffer to bring the final solution to 250 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.7% Triton X-100, and 0.1% 2-mercaptoethanol. Glycosidase F from *F. meningosepticum* (EC 3.2.2.18) (0.6 units) was added, and the samples were incubated at 37°C for 5 hr. Digestion was stopped by trichloroacetic acid precipitation. After centrifugation, the pellets were washed with acetone and analyzed by SDS-PAGE.

Gel Electrophoresis

Samples were dissolved in buffer containing 50 mM Tris-HCl, pH 6.8, 5% glycerol, 2% SDS, 20% 2-mercaptoethanol, 100 mM DTT, and 0.01% bromophenol blue, and separated by SDS-PAGE, according to Laemmli (1970) or to Schaegger and von Jagow (1987). After electrophoresis, gels were prepared for fluorography as described by Skinner and Griswold (1983). To quantify radiolabeled proteins of the fluorographed gels, the corresponding bands were cut out, and the radioactivity was determined by liquid scintillation counting.

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