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Predicted Membrane Topology of the Coronavirus Protein E1

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ABSTRACT: The structure of the envelope protein E1 of two coronaviruses, mouse hepatitis virus strain A59 and infectious bronchitis virus, was analyzed by applying several theoretical methods to their amino acid sequence. The results of these analyses combined with earlier data on the orientation and protease sensitivity of E1 assembled in microsomal membranes lead to a topological model. According to this model, the protein is anchored in the lipid bilayer by three successive membrane-spanning helices present in its N-terminal half whereas the C-terminal part is thought to be associated with the membrane surface; these interactions with the membrane protect almost the complete polypeptide against protease digestion. In addition, it is predicted that the insertion of E1 into the membrane occurs by the recognition of the internal transmembrane region(s) as a signal sequence.

Proteins synthesized in eukaryotic cells have divergent destinations. Either they stay in the cytoplasm, or they are transported to organelles, to the plasma membrane, or out of the cell. Little is known about the determinants that direct a protein to its particular destination. It is generally assumed that such determinants reside in the protein's specific structure. Recently, we have presented the E1 glycoprotein of coronavirus mouse hepatitis virus A59 (MHV-A59)[1] as a model intracellular protein, the study of which might give insight into the targeting principles of such proteins (Rottier et al., 1984, 1985; Armstrong et al., 1984).

In contrast to most other enveloped RNA viruses, coronaviruses bud into the endoplasmic reticulum (ER) (Holmes & Behnke, 1981; Niemann et al., 1982; Tooze et al., 1984). This particular budding site is determined by the envelope glyco-

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[1] Abbreviations: ER, endoplasmic reticulum; IBV, infectious bronchitis virus; MHV-A59, mouse hepatitus virus strain A59; SRP, signal-recognition particle.

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protein E1, which accumulates in internal membranes after its synthesis in infected cells; it can be transported out of the cell but only as part of virions (Dubois-Dalcq et al., 1982; Niemann et al., 1982; Tooze et al., 1984). E1 of MHV-A59 is an O-glycosylated protein (Rottier et al., 1981; Niemann & Klenk, 1981; Holmes et al., 1981). Its integration into membranes occurs without cleavage of an N-terminal signal sequence (Rottier et al., 1984); nevertheless, it requires a signal-recognition particle (SRP) (Rottier et al., 1985).

A better understanding of the features of E1 is not possible without knowledge of its disposition in the membrane. Protease digestion of E1 of in vitro synthesized MHV-A59 removed only small portions from the NH_2 and COOH termini if the translation had been carried out in the presence of dog pancreatic microsomes, suggesting that the protein is largely buried within the lipid bilayer (Rottier et al., 1984). Subsequent sequencing of the E1 gene of MHV-A59 revealed that the N-terminal part of the protein is, indeed, very hydrophobic. However, it remained unclear how the quite hydrophilic COOH terminal part of the protein is protected from digestion (Armstrong et al., 1984).

Here, we study the disposition of E1 in the membrane by combining biochemical data with an analysis of its primary structure. We also included in our study the E1 sequence of another coronavirus, the avian infectious bronchitis virus (IBV) (Boursnell et al., 1984). MHV and IBV are supposed to be only distantly related viruses. This is underlined by the facts that E1 of IBV is glycosylated through N linkages (Stern & Sefton, 1982; Cavanagh, 1983) and that in IBV-infected cells five subgenomic mRNAs are synthesized instead of six (Stern & Sefton, 1984). Nevertheless, it may be expected that the E1 proteins of both viruses have essentially the same membrane topology. This provides a check on the outcome of our analyses and may add to our knowledge of the interaction of proteins and membranes.

Experimental Procedures

The methods used in this study have been published elsewhere and are referred to at the appropriate places in the text.

Results

Comparison of MHV-A59 and IBV E1 Primary Structures. Comparison of the sequences of MHV-A59 and IBV (Beaudette strain) E1 at their gene's nucleic acid level revealed no appreciable homology (data not shown). At the amino acid level, however, the sequence could be aligned so as to reach a homology of about 27% (Figure 1). Some conservation of the protein appears thus to have occurred during the evolution of these distinct viruses. This conservation becomes more significant when its location within the proteins is considered. Inspection of Figure 1 reveals that the homology is not generally scattered over the proteins but is concentrated largely in their N-terminal half. In the 123 N-terminal amino acids of MHV, the homology is 41%, while in the remainder of the protein it is only 17%. In more detail, it appears that the homology of the N-terminal parts of MHV and IBV is clustered within two regions (residues 34–68 and residues 101–123), which are almost 70% homologous.

Disposition of E1 in the Membrane. Of the numerous methods to predict secondary structures of proteins, only a few, recent ones were designed specifically for membrane proteins. One simple method to make predictions about the disposition of various parts of membrane proteins involves the calculation of the free-energy cost of burying successive stretches of amino acids of a protein from an aqueous environment into the hydrophobic interior of a membrane (von Heijne, 1981a; Engelman & Steitz, 1981; Steitz et al., 1982). In the case of bacteriorhodopsin, this method confirmed the existence of the seven transmembrane segments previously established by biophysical methods (Henderson & Unwin, 1975; Engelman & Zaccai, 1980). Figure 2 shows the results of such calculations with MHV and IBV E1s on a segment length of 21 residues and with the criteria of von Heijne (1981a). Despite the differences in primary structure, the profiles of both proteins are remarkably similar. In their N-terminal halves, the plots resemble those of bacterial rhodopsin (Steitz et al., 1982) and bovine rhodopsin (Nathans & Hogness, 1983). The obvious interpretation here is the existence of three membrane-spanning segments. Interestingly, this approach was in part developed to analyze signal sequences (von Heijne, 1981a,b, 1982) but yields highly positive peptide-membrane free energies for the N-terminal extreme of E1, rendering a functioning as insertion signal virtually impossible. Another significant result concerns the C-terminal half of E1. In both proteins, the free-energy value of this region is also positive. This indicates that for this part of the polypeptide a location within the lipid bilayer would be unfavorable on energetic grounds.

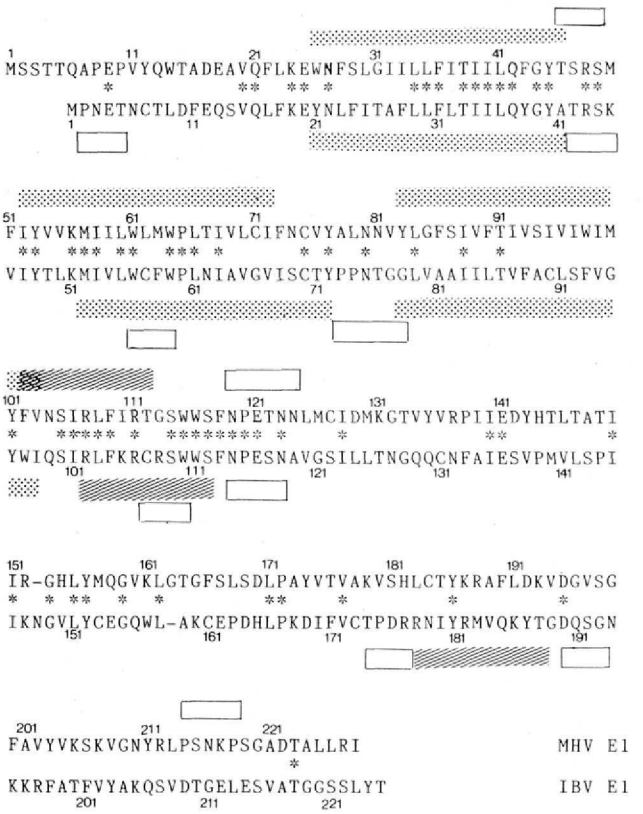

FIGURE 1: Amino acid sequences of MHV protein E1 (top sequence) and IBV protein E1 (bottom sequence) aligned according to maximal homology. Identical amino acids are indicated by asterisks. Dotted and hatched areas represent regions for which transmembrane and surface helices are predicted, respectively. Regions with high β-bend probability are symbolized by open boxes.

Another elegant approach to identity and characterize in membrane proteins the segments interacting with the lipid bilayer was recently presented by Eisenberg and co-workers (Eisenberg et al., 1984). These authors developed an algorithm in which the hydrophobicity of a 21-residue moving segment run over the entire protein is used to detect and locate, by a repeated process of selection and disqualification, the transmembrane α-helices. Application of this algorithm to the E1 sequences of MHV and IBV resulted in the localization in both proteins of three such helices. Their positions, together with

Table I: Transmembrane and Surface Helices in the E1 Protein of MHV and IBV

	MHV					IBV				
	21-residue segment with highest $\langle H \rangle$[a]	$\langle H \rangle$[b]	11-residue segment with max $\langle \mu_H \rangle$[c]	$\langle H \rangle$[d]	$\langle \mu_H \rangle$[e]	21-residue segment with highest $\langle H \rangle$[a]	$\langle H \rangle$[b]	11-residue segment with max $\langle \mu_H \rangle$[c]	$\langle H \rangle$[d]	$\langle \mu_H \rangle$[e]
first helix	26–46	0.71	36–46	0.67	0.38	21–41	0.66	31–41	0.62	0.29
second helix	52–72	0.78	53–63	0.72	0.30	52–72	0.67	60–70	0.59	0.25
third helix	83–103	0.85	86–96	0.85	0.34	78–98	0.80	81–91	0.84	0.23
first surface helix			102–112	0.11	0.73			102–112	−0.48	0.91
second surface helix								178–188	−0.42	0.83

[a] Transmembrane helices localized on the basis of maximal mean hydrophobicity ($\langle H \rangle$) according to Eisenberg et al. (1984); numbers represent the residue numbers in the proteins. [b] Corresponding mean hydrophobicity values. The minimal value for a transmembrane segment is 0.42, provided that there are two segments with a summed value of 1.10. [c] Regions with maximal mean hydrophobic moment. [d] Corresponding mean hydrophobicity values. [e] Corresponding mean hydrophobic moment values.

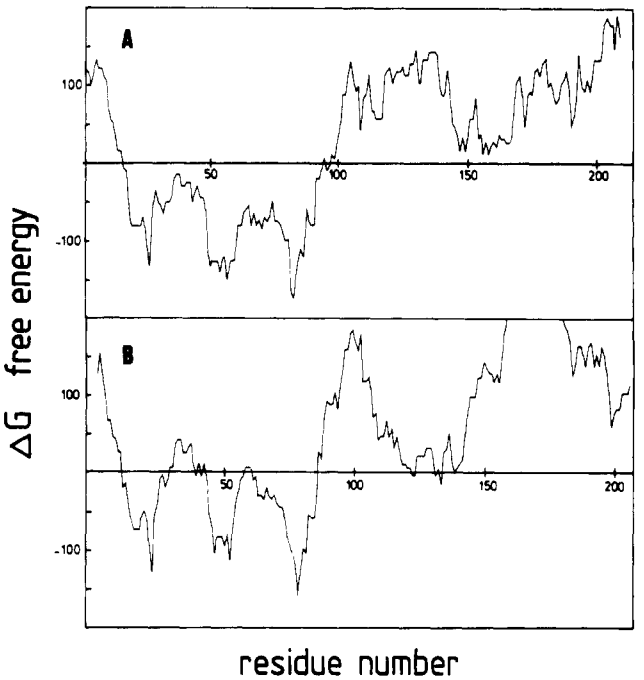

FIGURE 2: Free energy [G (kJ/mol)] of insertion of successive 21 amino acid segments as described by von Heijne (1981a). The energy is plotted as a function of the first amino acid in a segment. (A) MHV E1; (B) IBV E1. The minima may represent the start of a membrane-spanning helix.

their mean hydrophobicities, are given in Table I. The positions of the helices coincide with the three minima in the free-energy plot of Figure 2. It is noteworthy that the first and the third helices are predicted at exactly equivalent positions in both polypeptides. Eisenberg et al. also introduced the hydrophobic moment plot to classify membrane-associated helices. With this approach, the nature of a helix can be read from its position in the plot as determined by the values of hydrophobicity and hydrophobic moment, each calculated now with an 11-residue window. The data thus obtained are included in Table I. When positioned in the hydrophobic moment plot of Eisenberg et al., the E1 transmembrane helices appear to lie in the region of the so-called multimeric helices, i.e., protein segments that occur cooperatively associated within the lipid bilayer. Interestingly, this method also predicted the existence of some so-called surface helices (Table I). In the C-terminal half of the E1 molecule, a few helices were found with the high hydrophobic moments that are characteristic of close interaction with the surface of the membrane.

Having identified the membrane-associated parts in the E1 molecule, we finally searched for additional secondary structure for the other parts of the protein using more classical prediction methods. Notably, the position of β-bends as calculated with the rules of Chou and Fasman (1978) and of Cid et al. (1982) supported previous conclusions. Figure 1 shows the position of seven β-bends predicted by one or both methods for the IBV molecule, two of them conspicuously separating each pair of transmembrane helices. Only one of these latter was found in MHV E1. Two other β-bends are located in close proximity at equivalent positions in both proteins in the conserved region next to the first surface helix. Besides the transmembrane segments, only a few α-helices were predicted by the method of Lim (1974). These predictions in IBV E1 (residues 178–190) and in the corresponding region of MHV E1 agreed with the localization of the surface helix found in the E1 protein of IBV around residue 183.

Discussion

Three main conclusions can be drawn from the analysis of the coronavirus E1 protein sequence presented in this paper. (i) The protein is anchored in the membrane through three successive transmembrane segments. (ii) The C-terminal half of the polypeptide is not incorporated within the lipid bilayer. (iii) The coronavirus E1 protein has an internal signal sequence.

Though the occurrence of multiple membrane-spanning segments seems not to be unusual among membrane proteins, to our knowledge coronavirus E1 presents the first viral example of this kind. Other comparable proteins have widely different origins, ranging from bacterial [e.g., bacteriorhodopsin (Henderson & Unwin, 1975) and *Escherichia coli* lactose carrier protein (Foster et al., 1983)] to eukaryotic proteins [e.g., cytochrome P-450 (Heinemann & Ozols, 1982) and acetylcholine receptor subunits (e.g., Noda et al., 1983)]. The methods we have used in our study have proven their usefulness in predicting directly the existence and precise locations of the transmembrane segments in these other proteins (Eisenberg et al., 1984). For the coronavirus E1 transmembrane segments, the values of hydrophobicity and hydrophobic moment suggest that the helices are associated cooperatively within the lipid bilayer.

An intriguing question is why E1 behaves as an intracellular protein accumulating mainly in reticular membranes in the absence of virus maturation. It seems that the occurrence of multiple spanning segments is not the explanation for this behavior since this type of structure also exists in plasma membrane proteins such as erythrocyte band III protein (Steck, 1978), rhodopsin (Nathans & Hogness, 1983; Dratz & Hargrave, 1983), and the acetylcholine receptor subunits [e.g., Noda et al. (1983)].

Insertion of E1 into membranes takes place without cleavage of a signal sequence (Rottier et al., 1984). Nevertheless, the requirement of SRP (Rottier et al., 1985) implies a specific recognition sequence. The N-terminus of the protein has none

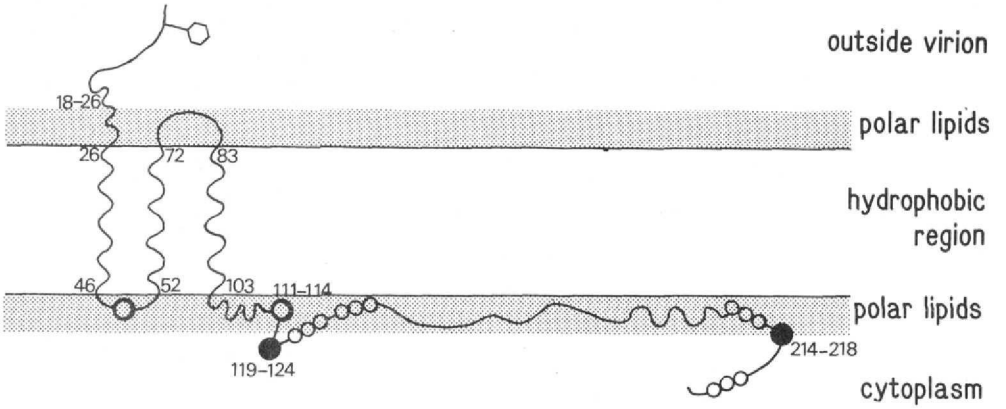

FIGURE 3: Suggested disposition of the MHV E1 polypeptide across the membrane. Symbols: (〰) α-helix (Eisenberg et al., 1984; Lim, 1974); (OOO) β-sheet (Lim, 1974); (◯) β-bend predicted by one method (Chou & Fasman, 1978); (●) β-bend predicted by two methods (Chou & Fasman, 1978; Cid et al., 1982); (○) location of the four glycosylation sites. Lumenal and cytoplasmic faces of the microsomal membrane are topologically equivalent to outer and inner faces of the virion membrane, respectively.

of the characteristics of such a sequence. Our previous experiments indicated that the signal for membrane insertion of MHV-A59 E1 could be located anywhere within its first 150 amino acids (Rottier et al., 1984, 1985). There is no sequence homology among published signal sequences, but unlike the extreme N-terminal sequences of E1, they are all hydrophobic and devoid of charged residues (von Heijne, 1981a). It is therefore likely that the SRP-specific membrane insertion signal resides in one of the membrane-spanning hydrophobic regions of E1. Possibly, the strong conservation in the first two membrane-spanning segments of the mammalian and avian viral E1 sequences pertains to such a function. The involvement of more than one functional insertion signal cannot be excluded, since the addition of SRP to an in vitro translation of E1 mRNA did not lead to the accumulation of a specific arrested peptide (Rottier et al., 1985). The occurrence of internal signal sequences is not without precedent. They have been postulated for several other membrane proteins (Braell & Lodish, 1982; Anderson et al., 1983; Chin et al., 1984; Holland et al., 1984) as well as for a secreted protein (Lingappa et al., 1979).

Combining the results of our theoretical predictions with the biochemical data on the MHV-A59 E1 protein assembled in microsomal membranes (Rottier et al., 1984), we arrive at the model shown in Figure 3. On the lumenal side of the microsomal membrane, which corresponds to the outside of the virion, an N-terminal region of about 2.5K is exposed and thus susceptible to proteolysis. The MHV sequence contains here potential O-glycosylation sites clustered at positions 2–5, which in the mature protein probably become positions 1–4 by the removal of the terminal methionine [see Housman et al. (1970)]. O-Linked sugars often appear in clusters near one end of the molecule [see Sharon & Lis (1981)]. In glycophorin, for example, all of the 15 O-glycosylated hydroxy amino acids are found in the first 50 N-terminal residues, particularly in the extreme terminal portion where each of residues 2–4 and 10–15 carries an oligosaccharide unit (Tomita et al., 1978). MHV E1 enters the lipid membrane around residue 26 as judged from the size of the protease-sensitive fragment and the primary structure at this point. Then, the polypeptide chain traverses the membrane three times. It emerges from the cytoplasmic face of the lipid bilayer with the surface helix around residue 109; this probably represents the domain that is slightly accessible to proteases (Rottier et al., 1984). Then, a remarkably long stretch of amino acids follows for which, apart from a few β-bends, no secondary structure is predicted. This part of the protein is again extremely protease resistant if synthesized in the presence of microsomal membranes (Rottier et al., 1984). Therefore, we postulate it to be embedded in the surface of the membrane, together with the second surface α-helix (residues 182–192). An alternative possibility is an interaction with the transmembrane α-helices. Finally, the polypeptide becomes exposed again, the β-bend at positions 214–218 probably marking the start of the protease-sensitive C-terminus.

The intracellular budding of coronaviruses is supposed to involve a highly specific recognition between nucleocapsids in the cytoplasm and E1 molecules accumulated in the endoplasmic reticular membranes. The C-terminal part of the E1 proteins is located at the cytoplasmic side of the ER and is thus available for interaction with the nucleocapsid protein and the genome. The presence of almost all basic amino acids in this very part of the protein supports this view and may explain the general affinity of E1 for RNA (Sturman et al., 1980).

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Radioactive Probes for Adrenocorticotropic Hormone Receptors†

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ABSTRACT: Our attempts to develop adrenocorticotropic hormone (ACTH) analogues that can be employed for ACTH receptor identification and isolation began with the synthesis of ACTH fragments containing N^{ϵ}-(dethiobiotinyl)lysine (dethiobiocytin) amide in position 25 to be used for affinity chromatographic purification of hormone–receptor complexes on Sepharose-immobilized avidin resins. Because labeling ACTH or ACTH fragments by conventional iodination techniques destroys biological activity due to oxidation of Met^4 and incorporation of iodine into Tyr^2, we have prepared [Phe^2,Nle^4]$ACTH_{1-24}$, [Phe^2,Nle^4,biocytin^{25}]$ACTH_{1-25}$ amide, and [Phe^2,Nle^4,dethiobiocytin^{25}]$ACTH_{1-25}$ amide by conventional synthetic techniques. The HPLC profiles and amino acid analyses of the final products indicate that the materials are of a high degree of purity. The amount of tertiary butylation of the Trp residue in the peptides was assessed by NMR and was found to be less than 0.5%. All three peptides are equipotent with the standard $ACTH_{1-24}$ as concerns their ability to stimulate steroidogenesis and cAMP formation in bovine adrenal cortical cells. Iodination of [Phe^2,Nle^4]$ACTH_{1-24}$, with iodogen as the oxidizing agent, has been accomplished without any detectable loss of biological activity. The mono- and diiodo derivatives of [Phe^2,Nle^4]$ACTH_{1-24}$ have been prepared, separated by HPLC, and assayed for biological activity. Both peptides have the full capacity to stimulate steroidogenesis and cAMP production in bovine adrenal cortical cells.

It is the ultimate aim of this investigation to gain information regarding the chemical nature of ACTH[1] receptors. Conventional approaches to this problem have thus far been unsuccessful; in particular, the receptor appears to lose its affinity for ACTH upon solubilization. We are exploring a combination of affinity labeling and affinity chromatographic techniques based on the avidin–biotin interaction to identify

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[1] Abbreviations: ACTH, adrenocorticotropic hormone; Bct, biocytin; Boc, *tert*-butoxycarbonyl; CCD, countercurrent distribution; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DIPEA, *N*-ethyldiisopropylamine; DMF, dimethylformamide; DTBct, dethiobiocytin; HPLC, high-pressure liquid chromatography; OBu^t, *tert*-butyl ester; OSu, *N*-hydroxysuccinimide ester; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; Z, benzyloxycarbonyl.

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