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Biosynthesis and Sorting of Proteins of the Endoplasmic Reticulum

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I. INTRODUCTION

A. Protein Sorting in General

How proteins are sorted to their proper location within the cell is one of the most intriguing questions in cell biology. The answers to this question will enable us to begin to understand how a cell maintains and regulates the structural and functional distinctiveness of its intracellular membrane compartments. A significant amount of progress has been made in understanding many aspects of the general problem of protein sorting. The mechanisms which discriminate between proteins that remain in the cytoplasm and those that are either to be secreted or to become integral membrane proteins continue to be elucidated in great detail (for review, see Lodish *et al.*, 1981; Sabatini *et al.*, 1982; Walter *et al.*, 1984; Wickner

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and Lodish, 1985). Recent studies have shown that many nuclear-encoded proteins destined for the mitochondria (for review, see Hay *et al.*, 1984) or the chloroplast (for review, see Schmidt *et al.*, 1980) are synthesized as preproteins with an amino-terminal extension which acts as a signal for the posttranslational uptake of the precursors into these organelles. In the case of lysosomal hydrolases in fibroblasts, it has been shown that appropriately oriented mannose 6-phosphate moieties on the enzymes act as one of the signals which results in the sorting of these proteins into lysosomes (for review, see Sly and Fischer, 1982; Sly, 1985).

It has been shown that the rate-limiting and distinctive step in the intracellular maturation of different secretory proteins is the movement of the proteins from their site of synthesis, the endoplasmic reticulum (ER),[1] to the Golgi (Lodish *et al.*, 1983). These results are incompatible with the hypothesis that vesicles bud off from a specialized region of the ER and merely contain a sample of the luminal content proteins and the ER membrane proteins. Therefore, it has been proposed that this type of sorting may also be a receptor-mediated process. In addition, Kalderon *et al.* (1984) have described a short amino acid sequence of SV40 T antigen, Pro-Lys-Lys-Lys-Arg-Lys-Val, which appears to specify the nuclear localization of that protein and which can mediate the transport to the nucleus of chimeric proteins which have been constructed to contain this sequence. This chapter describes experiments which have as their ultimate goal to test the hypothesis that the proteins of the internal membranes of the cell have analogous sequence or structural features which enable the cell to sort them to their correct subcellular compartments (Nelson and Robinson, 1983).

The work of many laboratories has made it clear that the biochemical tools already exist to begin a detailed investigation of the sorting problem. Experiments involving the ability to express the coding sequences of membrane proteins in suitable vector–host systems, coupled with the ability to modify these sequences through the use of *in vitro* mutagenesis techniques, provide a powerful way to approach the answers to these questions (for review, see Garoff, 1985; Schekman, 1985). This type of

[1] Abbreviations: ER, endoplasmic reticulum; VSV, vesicular stomatitis virus: ERp, endoplasmic reticulum protein; endo H, endo-β-*N*-acetylglucosaminidase H; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; MOPC-315, mineral oil-induced plasmacytoma 315; RER, rough endoplasmic reticulum; EDTA, ethylenediaminetetraacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; LPS, bacterial lipopolysaccharide (*Escherichia coli* serotype B5:055); H^{M315}, heavy chain of MOPC-315 immunoglobulin A (IgA) molecule; MDBK, Madin–Darby bovine kidney; HPLC, high-performance liquid chromatography; CHO, carbohydrate; bp, base pairs.

approach has provided a valuable way to identify possible protein sorting signals in plasma membrane proteins, such as VSV glycoprotein (Rose and Bergmann, 1982, 1983; Guan and Rose, 1984, Guan *et al.*, 1985; Adams and Rose, 1985a,b), the influenza virus hemagglutinin (Gething and Sambrook, 1982; Doyle *et al.*, 1985; Gething *et al.*, 1986), and the polymeric immunoglobulin receptor (Mostov *et al.*, 1986).

The work of Rose and his co-workers provides an excellent example of such studies as applied to the intracellular transport of plasma membrane proteins. Rose and colleagues were able to examine the effects of alterations in various regions of VSV G protein on G protein sorting. For example, it was demonstrated that alteration of the cytoplasmic domain produced two classes of mutations: Class I, in which G protein transport was arrested and G protein accumulated in an early subcellular compartment, and Class II, in which a reduced rate of transport was observed with G protein eventually reaching the cell surface. The existence of the Class I alterations was interpreted to provide preliminary evidence for the existence of an ''appropriate'' cytoplasmic domain leading to ER localization.

Additional studies (Puddington *et al.*, 1986), utilizing chimeric cDNAs that encode the extracellular and transmembrane domains of VSV G protein linked to the cytoplasmic domain of other plasma membrane proteins, further emphasized the importance of the cytoplasmic domain for efficient transport to the cell surface. Furthermore, in studies of the transmembrane region, it was demonstrated the deletion of 2,4, or 6 amino acids in the 20 amino acid transmembrane region had no significant effect on transport, while deletions of 8 or 12 amino acids resulted in a G protein whose transport was blocked in the Golgi. Deletion of the entire transmembrane region resulted in a G protein which accumulated in the ER and was secreted only slowly. In other studies, it was found that the incorporation of a charged amino acid in the middle of the transmembrane region blocked transport of G protein to the surface, but not membrane insertion. Finally, studies in which a chimeric protein was created, containing the coding sequences for growth hormone fused to the transmembrane and cytoplasmic domains of G protein, demonstrated that this construction was only transported to the Golgi and that the signal for secretion of growth hormone was not operative when it was membrane bound.

Rizzolo *et al.* (1985) and Guan *et al.* (1985) have studied growth hormone–viral glycoprotein chimeric molecules to gain insights into the role of both luminal and cytoplasmic protein sequences in protein sorting. Although some of the results from these studies are not easily interpretable, it is clear that both luminal and cytoplasmic sequences influence

protein sorting either directly as signals or indirectly through altered conformations of the chimeric molecules. In general, a major difficulty encountered in carrying this work further has been that alterations in protein sequences of membrane proteins have produced negative effects, i.e., the transport of the protein is blocked at an intermediate step in the transport pathway. It is difficult, therefore, to determine the direct cause of this transport lesion. It could be that sorting signals have been specifically altered and that the altered protein is no longer recognized by the cellular sorting machinery. It also could be the case, however, that the altered protein merely precipitates more easily in the cell and thus becomes physically unable to be transported. In this regard, it now appears that the retention in the ER of the altered forms of many normally exported proteins is due to their inability to fold properly. In this state, they are retained in the ER by their association with the immunoglobulin heavy chain binding protein (BiP) (Haas and Wabl, 1983; Bole *et al.*, 1986; Gething *et al.*, 1986).

Guan *et al.* (1985) have observed that the addition of functional carbohydrate attachment sites to the growth hormone sequence of a growth hormone–VSV G protein chimera, which accumulated in the Golgi apparatus, provided this molecule with a signal for protein transport to the plasma membrane. Thus, these workers have produced positive effects on transport by altering the luminal portion of a hybrid molecule.

While these data indicate that all domains of a membrane protein contribute to its sorting properties, they also underscore the need for more experiments to determine the sorting behavior of different types of membrane proteins before a truly coherent picture of protein transport signals can be developed. The design of systems in which positive effects on transport are created should be of particular value. The application of recombinant DNA technology to the study of ERp sorting should provide such model systems in that the deletion or mutation of signals that lead to ER localization could allow the ERps to traverse the complete protein transport pathway.

B. Sorting of ER Proteins in Particular

1. *Site of Sorting of ER Proteins in the ER*

One of the early questions asked about ERp sorting was where the sorting took place. Were the ERps preferentially retained in the ER or did they leave the ER with the rest of the proteins being transported from the ER only to be returned by a recycling mechanism? Based on the composition and structural organization of the Golgi apparatus and its well-estab-

lished role in the sorting of other membrane proteins, it had been postulated (Rothman, 1980) that ERps are sorted in a process analogous to fractional distillation. This idea has been invoked recently by Munro and Pelham (1987) to explain their observations concerning a retention signal for a luminal ER protein (see below). According to this model, the ER is not a site for protein sorting. Instead, all proteins are first transported to the Golgi, and those proteins which are destined for the ER are recovered from the cis-most stacks of this organelle by recycling. Our studies investigating the sorting of six major ERps from murine plasmactyoma cells, ERp49, ERp59, ERp60, ERp61, ERp72, and ERp99, have produced no evidence to indicate that these proteins ever leave the ER (Lewis *et al.*, 1985b, 1986). Similar conclusions have been reached in studies of hexose-6-phosphate dehydrogenase (Brands *et al.*, 1985), glucosidase II (Brands *et al.*, 1985), and ribophorin I (Rosenfeld *et al.*, 1984) and in studies utilizing tripeptides containing the acceptor sequence for Asn-linked glycosylation to examine the rate of bulk flow from the endoplasmic reticulum to the cell surface (Wieland *et al.*, 1987).

The evidence against a recycling mechanism for ER localization can be summarized by three general observations. For those ERps with N-linked oligosaccharides, it has been determined that the oligosaccharides are of the high-mannose type and are sensitive to endo H. This is consistent with the hypothesis that the ERps never leave the ER for the Golgi where the oligosaccharides can be processed into complex, endo H-resistant moieties. In addition, in experiments where protein transport was assayed directly by sucrose gradient fractionation of pulse-labeled cell extracts, no movement of the proteins from the ER to the Golgi could be detected. Finally, the secretion of glycopeptides is faster than that of any known secretory proteins (Wieland, 1987). Since much evidence suggests that oligosaccharides are not signals for transport (Hickman and Kornfeld, 1978; Keller and Swank, 1978; Olden *et al.*, 1978; Struck *et al.*, 1978; Bell-Quint *et al.*, 1981; Sidman *et al.*, 1981; Gotlieb and Wallace, 1982), it appears that no signal is required for rapid and efficient transport from the ER to the cell surface. Thus, the unique protein composition of the ER appears to be established and maintained by the selective export of non-ER proteins. This suggests that the ER is the initial site for the sorting of ER membrane proteins and that ERps must contain specific retention signals.

2. *Signals Directing the Sorting of ER Proteins*

Given the conclusion that ERps are specifically retained in the ER, the problem becomes the elucidation of the structural features which identify newly synthesized membrane proteins as ERps. Many ER proteins and

enzymes have been studied in a large number of laboratories. ER proteins are involved in the cotranslational insertion of membrane proteins into the ER membrane (for review, see Walter *et al.*, 1984) as well as the cotranslational translocation of lysosomal and secretory proteins across the ER membrane (Gilmore and Blobel, 1985). In the majority of cases, the cotranslational insertion or translocation of a protein in or through the membrane is accompanied by the removal of an amino-terminal signal peptide by the ER signal peptidase. Evans *et al.* (1986) have recently described the purification of a complex of microsomal proteins which contain signal peptidase activity. Other ER-associated activites include the enzymes of glycerolipid synthesis (for review, see Bell and Coleman, 1980; Bell *et al.*, 1981), the enzymes responsible for the synthesis and transfer of the lipid-linked oligosaccharide involved in N-linked glycosylation of nascent proteins (Snider and Robbins, 1982; Snider and Rogers, 1984), and the enzymes which process the newly transferred oligosaccharide, such as glucosidase I, glucosidase II, and the ER α-mannosidase (for review, see Kornfeld and Kornfeld, 1985). In addition, protein disulfide isomerase (for review, see Freedman and Hillson, 1980) and prolyl hydroxylase (for review, see Kivirikko and Myllyla, 1980) are confined to the ER.

For the purpose of this chapter, we confine our attention to those ERps whose structure and/or sorting are the best characterized. The structural features of these ERps are given in Table I. Cytochrome b_5 and NADH–cytochrome b_5 reductase have been included in Table I because both components have been shown to be enriched in the ER. They are, however, present in other cell fractions including the Golgi apparatus, the plasma membrane, mitochondria, and peroxisomes. It is possible that the reason for this wide distribution can be found in the way in which these proteins are made and the way in which they subsequently interact with the intracellular membranes. Cytochrome b_5 and NADH–cytochrome b_5 reductase are synthesized on free polysomes, released into the cytoplasm,a nd the completed polypeptides are then incorporated into membranes posttranslationally (Okada *et al.*, 1982; Anderson *et al.*, 1983). Analysis of the amino acid sequence of cytochrome b_5 has led to the idea that this protein interacts with the membrane bilayer by virtue of its hydrophobic carboxy-terminal region (Ozols and Gerard, 1977). What is known about the structure of NADH–cytochrome b_5 reductase is consistent with a similar conclusion for this membrane component (Tajima *et al.*, 1979). Thus, the finding that cytochrome b_5 and NADH–cytochrome b_5 reductase are enriched in the ER may be the result of the interaction of these proteins with proteins of the ER membrane before they themselves are incorporated into the membrane.

TABLE I

Properties of Endoplasmic Reticulum Membrane Proteins[a]

Protein	Molecular weight (Ref.)	Number of N-linked oligosaccharides	Cleaved signal sequence	Orientation[b]
Cellular proteins				
Cytochrome b_5	17,000 (ref. 1)	—	No	Cytoplasmic
Cytochrome P-450 (induced by phenobarbital)	56,000 (ref. 2)	—	No	Cytoplasmic
Egasyn (monomer → tetramer)	64,000 (ref. 3) (monomer)	+[c]	?	Luminal
Epoxide hydrolase	53,000 (ref. 4)	—	No	Cytoplasmic
ERp49	49,000	—	No	Transmembrane
ERp60	60,000	—	Yes	Luminal
ERp61	61,000	—	Yes	Luminal
ERp72	72,000	—	Yes	Luminal
ERp99 (also GRP94) (monomer → dimer)	92,500 (ref. 5–9) (monomer)	1	Yes	Transmembrane
Glucosidase II	123,000	+[c]	?	Luminal
Hexose-6-phosphate dehydrogenase	108,000	+[c]	?	Luminal
Heavy chain binding protein (BiP; also GRP78)	70,000 (ref. 10)	–	Yes	Luminal
3-Hydroxy-3-methylglutaryl-coenzyme A reductase	97,000 (ref. 11)	1	No	Transmembrane
NADH–cytochrome b_5 oxidoreductase	31,000 (ref. 12)	—	No	Cytoplasmic
NADPH–cytochrome P-450 oxidoreductase	77,000 (ref. 13)	—	No	Cytoplasmic
Prolyl hydroxylase (tetramer)	230,000 (ref. 14)	—	—	Luminal
α subunit	64,000 (refs. 15,16)	1	?	
α' subunit	64,000 (refs. 15,16)	2	?	
β subunit	60,000 (ref. 15)	—	Yes	
(Note: β subunit is PDI)	(refs. 16–18)			
Protein Disulfide Isomerase (PDI; also ERp59)	55,000 (ref. 19)	—	Yes	Luminal
Ribophorin I	65,000	1	Yes	Transmembrane

continued

TABLE I (*Continued*)

Protein	Molecular weight (Ref.)	Number of N-linked oligosaccharides	Cleaved signal sequence	Orientation[b]
Ribophorin II	63,000	1	Yes	Transmembrane
Signal recognition particle receptor ("docking protein")	70,000 (ref. 20)	—	No	Cytoplasmic
Viral Proteins				
Adenovirus E19	19,000 (ref. 21)	2	Yes	Transmembrane
Coronavirus E1	26,000 (ref. 22)	—[d]	No	Transmembrane
Rotavirus NCVP5	29,000	2	No	Transmembrane
Rotavirus VP7	38,000	1	No	Transmembrane

[a] The information was compiled from the references given here and those cited in the text. Key to references: (1) Ozols and Heinemann (1982), (2) Fujii-Kuriyama *et al.* (1982), (3) Swank and Paigen (1973), (4) Heinemann and Ozols (1984), (5) Sorger and Pelham, 1987, (6) Mazzarella and Green (1987), (7) Kulomaa *et al.* (1986), (8) Sargan *et al.* (1986), (9) Kleinsek *et al.* (1986), (10) Munro and Pelham (1986), (11) Chin *et al.*, 1984, (12) Yubisui *et al.* (1984), (13) Porter and Kasper (1985), (14) Berg and Prockop (1973), (15) Berg *et al.* (1980), (16) Koivu and Myllyla (1986), (17) Koivu and Myllyla (1987), (18) Koivu *et al.* (1987), (19) Edman *et al.* (1985), (20) Lauffer *et al.* (1985), (21) Herrisse' *et al.* (1980), and (22) Armstrong *et al.* (1984).

[b] The classification of a protein as either cytoplasmically or luminally oriented is based on the protein's activity beirg either sensitive or insensitive to proteases in the absence of detergent in intact microsomes. A protein is classified as transmembrane if the protein is shown to be available to protease in intact microsomes and the protein possesses an oligosaccharide or if a substantial proteolytic fragment which is detected in the absence of detergent is also degraded in the presence of detergent.

[c] The number of endo H-sensitive oligosaccharides is not known.

[d] Glycosylation of E1 is a posttranslational event. It is not inhibited by tunicamycin, 2-deoxyglucose, or 2-deoxy-2-fluoroglucose, all of which are inhibitors of asparagine-linked glycosylation. Analysis of the E1 carbohydrate and its sensitivity to mild alkaline treatment suggest that there is an O-glycosidic linkage between the oligosaccharide and the polypeptide. O-Linked glycosylation of E1 presumably occurs after the virus assembles and subsequently migrates to the Golgi apparatus. Glycosylation of E1 increases its molecular weight by about 3,000 (Niemann and Klenk, 1981).

While this is a type of protein sorting, it is not the kind that is the main focus of this chapter. We are interested in those ER proteins that are cotranslationally incorporated into the ER membrane and remain there in spite of the continuous export of proteins from the ER compartment. These proteins begin the transport pathway like all other membrane proteins and secretory proteins, but for some reason they are normally arrested at an early stage of the process. We are interested in defining those

features of ER proteins which are responsible for this arrest and determining how these signals accomplish the ER localization of these proteins.

Drawing on the work of other laboratories investigating the transport of plasma membrane proteins, it is reasonable to assume that sorting information, or at least structural features which could influence sorting, could be located in all three domains of a typical ER membrane protein: the luminal domain, the transmembrane domain(s), and the cytoplasmic domain. Two common features of the luminal domain which could influence sorting are the presence of an uncleaved signal peptide or the presence of an N-linked oligossaccharide moiety. It has been reported that HMG-CoA reductase (Brown and Simoni, 1984), cytochrome *P*-450 (Bar-Nun *et al.*, 1980), NADPH–cytochrome *P*-450 reductase (Okada *et al.*, 1982), epoxide hydrolase (Okada *et al.*, 1982), rotavirus NCVP5 (Both *et al.*, 1983), and coronavirus E1 (Rottier *et al.*, 1984) lack cleavable signal peptides, while the signal peptides of rotavirus VP7 (Ericson *et al.*, 1983), adenovirus E19 (Persson *et al.*, 1980; Wold *et al.*, 1985), and ribophorin I and II are cleaved (Rosenfeld *et al.*, 1984). Our own experiments have shown that ERp99, ERp72, ERp61, ERp60, and protein disulfide isomerase (ERp59) have cleavable signal peptides (R. Mazzarella, unpublished results). Thus, it appears that the mere possession of an uncleaved signal peptide is not sufficient to determine the sorting properties of ER proteins. In addition, our finding that, of the six ER proteins we have studied, only ERp99 is a glycoprotein (Lewis *et al.*, 1985a,b, 1986) taken together with analogous studies of ER glycoproteins such as HMG-CoA reductase (Liscum *et al.*, 1983), the ribophorins (Kreibich *et al.*, 1983a; Rosenfeld *et al.*, 1984), hexose-6-phosphate dehydrogenase (Hino and Minakami, 1982; Brands *et al.*, 1985), glucosidase II (Hino and Rothman, 1985), prolyl hydroxylase (Kedersha *et al.*, 1985a,b), egasyn (Swank and Paigen, 1973), adenovirus E19 (Kornfeld and Wold, 1981), and rotavirus VP7 (Kabcenell and Atkinson, 1985) and NCVP5 (Both *et al.*, 1983) indicates that the mere presence or absence of an N-linked oligosaccharide is not sufficient to define an ER protein.

As the amino acid sequences of ER proteins have become available from the sequencing of cDNA clones encoding them, it has become possible to examine the transmembrane regions of these proteins with regard to their possible role in ER localization. Liscum *et al.* (1985) have proposed that the seven membrane spanning domains of HMG-CoA reductase may be involved in the localization of this protein to the cholesterol-poor membranes of the ER. Recently, Kabcenell and Atkinson (1985) and Poruchynsky *et al.* (1985) have described studies investigating the subcellular localization and intracellular sorting of the VP7 glycoprotein of rotavirus SA11. These workers have shown that while wild-type VP7 is

confined to the ER in infected cells or in cells transfected with expression vectors containing the VP7 gene, it is possible to construct mutants of VP7 which are transported to the Golgi and eventually secreted. Specifically, it appears that alterations in the second hydrophobic region near the amino terminus will abolish the ER localization of VP7. Thus, these authors conclude that this hydrophobic domain provides both a membrane anchor and a positive signal for ER localization. It will be interesting to see if this conclusion can be extended to the membrane-spanning regions of other ERps as they are cloned and subjected to mutational analysis.

As more information has become available about ER proteins, it has been observed that one relatively common feature of the ER membrane proteins was the fact that a significance part of the protein was exposed on the cytoplasmic face of the ER membrane. This type of orientation is exhibited by cytochrome *P*-450 (Matsuura *et al.*, 1978; Nilsson *et al.*, 1978; Heinemann and Ozols, 1982), NADPH–cytochrome *P*-450 reductase (Black *et al.*, 1979), HMG-CoA reductase (Liscum *et al.*, 1983, 1985), epoxide hydrolase (Griffen and Noda, 1980; Galteau *et al.*, 1985), signal recognition particle receptor (docking protein) (Walter *et al.*, 1979; Meyer and Dobberstein, 1980a,b), ERp99 (Lewis *et al.*, 1985b), and rotavirus NCVP5 (Kabcenell and Atkinson, 1985). Our results with ERp72, ERp60, ERp61, and ERp59 (Lewis *et al.*, 1985b, 1986) taken together with studies of protein disulfide isomerase (Lambert and Freedman, 1985), prolyl hydroxylase (Olsen *et al.*, 1973), egasyn (Swank and Paigen, 1973), and immunoglobulin heavy chain binding protein (Haas and Wabl, 1983; Munro and Pelham, 1986), all of which exhibit a predominantely luminal orientation, indicate that a cytoplasmic or luminal orientation is also not the sole property by which an ERp is defined.

Recent findings have demonstrated that ER sorting signals will, in all likelihood, not be bulk properties of the protein. Experiments by Medda *et al.* (1987) implicate the egasyn–esterase active site in attachment of microsomal β-glucononidase to egasyn by a novel mechanism that, in turn, compartmentalizes β-glucuronidase within the endoplasmic reticulum. In addition, Munro and Pelham (1987) have shown that the C-terminal KDEL sequence is necessary to retain the luminal protein GRP78 in the ER compartment. Finally studies by Paabo *et al.* (1987) on the transmembrane protein E19 encoded by the adenovirus genome illustrate the importance of cytoplasmic domains in the retention of ER proteins. The E19 protein has only a 15-residue cytoplasmic tail, whereas the luminal domain consists of 104 amino acids and two N-linked carbohydrates. Shortening the cytoplasmic tail to 7 amino acids abolishes the ER localization of this protein and this mutant E19 protein becomes transported to the plasma membrane.

The results that have been obtained so far are helpful in formulating general ideas about the assembly of the ER. It is clear, however, that the elucidation of the signal or groups of signals necessary to define an ER membrane protein will be a challenging problem that will, in all likelihood, be solved only by the investigation and comparison of the structural features of a large number of ERps. Much more must be done in order to determine the nature of the ERp sorting signals and the way in which they function. We need to know if the signals act in a positive manner to accomplish the retention of the ERp in the ER or in a negative way to prevent movement from the ER.

The question of why a protein is localized to the ER can have at least three answers. The protein may have structural features which keep it in the ER membrane. It may possess, for example, transmembrane domains which favor the cholesterol-poor ER membranes, as has been postulated for HMG-CoA reductase (Liscum *et al.*, 1985), or it may interact with cytoskeletal elements which prevent its movement to other membrane compartments, as may be the case for the ribophorins (Kreibich *et al.*, 1978). On the other hand, the protein may lack features which allow it to be transported out of the ER. It may be missing a suitably positioned or oriented oligosaccharide or it may lack cytoplasmically exposed structural features that are recognized by the cellular sorting machinery. Of course, it is always possible that for any given protein, the answer may be a combination of both situations. For these reasons, it is critical that several ERps be analyzed in order to formulate sorting rules.

II. STRUCTURE AND SORTING OF ERp99, AN ABUNDANT, CONSERVED ER GLYCOPROTEIN

A. Preparation of ERps and Anti-ERp Antibodies

Our studies of ERp synthesis and sorting began with attempts to characterize major components of purified RER from murine plasmacytoma tissue. RER was isolated from MOPC-315 plasmacytoma tissue according to the procedures of Gold and Green (1983). Analysis of the RER by electron microscopy showed ribosome-studded membrane vesicles typical of these types of preparations. Treatment of the RER with EDTA removed greater than 95% of the bound ribosomes from the vesicles. Incubation of the EDTA-treated RER preparations with 0.03% (w/v) Triton X-100 allowed the removal of the RER lumen proteins while having no significant effect on RER membrane proteins. A detergent-soluble extract

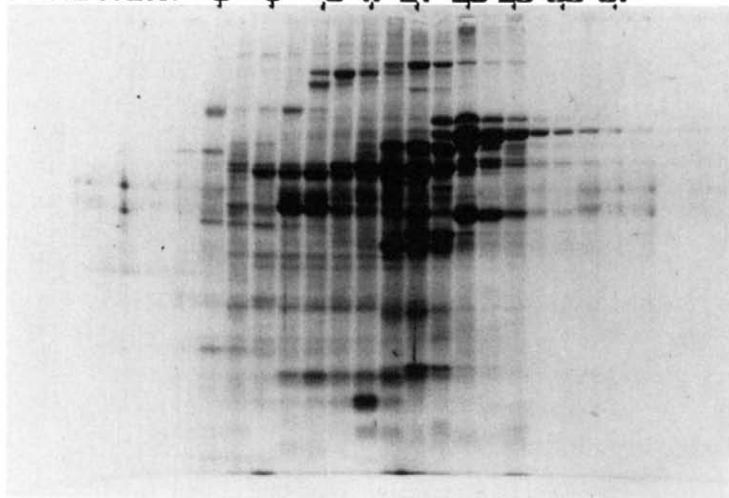
was prepared from the RER preparation obtained after these manipulations by treating the washed vesicles with 0.5% (w/v) Triton X-100. Six of the major proteins in this extract, ERp49, ERp59, ERp60, ERp61, ERp72, and ERp99, were purified by a combination of DEAE–cellulose chromatography and preparative SDS–PAGE (Fig. 1).

After elution from the preparative slab gel, the purified proteins were used to prepare monospecific rabbit antibodies. Before using these preparations in our experiments, the specificity of the antibodies was assessed in immunoprecipitation experiments using three different radiolabeled antigen preparations. The first sample was obtained by lactoperoxidase-mediated radioiodination of sonicated EDTA-treated RER. Labeled proteins extracted from this sample with 0.5% (w/v) Triton X-100 were used as the antigen. The second sample was obtained by the chemical radioiodination of the 0.5% (w/v) Triton X-100 extract of EDTA-treated RER with ^{125}I-labeled Bolton–Hunter reagent. The third sample was a detergent-soluble lysate prepared from [^{35}S]methionine-labeled MOPC-315 plasmacytoma cells which had been cultured for 4 hr in the presence of the radioactive amino acid. In all cases, treatment of the samples with each antibody resulted in the immunoprecipitation of only one major radioactive protein. In each case, the labeled proteins comigrated with the purified, unlabeled proteins used to prepare the antibody.

In the following sections, we present and discuss experiments focusing on the structure and sorting of ERp99. There are several reasons that make ERp99 a particularly interesting focal point for our discussions. We have shown ERp99 to be an abundant ER protein in murine tissues and to be antigenically conserved across a wide range of mammalian species. These data indicate that ERp99 may play an important structural and/or functional role in the ER membrane. In addition, we should be able to obtain amounts of purified ERp99 which would be sufficient for physical and chemical studies designed to follow up our observations concerning the sorting properties of normal and mutated ERp99. We have shown that ERp99 is a transmembrane glycoprotein. Thus, we will be able to compare the information we obtain about its structure and sorting with that

Fig. 1. Fractionation and purification of the ERps. RER was purified from MOPC-315 plasmacytoma tissue and treated with 20 m*M* EDTA and 0.03% (w/v) Triton X-100. The RER-derived membrane was solubilized in 0.5% (w/v) Triton X-100 and fractionated by DEAE–cellulose chromatography. The bound protein was eluted with a 0–0.4 *M* linear NaCl gradient. SDS–PAGE analysis of the indicated column fractions was performed on a 10% polyacrylamide gel, and the designated fractions were pooled and again analyzed by SDS–PAGE. The indicated ERps were recovered from the appropriate regions of preparative SDS gels by electroelution and used for the inoculation of rabbits. From Lewis *et al.* (1986).

FRACTION: 5 9 13 17 21 25 29 33 37



1-9
10-17
18-23
24-31

ERp61

ERp72
ERp59
ERp49

ERp99
ERp60

obtained about other transmembrane glycoproteins which either remain in the ER or are transported to the plasma membrane. Finally, we have observed that the specific synthesis and accumulation of ERp99 increases approximately 10-fold on LPS-induced differentiation of murine B cells (Lewis *et al.*, 1985a). These results are also consistent with the possibility that ERp99 has an important role in ER structure and/or function as the resting B lymphocyte differentiates into an actively secreting lymphoblast and plasma cell. Finally, our initial cDNA cloning experiments have enabled us to obtain several ERp99 cDNA clones. With these clones, we can begin experiments designed to apply recombinant DNA technology to the investigation of ERp structure and sorting.

B. Subcellular Distribution of ERp99

Experiments performed to determine the subcellular distribution of ERp99 employed two different approaches. In the first type of experiment (Table II), various subcellular fractions of MOPC-315 plasmacytoma tissue were obtained by standard methods. The proteins in each of these fractions were enzymatically labeled with ^{125}I, and a 0.5% (w/v) Nonidet P-40 extract was prepared from each of the fractions. ERp99 was then sequentially immunoprecipitated from 250-ng aliquots of each iodinated fraction and analyzed by SDS–PAGE. The amount of ERp99 in each

TABLE II

Relative Enrichment of ERp99 in MOPC-315 Subcellular Fractions[a]

ERp	Homogenate	Mitochondria	RER	SER	Golgi	PM	Soluble
ERp99	1.0	1.0	18.1	1.6	1.1	ND[b]	5.1
Glu-6-Pase	1.0	1.2	4.4	1.2	2.1	0.9	0.6

[a] ERp99 was immunoprecipitated from iodinated preparations of the total homogenate and six subcellular fractions (250-ng aliquots of protein) and analyzed by SDS–PAGE and autoradiography. The quantity of ERp99 in the different fractions was determined from a densitometric scan of the autoradiograph. The results obtained with each subcellular fraction were normalized to the amount of ERp99 in total homogenate. The specific activity of glucose-6-phosphatase (Glu-6-Pase) in each subcellular fraction was normalized to that of total homogenate and is presented for comparison. The purity of each subcellular fraction was determined by marker enzyme analysis, and the enrichment achieved over total homogenate was as follows: mitochondria, 2.77-fold (monoamine oxidase); RER, 4.44-fold (glucose-6-phosphatase); smooth endoplasmic reticulum (SER), 1.24-fold (glucose-6-phosphatase); Golgi, 31.5-fold (sialyltransferase); plasma membrane (PM), 1.30-fold (5′-nucleotidase). Adapted from Lewis *et al.* (1985a).

[b] ND, No peak detected.

fraction was quantitated from densitometric scans of the autoradiograph. ERp99 was almost completely localized to the RER.

In the second type of experiment, the biosynthetic sorting of ERp99 was investigated by a procedure which allowed the transport of ERp99 to different intracellular membrane compartments to be measured directly (Fig. 2). The procedure employed was a modification of a method used to subfractionate the Golgi apparatus (Goldberg and Kornfeld, 1983). Intracellular membranes were isolated from pulse-labeled MOPC-315 cells and fractionated on linear sucrose gradients. An analysis of the fractionation of glucose-6-phosphatase activity, an ER marker, and sialyltransferase

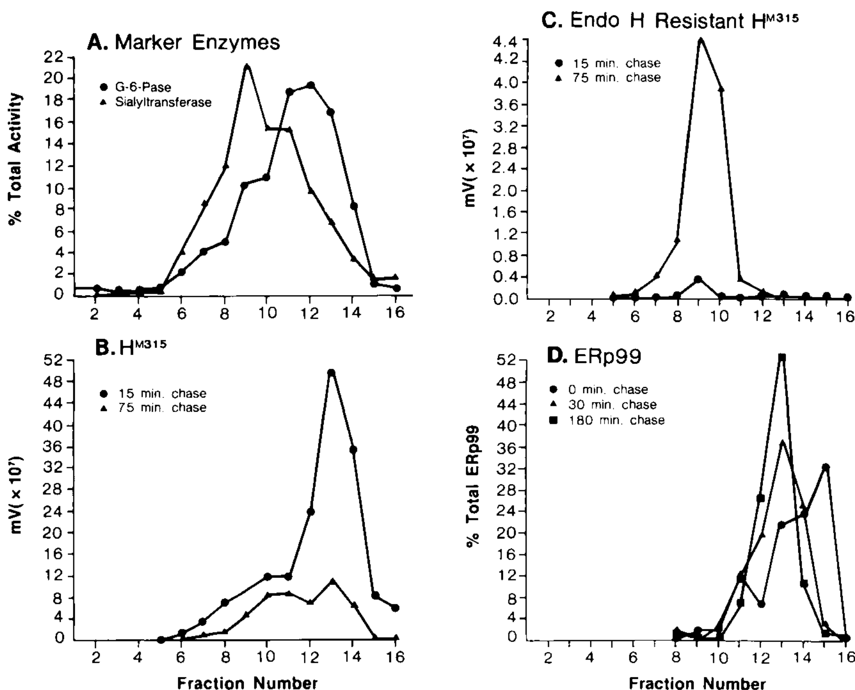


Fig. 2. Biosynthetic sorting of ERp99. Intracellular membranes were isolated from a postnuclear supernatant of unlabeled MOPC-315 plasmacytoma cells and fractionated on 15–45% linear sucrose gradients. Sixteen 1-ml fractions were collected from the gradient, and the membranes were isolated from each fraction by centrifugation at 200,000 *g*. The membranes were resuspended in buffer and used for marker enzyme assays. The distributions of glucose-6-phosphatase activity and sialyltransferase activity are depicted in (A). The results presented in (B–D) depict the distributions of total [^{35}S]methionine-labeled H^{M315}, endo H-resistant H^{M315}, and ERp99. The quantity of the appropriate protein in each fraction was calculated from a densitometric scan of autofluorographs. In (D) the quantity of ERp99 in each fraction is presented as a percent of the total ERp99 in the gradient. Adapted from Lewis *et al.* (1985b).

activity, a Golgi marker, as well as an analysis of the intracellular transport of H^{M315}, demonstrated that the procedure was suitable for separating these two intracellular membrane compartments. The amount of ERp99 in the gradient fractions was assayed by immunoprecipitation. Nearly all of ERp99 was always recovered from the densest of the ER fractions, well separated from the Golgi region. This restricted localization of ERp99 was entirely consistent with the results obtained from subcellular fractionation studies. These results suggest that ERp99 may be exclusively localized in the RER. There was no evidence from this experiment that ERp99 ever leaves the ER, in that the gradient distribution of ERp99 was consistent throughout the 180-min chase. We feel, therefore, that it is unlikely that transport to and recycling from the Golgi apparatus plays a major role in the sorting of ER proteins. It is, of course, impossible to exclude this mechanism for all ER proteins, and it would be useful to apply these tests to newly identified ERps as they become available.

C. Tissue Distribution and Antigenic Conservation of ERp99

Using our standard immunoprecipitation methods, we have demonstrated that ERp99 is present in normal mouse tissues (Table III). These results indicate that ERp99 is a significant component of the microsomes

TABLE III

Tissue Distribution of ERp99[a]

	Quantity of ERp99 per 500 ng of total microsomal protein	
Tissue	Total amount	Relative amount
Plasmacytoma	1.92	1
Spleen	0.35	0.18
Liver	4.44	2.3
Kidney	0.38	0.20
Muscle	Not detected	—

[a] ERp99 was immunoprecipitated from ^{125}I-labeled preparations of total microsomes of various mouse tissues. ERp99 was immunoprecipitated from 500-ng aliquots of protein and analyzed by SDS–PAGE and autoradiography. The quantity of ERp99 was determined from a densitometric scan of the autoradiograph and is expressed in arbitrary density units.

of several tissues, especially liver, and serve to emphasize that the results we obtain by studying the sorting of ERp99 in plasmacytoma cells will be relevant to the assembly of intracellular membranes in other tissues. Proteins which are immunologically related to and of similar size to the ERp99 were present in detergent extracts of various cell types from other eukaryotic species (Fig. 3). Proteins were metabolically labeled for 4 hr with [^{35}S]methionine, and aliquots of a detergent-soluble extract were treated with anti-ERp99 antibody. The immunoabsorbed proteins were treated overnight with endo H and analyzed by SDS–PAGE. A prominent band which comigrated with murine ERp99 was detected in rat, hamster,

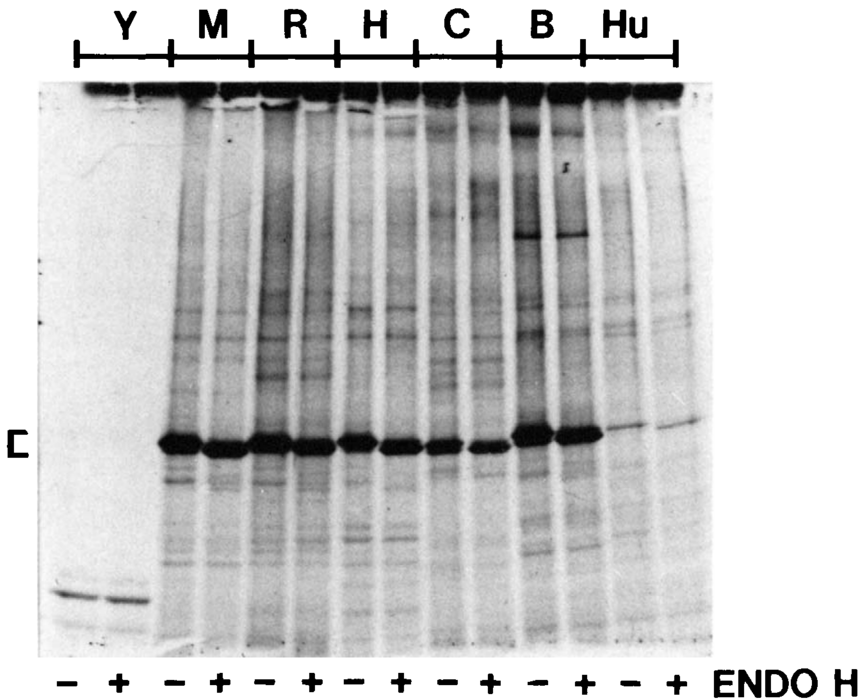


Fig. 3. Proteins immunologically related to ERp99 are found in several eukaryotic species. Seven eukaryotic cell lines were labeled with [^{35}S]methionine. Detergent-soluble lysates were prepared from the labeled cells and treated with anti-ERp99 antibody. Immunoprecipitated proteins were incubated in the absence and presence of endo H and analyzed by SDS–PAGE on a 7.5% polyacrylamide gel. The cell lines used were as follows: yeast cells (Y), MOPC-315 cells (M), rat Y3 myeloma cells (R), Chinese hamster ovary cells (H), chick embryo fibroblasts (C), MDBK (bovine) cells (B), and HeLa cells (human) (Hu). The position of the authentic ERp is indicated by the bracket. Adapted from Lewis *et al.* (1985a).

chicken, and bovine cells. On prolonged exposure of the gel, a comigrating species was also detected in HeLa cells. Nearly all of each ERp99-related protein was sensitive to endo H, indicating that ERp99 contains at least one high-mannose oligosaccharide. Based on comigration, no ERp99-related proteins were detected in yeast cells. Recent results in our laboratory, using ERp99 cDNA clones to detect ERp99 mRNA in various cell lines, have shown that the high degree of immunological relatedness observed using our anti-ERp99 antibody is reflected in the sequence homology observed between the ERp99 mRNAs (data not shown). These studies indicate that ERp99 is present in higher eukaryotic cells of different species, cell types, and stages of differentiation and that ERp99 is a highly conserved ER membrane protein.

D. Metabolic Fate of ERp99

A pulse–chase analysis was used to determine the nature and amount of posttranslational processing involved in the synthesis of ERp99 (Fig. 4). No precursor forms of ERp99 were detected. ERp99 contained, however, at least one, endo H-sensitive oligosaccharide. This carbohydrate moiety could be detected at the earliest sampling time and remained endo H sensitive throughout the chase. The nature of the ERp99 carbohydrate was determined by HPLC analysis (Fig. 5). The majority of [^{3}H]mannose

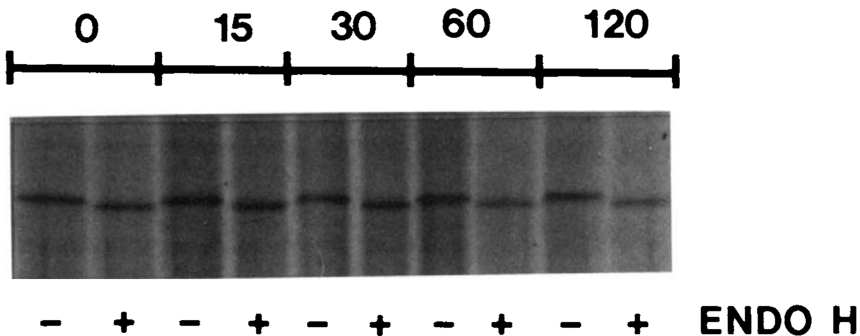


Fig. 4. Pulse–chase analysis of the processing of ERp99. ERp99 was metabolically labeled with [^{35}S]methionine during a 15-min incubation of MOPC-315 plasmacytoma cells with the radioactive amino acid. To begin the chase, cells were diluted 20-fold in complete RPMI-1640 medium supplemented with 1 m*M* methionine. At the indicated times, samples were removed and the cells collected by centrifugation. The cell pellets were washed once and resuspended in lysing buffer at 5×10^{6} cells/ml. The ERp99 was isolated by immunoprecipitation from the detergent-soluble cell lysates, incubated in the absence (−) and presence (+) of endo H and analyzed by SDS–PAGE on a 7.5% polyacrylamide gel. The radioactive protein was visualized by autofluorography. Adapted from Lewis *et al.* (1985b).

was incorporated into a species which comigrated with the mannose$_8$ *N*-acetylglucosamine standard. Smaller amounts of oligosaccharides coeluting with larger and smaller size standards were also detected. The extent of processing of asparagine-linked oligosaccharides has been used to follow the intracellular transport of glycoproteins (for review, see Hubbard and Ivatt, 1981). The conversion of the oligosaccharide from an endo H-sensitive structure to an endo H-resistant structure has been used to indicate that the protein has been transported from the site of oligosaccharide addition in the RER to the site of the GlcNAc transferase I and GlcNAc-dependent α-mannosidase activities in the Golgi. The ERp99 carbohydrate moiety was never converted to an endo H-resistant structure. These results and those reported for the HMG-CoA reductase (Liscum *et al.*, 1983) ribophorins (Rosenfeld *et al.*, 1984), glucosidase II (Hino and Rothman, 1985; Brands *et al.*, 1985), hexose-6-phosphate dehydrogenase (Hino and Minakami, 1982; Brands *et al.*, 1985), prolyl hydroxylase (Kedersha *et al.*, 1985a,b), adenovirus E19 (Kornfeld and Wold, 1981), and rotavirus VP7 and NCVP5 (Kabcenell and Atkinson, 1985) are

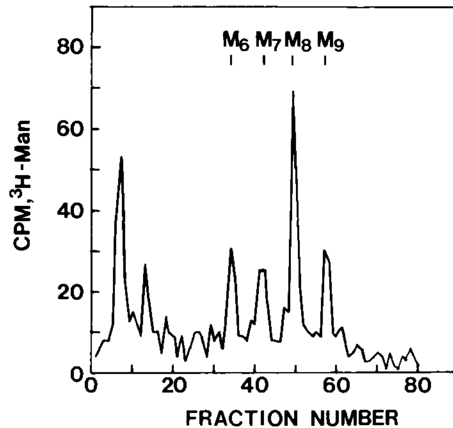


Fig. 5. HPLC analysis of ERp99-derived oligosaccharides. ERp99 was metabolically labeled with [^{3}H]mannose during a 30-min incubation of MOPC-315 plasmacytoma cells with the radiolabeled sugar. Labeled cells were isolated by centrifugation and resuspended in complete RPMI-1640 medium. After a 3-hr chase, cells were washed once and resuspended in lysing buffer at 5×10^6 cells/ml. The detergent-soluble cell lysate was preabsorbed extensively with rabbit anti-H^{M315} before treatment with anti-ERp99. The immunoprecipitated proteins were digested with pronase, and the resultant glycopeptides were treated with endo H. The products of endo H digestion were reduced with sodium borohydride and desalted before HPLC analysis. The indicated standard oligosaccharides refer to the following moieties: M_6, Man$_6$GlcNAc; M_7, Man$_7$GlcNAc; M_8, Man$_8$GlcNAc; and M_9 Man$_9$GLcNAc. Taken from Lewis *et al.* (1985b).

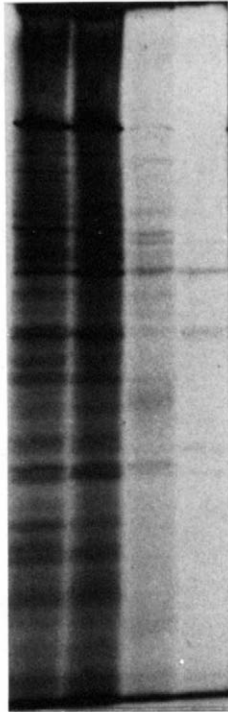
consistent with the idea that these proteins are not transported from the ER to the Golgi. In addition, these data taken together with the data obtained from subcellular fractionation and biosynthetic sorting experiments confirm the localization of ERp99 to the RER membrane.

E. Membrane Topography of ERp99

The results described above provide a framework for a study of the structural features of ERp99 which result in its sorting to the ER membrane. One of the initial questions which must be answered is the manner in which ERp99 is oriented in the membrane. We were able to obtain a general picture of the membrane topography of ERp99 by the use of proteolysis experiments (Fig. 6). ERp99 was completely sensitive to both papain and trypsin digestion in the presence of detergent. Interestingly, ERp99 was completely degraded by papain and significantly degraded by trypsin even in the absence of detergent. These results indicate that the bulk of ERp99 is exposed on the cytoplasmic side of the ER membrane.

An additional piece of evidence which helped us to create a picture of the way in which ERp99 interacts with the membrane came from the results of experiments performed to investigate the synthesis and processing of ERp99 in a cell-free system (Fig. 7). The 15–19 S fraction of MOPC-315 poly(A)$^+$ mRNA, which had been isolated by standard procedures (McCandliss *et al.*, 1981), was incubated in reticulocyte extracts in the absence or presence of intracellular membranes isolated from Krebs II ascites tumor cells (Szczesna and Boime, 1976; Green, 1979; Green and Gleiber, 1980). The membranes were added either cotranslationally or posttranslationally. ERp99-related cell-free products were isolated by immunoprecipitation, incubated in the presence or absence of endo H to detect N-linked glycosylation, and analyzed by SDS–PAGE. ERp99 was proteolytically cleaved by the membranes when they were added cotranslationally but not posttranslationally. In addition, ERp99 was cotranslationally, but not posttranslationally, N-glycosylated by the oliogsaccharyltransferase of the ascites membranes.

From these data, we have formulated a general model of the interaction of ERp99 with the ER membrane. Since ERp99 has a cleavable signal peptide, we propose that the mature amino terminus is inside the lumen of the ER. Since ERp99 appears to be completely sensitive to papain and trypsin in intact microsomes, showing no evidence of a major membrane-protected fragment, we propose that the bulk of the protein is exposed on the cytoplasmic face of the ER. Since, however, ERp99 possesses an N-linked oligosaccharide moiety, we propose that this moiety is attached at a site in the amino-terminal end of the molecule and that enough of this



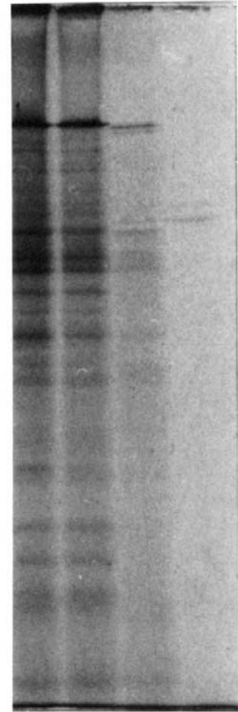


Fig. 6. Orientation of the ERp99 in the ER membrane. MOPC-315 cells were labeled for 60 min with [^{35}S]methionine, and cell homogenates were prepared as described by Goldberg and Kornfeld (1983). (A) Papain digestion. Where indicated, papain was added to a final concentration of 40 μg/ml and Triton X-100 added to a final concentration of 0.067% (w/v). All samples were incubated for 30 min at 37°C. (B) Trypsin digestion. Where indicated, trypsin was added to a final concentration of 250 μg/ml and Triton X-100 added to a final concentration of 0.067% (w/v). All samples were incubated for 30 min at room temperature. After inactivation of the enzymes, ERp99 was isolated by immunoprecipitation and analyzed on 10% polyacrylamide gels by SDS–PAGE and autofluorography. The arrows indicate the position of ERp99. Adapted from Lewis *et al.* (1985b).

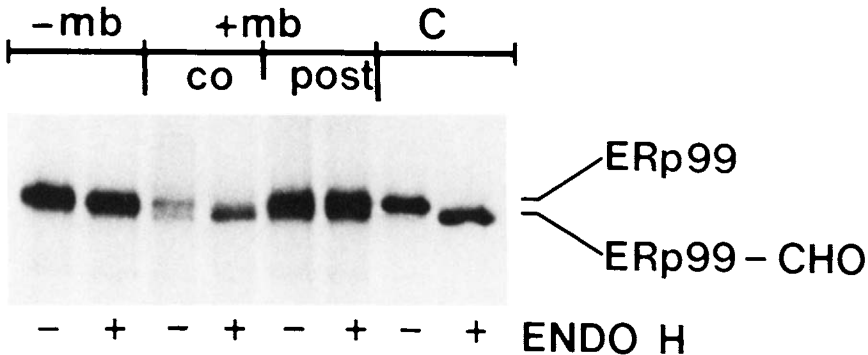


Fig. 7. Cell-free synthesis of ERp99. The 15–19 S fraction of MOPC-315 poly(A)$^+$ mRNA was incubated in reticulocyte extracts in the absence (−mb) or in the presence (+mb) of intracellular membrane isolated from Krebs II ascites tumor cells. The membranes were added either cotranslationally (co) or posttranslationally (post). In the case of posttranslational addition, protein synthesis was carried out for 1 hr, cycloheximide was added to 5 μg/ml to stop further protein synthesis, membranes were added, and the incubation was continued for 1 hr at 30°C. Cell-free products were analyzed by immunoprecipitation of the reaction mixtures followed by SDS–PAGE analysis of the immunoabsorbed proteins. The cell-free products were incubated in the absence or presence of endo H to test for the presence of an oligosaccharide moiety. The cell-free products were compared to native ERp99 immunoprecipitated from lysates of [^{35}S]methionine-labeled MOPC-315 cells (C lanes). The positions of the cellular ERps, ERp99 and ERp99—CHO, are indicated. Taken from Mazzarella and Green (1987).

end of the molecule has passed through the membrane into the lumen to make this oligosaccharide addition site available for the oligosaccharyltransferase.

F. Molecular Cloning and Expression of ERp99 cDNA

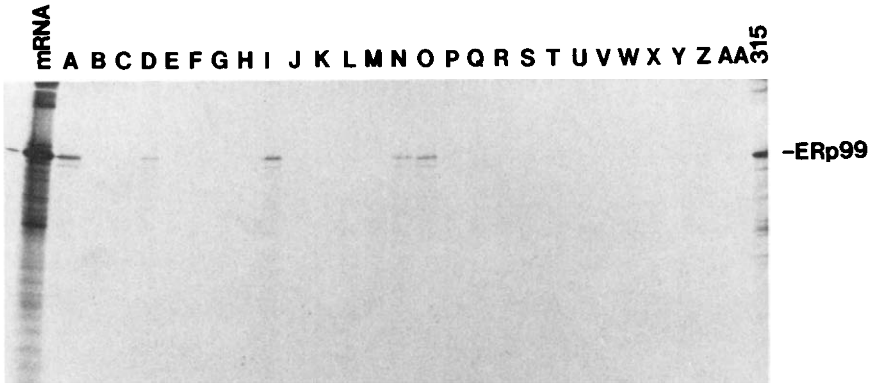
The ultimate goal of our studies is to determine, in detail, the way in which ERp99 is oriented in the membrane. We also wish to test the hypothesis that ER membrane proteins possess structural features that may act as signals to direct their localization to the ER. A first step in the realization of these goals is the determination of the primary sequence of ERp99. The most rapid way of determining the amino acid sequence of a protein is to obtain and sequence a full-length cDNA clone encoding the protein. From calculations we had made from the results of our cell-free translation experiments, we reasoned that sucrose gradient fractionation would be sufficient to enrich our mRNA preparations to the point where ERp99 mRNA was approximately 0.5% of the mRNA. A cDNA library prepared from this mRNA fraction could be screened by hybridization

selection procedures which, although laborious, promised a reasonable chance of identifying an ERp cDNA clone.

Our search for ERp99 cDNA clones using hybrid selection to screen a cDNA library prepared in pBR322 from a 15–19 S fraction of plasmacytoma poly(A)$^+$ mRNA was quite successful (Fig. 8). In a screen of 800 independent transformants, 7 (0.9%) ERp99 clones were found. The first ERp99 cDNA clone that was analyzed (I-14 in Fig. 8) contained an insert of 500 bp. We used this insert to screen the members of the other transformant pools which were positive for ERp99 cDNA clones (pools A, D, N, and O in Fig. 2 and pools from another screening experiment). Three larger cDNA clones were identified containing overlapping cDNAs of 2100, 2500, and 2600 bp. Measurement of the size of murine ERp99 mRNA by Northern blot analysis indicated that ERp99 mRNA is approximately 3200 nucleotides in length (Fig. 9). An mRNA of the same length was also detected in human (HeLa) cells. This observation is consistent with our earlier results demonstrating a striking degree of immunological relatedness between the ERp99 identified in different cells and tissues (1985a). In addition, the probe hybridized to an RNA species of about 5800 nucleotides present in the HeLa cells.

A fragment of one of the original ERp99 cDNA clones was used to screen a cDNA libary prepared in the pcD shuttle vector (Okayama and Bery, 1982) using mRNA isolated from transformed mouse fibroblasts. Two clones containing ERp99 sequences, pcD99-1 and pcD99-2, were identified containing *Bam*HI inserts of approximately 1700 and 3000 bp, respectively. Plasmid DNA was purified from these transformants and used to transfect COS cells (Fig. 10). In mock-transfected cells (lanes 1,2), in untransfected cells (lanes 7,8), or in cells transfected with pcD99-1 (lanes 3,4), the only ERp99-related band observed was a small amount of cross-reacting monkey ERp99. This was an expected finding based upon our earlier observations concerning the antigenic relatedness of ERp99 from cells of various animal species. The monkey ERp99 contained an endo H sensitive oligosaccharide moiety and was slightly larger than murine ERp99. Transfection with pcD99-2 resulted in the synthesis of a significant amount of murine ERp99 which, like the ERp99 made in plasmacytoma cells, was completely endo H sensitive (lanes 5,6). In addition to normally sized ERp99, a species migrating more slowly was detected when the immunoprecipitates from pcD99-2 transfected cells were not treated with endo H (lane 5). Upon treatment with endo H (lane 6), this band migrated at the ERp99 (—CHO) position. These results indicate that the altered migration of this ERp99-related species is a function of its N-linked oligosaccharide residue(s).

The strategy used to determine the complete nucleotide sequence of the



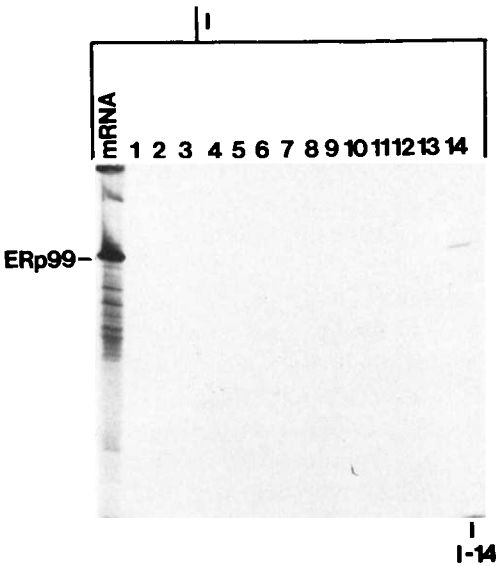


Fig. 8. Screening a murine plasmacytoma 15–19 S poly(A)$^+$ mRNA library for ERp99 cDNA clones by hybridization selection. Transformants (tet^R *E. coli* RR1) were grouped into 27 colony pools (A–AA) of approximately 15 transformants each. Plasmid DNA was obtained from these pools by standard procedures, bound to nitrocellulose filters, and incubated with plasmacytoma poly(A)$^+$ mRNA. The mRNA eluted from each filter was translated in a reticulocyte lysate-derived cell-free system supplemented with [^{35}S]methionine, and the cell-free products were assayed by immunoprecipitation with anti-ERp99 antibody followed by SDS–PAGE and autofluorography. The result of the first screen for ERp99 is shown at the top. One pool (I) containing an ERp99 clone was analyzed further. DNA was isolated from individual pool members for use in hybridization selection experiments. The products of the cell-free translation of the eluted mRNA were analyzed as described above. The results for the final screen of the pool is shown at the bottom. The products immunoprecipitated from the cell-free products of the starting mRNA fraction are included in the gels for reference. As can be seen, clone I-14 was identified as a cDNA clone for ERp99. Taken from Mazzarella and Green (1987).

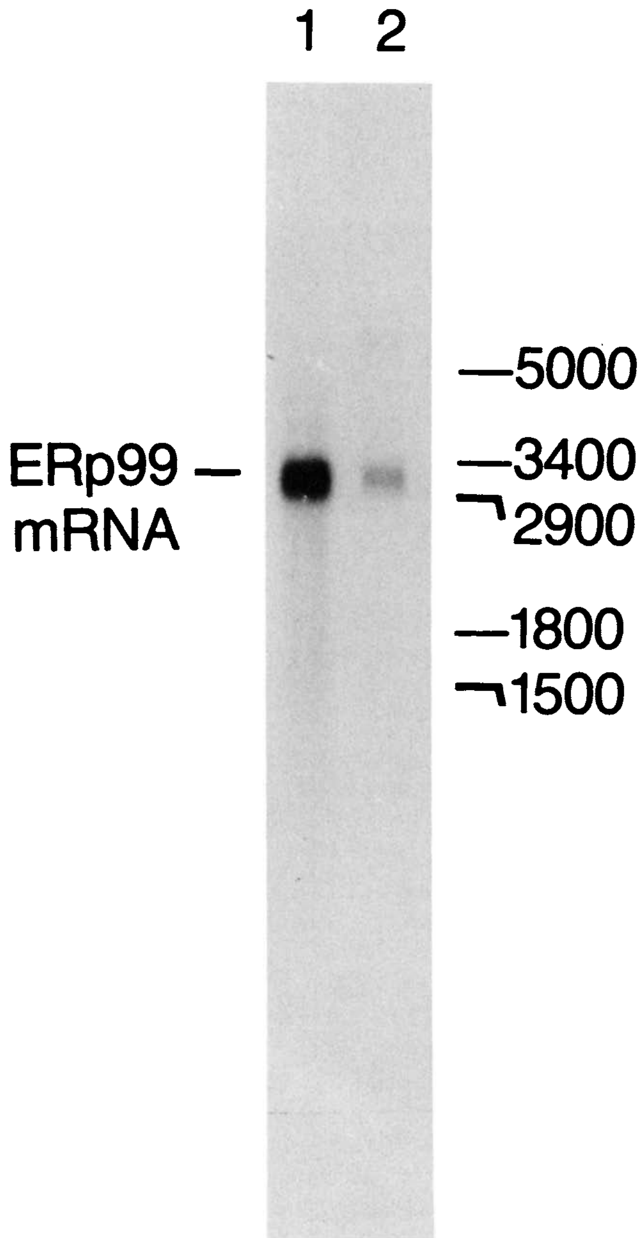


Fig. 9. Northern analysis of ERp99 mRNA. Poly(A)$^+$ mRNA was isolated from mouse liver (Lane 1) and HeLa cells (Lane 2) by standard procedures. RNA (2 μg of each sample) was fractionated by electrophoresis through 1.4% agarose gels containing 2.2 *M* formaldehyde as described in the text. After electrophoresis, the RNA was transferred to nitrocellulose paper by blotting overnight. The nitrocellulose filter was dried by baking for 2 hr at 80°C in a vacuum oven and ERp99-related mRNAs on the filter were detected by hybridization with the ^{32}P-nick-translated *Pst* I–*Hin*dIII internal fragment of the ERp99 clone (see Fig. 11). The positions of marker RNAs (reticulocyte, yeast, and *E. coli* ribosomal RNAs) fractionated on the same gel are indicated on the right. Taken from Mazzarella and Green (1987).

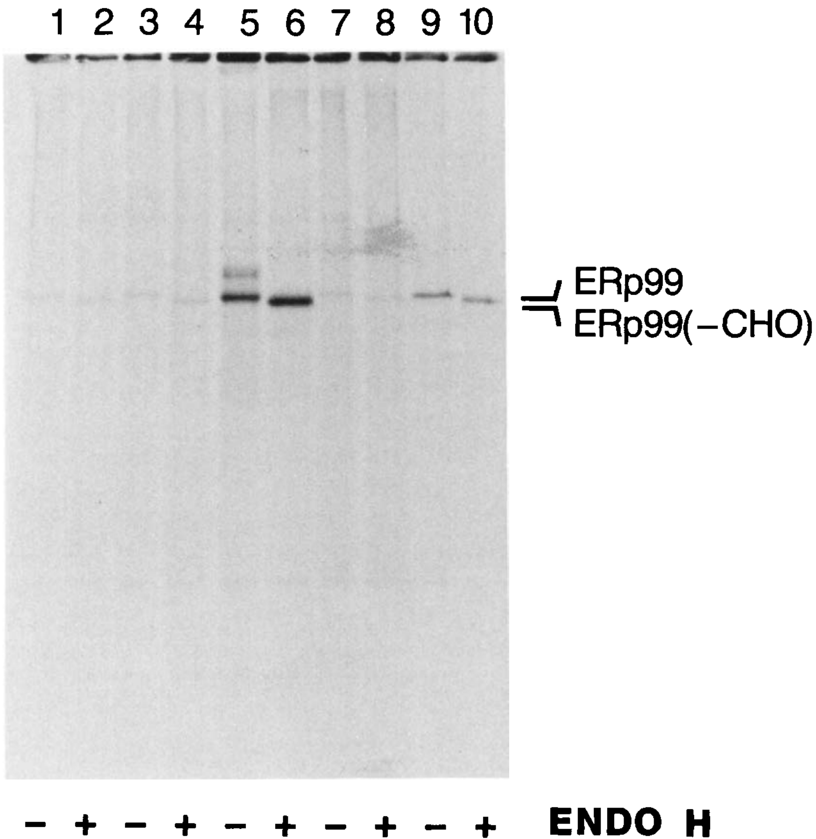


Fig. 10. Expression of cloned murine ERp99 cDNA in COS cells. Two clones containing ERp99 cDNA, pcD99-1 and pcD99-2, were obtained from a transformed mouse fibroblast cDNA library prepared in the pcD shuttle vector. Plasmid DNA was purified from the clones and used to transfect COS cells according to standard procedures. After 72 hr of culture, the cells were labeled with [^{35}S]methionine for 4 hr, washed, and lysed with detergent. Aliquots (2×10^6 TCA precipitable cpm) of the cell lysates were assayed for the presence of murine ERp99 by immunoprecipitation with anti-ERp99 antibody. The immunoprecipitated ERp99 was divided into two aliquots and incubated in the presence (+) or absence (−) of endo H. After this incubation, the products were analyzed by SDS–PAGE. The samples are as follows: Lanes 1,2—mock transfected COS cells; Lanes 3,4—COS cells transfected with pcD99-1 DNA; Lanes 5,6—COS cells transfected with pcD99-2 DNA; Lanes 7,8—untransfected COS cells; Lanes 9,10—authentic ERp99 immunoprecipitated from murine plasmacytoma cells labeled for 4 hr with [^{35}S]methionine. The migration of endoH treated ERp99 is indicated by ERp99-CHO. Taken from Mazzarella and Green (1987).

ERp99 cDNA clone pcD99-2, is depicted in Fig. 11. The entire sequence of both DNA strands of the insert was completely determined by isolating a nested set of subclones for each strand (Dale *et al.*, 1985). The ERp99 amino acid sequence derived from the nucleotide sequence is presented in Fig. 12. The ERp99 precursor encoded by the cDNA consists of 802 amino acids and has a calculated molecular weight of 92,475. The first AUG codon in the cDNA sequence (position −21 of the protein) is preceded by a purine nucleotide three nucleotides upstream of the coding sequence. This is typical of the large majority of functional initiation sites in eukaryotic mRNA (Kozak, 1983). The consensus poly(A) addition signal (Moldave, 1985) is present 14 nucleotides from the poly(A) tail. A comparison of the derived amino acid sequence to the N-terminal sequence obtained directly from the mature protein indicated that the site of

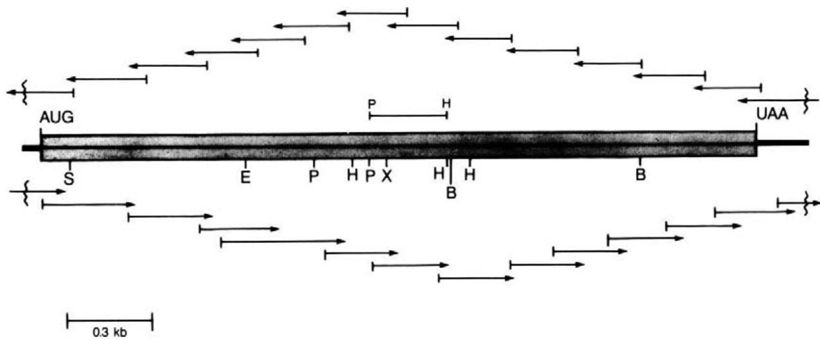


Fig. 11. Sequencing strategy for full-length ERp99 cDNA clone (pcD99-2 insert). The arrows depict the sequences determined from the overlapping set of clones obtained from each strand by the method described in the text. The sequence of each strand was completely determined. The thick line in the middle depicts the relative position of the ERp99 coding region within the insert. The *Pst*I–*Hin*dIII fragment used as a probe in these studies is indicated above the coding region (P—H). Some six-base restriction enzyme sites are indicated: B, *Bcl*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; X, *Xho*I. Taken from Mazzarella and Green (1987).

Fig. 12. Nucleotide sequence and deduced amino acid sequence of murine ERp99 (pcD99-2 cDNA insert). Nucleotides are numbered on the right of each row. The deduced amino acid sequence is shown below the nucleotide sequence. Amino acids are numbered positively beginning with the N-terminus of mature ERp99 and negatively from the site of signal peptide cleavage (▲). The underlined amino acid sequence indicates the sequence confirmed by the direct amino acid sequencing of the N-terminus of mature ERp99. Potential N-linked glycosylation sites are identified by the asterisks. The putative polyadenylation signal is indicated by the box (end of sequence). A poly(A) tail of about 100 nucleotides was found following the T at position 2759. Taken from Mazzarella and Green (1987).

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                                                   TGGAGGTGTGAGGAGCTTAGACTCGGGATTGGGGGGGTGGAGGCGGCTCCTGAG   54

                                   -21                                         -10                                      -1
ACCGAAAAGGACTTGCGACTCGCCGGCCACGCACC ATG AGG GTC CTG TGG GTG TTG GGC CTC TGC TGT GTC CTG CTG ACC TTC GGG TTC GTC AGA GCT  152
                                   Met Arg Val Leu Trp Val Leu Gly Leu Cys Cys Val Leu Leu Thr Phe Gly Phe Val Arg Ala

 1                                       10                                      20                                      30
GAT GAT GAA GTC GAC GTG GAT GGC ACA GTG GAA GAG GAC CTG GGT AAA AGC CGA GAA GGC TCA AGG ACA GAT GAT GAA GTT GTG CAG AGA  242
Asp Asp Glu Val Asp Val Asp Gly Thr Val Glu Glu Asp Leu Gly Lys Ser Arg Glu Gly Ser Arg Thr Asp Asp Glu Val Val Gln Arg
____________________________________________________________________________

                                         40                                      50                                      60
GAG GAA GAA GCT ATT CAG TTG GAT GGG TTA AAC GCA TCA CAG ATA AGA GAA CTT AGA GAA AAA TCT GAA AAG TTC GCC TTC CAA GCT GAA  332
Glu Glu Glu Ala Ile Gln Leu Asp Gly Leu Asn Ala Ser Gln Ile Arg Glu Leu Arg Glu Lys Ser Glu Lys Phe Ala Phe Gln Ala Glu
                                        ********

                                         70                                      80                                      90
GTG AAC AGG ATG ATG AAA CTT ATC ATC AAT TCT TTG TAT AAA AAT AAA GAG ATT TTC CTG AGA GAA CTG ATT TCA AAT GCT TCT GAT GCT  422
Val Asn Arg Met Met Lys Leu Ile Ile Asn Ser Leu Tyr Lys Asn Lys Glu Ile Phe Leu Arg Glu Leu Ile Ser Asn Ala Ser Asp Ala
                                                                                                  ********

                                        100                                     110                                     120
TTA GAC AAG ATA AGG CTC ATC TCC CTA ACT GAT GAA AAT GCA CTC GCT GGA AAT GAG GAG TTA ACG GTC AAG ATT AAG TGT GAC AAA GAG  512
Leu Asp Lys Ile Arg Leu Ile Ser Leu Thr Asp Glu Asn Ala Leu Ala Gly Asn Glu Glu Leu Thr Val Lys Ile Lys Cys Asp Lys Glu

                                        130                                     140                                     150
AAA AAC CTG CTG CAT GTC ACA GAC ACG GGT GTA GGA ATG ACT AGA GAG GAG TTG GTT AAA AAT CTC GGC ACC ATA GCC AAA TCT GGA ACA  602
Lys Asn Leu Leu His Val Thr Asp Thr Gly Val Gly Met Thr Arg Glu Glu Leu Val Lys Asn Leu Gly Thr Ile Ala Lys Ser Gly Thr

                                        160                                     170                                     180
AGC GAG TTT TTA AAC AAA ATG ACA GAA GCT CAA GAA GAT GGT CAG TCA ACC TCT GAA CTG ATT GGC CAG TTT GGT GTC GGT TTT TAT TCT  692
Ser Glu Phe Leu Asn Lys Met Thr Glu Ala Gln Glu Asp Gly Gln Ser Thr Ser Glu Leu Ile Gly Gln Phe Gly Val Gly Phe Tyr Ser

                                        190                                     200                                     210
GCC TTC CTT GTA GCA GAT AAG GTC ATT GTC ACA TCG AAA CAC AAC AAT GAT ACC CAG CAC ATC TGG GAA TCA GAC TCC AAT GAA TTC TCT  782
Ala Phe Leu Val Ala Asp Lys Val Ile Val Thr Ser Lys His Asn Asn Asp Thr Gln His Ile Trp Glu Ser Asp Ser Asn Glu Phe Ser
                                                            ********

                                        220                                     230                                     240
GTA ATT GCT GAC CCA AGA GGA AAC ACA CTA GGT CGT GGA ACA ACA ATT ACT CTT GTC TTA AAA GAA GAA GCA TCT GAT TAC CTT GAA TTG  872
Val Ile Ala Asp Pro Arg Gly Asn Thr Leu Gly Arg Gly Thr Thr Ile Thr Leu Val Leu Lys Glu Glu Ala Ser Asp Tyr Leu Glu Leu

                                        250                                     260                                     270
GAC ACA ATT AAA AAT CTT GTC AGG AAG TAC TCT CAG TTC ATC AAC TTT CCC ATC TAC GTG TGG AGT AGC AAG ACA GAG ACT GTT GAG GAG  962
Asp Thr Ile Lys Asn Leu Val Arg Lys Tyr Ser Gln Phe Ile Asn Phe Pro Ile Tyr Val Trp Ser Ser Lys Thr Glu Thr Val Glu Glu

                                        280                                     290                                     300
CCC TTG GAA GAA GAT GAA GCA GCA AAA GAA GAG AAA GAA GAA TCT GAT GAT GAA GCT GCA GTA GAG GAG GAA GAA GAA GAA AAG AAA CCA 1052
Pro Leu Glu Glu Asp Glu Ala Ala Lys Glu Glu Lys Glu Glu Ser Asp Asp Glu Ala Ala Val Glu Glu Glu Glu Glu Glu Lys Lys Pro

                                        310                                     320                                     330
AAA ACT AAG AAA GTT GAA AAA ACT GTG TGG GAT TGG GAA CTT ATG AAT GAT ATC AAA CCA ATA TGG CAG AGA CCA TCC AAA GAA GTA GAA 1142
Lys Thr Lys Lys Val Glu Lys Thr Val Trp Asp Trp Glu Leu Met Asn Asp Ile Lys Pro Ile Trp Gln Arg Pro Ser Lys Glu Val Glu

                                        340                                     350                                     360
GAA GAC GAA TAC AAA GCT TTC TAC AAA TCA TTT TCA AAG GAA AGT GAT GAC CCC ATG GCT TAT ATC CAC TTC ACT GCA GAA GGG GAG GTC 1232
Glu Asp Glu Tyr Lys Ala Phe Tyr Lys Ser Phe Ser Lys Glu Ser Asp Asp Pro Met Ala Tyr Ile His Phe Thr Ala Glu Gly Glu Val

                                        370                                     380                                     390
ACC TTC AAG TCG ATT TTG TTT GTA CCC ACA TCT GCA CCT CGA GGT CTG TTT GAT GAA TAT GGA TCT AAG AAG AGT GAT TAT ATT AAG CTG 1322
Thr Phe Lys Ser Ile Leu Phe Val Pro Thr Ser Ala Pro Arg Gly Leu Phe Asp Glu Tyr Gly Ser Lys Lys Ser Asp Tyr Ile Lys Leu

                                        400                                     410                                     420
TAT GTA CGC CGC GTA TTC ATC ACA GAT GAC TTC CAT GAT ATG ATG CCC AAA TAC CTT AAT TTT GTC AAA GGT GTT GTG GAT TCC GAT GAT 1412
Tyr Val Arg Arg Val Phe Ile Thr Asp Asp Phe His Asp Met Met Pro Lys Tyr Leu Asn Phe Val Lys Gly Val Val Asp Ser Asp Asp
```

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                                            430                                      440                                      450
CTC CCC CTC AAT GTT TCC CGT GAG ACT CTT CAG CAA CAT AAA TTG CTC AAG GTG ATT AGG AAG AAG CTT GTC CGA AAA ACT CTG GAC ATG 1502
Leu Pro Leu Asn Val Ser Arg Glu Thr Leu Gln Gln His Lys Leu Leu Lys Val Ile Arg Lys Lys Leu Val Arg Lys Thr Leu Asp Met
            ********
                                            460                                      470                                      480
ATC AAG AAG ATT GCT GAT GAG AAG TAT AAC GAC ACT TTC TGG AAG GAG TTC GGC ACG AAT ATC AAG CTT GGT GTG ATT GAA GAC CAC TCA 1592
Ile Lys Lys Ile Ala Asp Glu Lys Tyr Asn Asp Thr Phe Trp Lys Glu Phe Gly Thr Asn Ile Lys Leu Gly Val Ile Glu Asp His Ser
                                    ********
                                            490                                      500                                      510
AAT CGA ACA CGG CTT GCT AAA CTT CTT AGG TTC CAG TCT TCT CAC CAT TCA ACT GAC ATT ACT AGT TTA GAC CAG TAT GTG GAA AGA ATG 1682
Asn Arg Thr Arg Leu Ala Lys Leu Leu Arg Phe Gln Ser Ser His His Ser Thr Asp Ile Thr Ser Leu Asp Gln Tyr Val Glu Arg Met
********
                                            520                                      530                                      540
AAG GAA AAA CAG GAC AAA ATC TAC TTC ATG GCT GGG TCA AGC AGA AAG GAG GCG GAA TCT TCT CCA TTT GTT GAG AGA CTT CTG AAG AAG 1772
Lys Glu Lys Gln Asp Lys Ile Tyr Phe Met Ala Gly Ser Ser Arg Lys Glu Ala Glu Ser Ser Pro Phe Val Glu Arg Leu Leu Lys Lys

                                            550                                      560                                      570
GGC TAT GAA GTC ATT TAT CTC ACA GAG CCT GTG GAT GAA TAC TGC ATT CAG GCT CTT CCC GAG TTT GAT GGG AAG AGG TTT CAG AAT GTT 1862
Gly Tyr Glu Val Ile Tyr Leu Thr Glu Pro Val Asp Glu Tyr Cys Ile Gln Ala Leu Pro Glu Phe Asp Gly Lys Arg Phe Gln Asn Val

                                            580                                      590                                      600
GCC AAA GAA GGG GTG AAG TTT GAT GAG AGT GAG AAA ACT AAA GAA AGT CGG GAA GCA ACA GAG AAG GAG TTT GAA CCT CTG CTC AAC TGG 1952
Ala Lys Glu Gly Val Lys Phe Asp Glu Ser Glu Lys Thr Lys Glu Ser Arg Glu Ala Thr Glu Lys Glu Phe Glu Pro Leu Leu Asn Trp

                                            610                                      620                                      630
ATG AAA GAT AAG GCC CTC AAG GAC AAG ATA GAA AAG GCT GTG GTG TCG CAG CGC CTC ACA GAG TCT CCC TGT GCT CTT GTG GCC AGT CAG 2042
Met Lys Asp Lys Ala Leu Lys Asp Lys Ile Glu Lys Ala Val Val Ser Gln Arg Leu Thr Glu Ser Pro Cys Ala Leu Val Ala Ser Gln

                                            640                                      650                                      660
TAT GGA TGG TCT GGC AAC ATG GAG AGG ATC ATG AAG GCA CAA GCA TAC CAG ACG GGC AAG GAC ATC TCT ACA AAT TAC TAT GCC AGT CAA 2132
Tyr Gly Trp Ser Gly Asn Met Glu Arg Ile Met Lys Ala Gln Ala Tyr Gln Thr Gly Lys Asp Ile Ser Thr Asn Tyr Tyr Ala Ser Gln

                                            670                                      680                                      690
AAG AAA ACG TTC GAA ATC AAT CCT AGA CAC CCA CTG ATC AGA GAC ATG TTG CGG CGG ATT AAG GAA GAT GAA GAT GAC AAG ACA GTC ATG 2222
Lys Lys Thr Phe Glu Ile Asn Pro Arg His Pro Leu Ile Arg Asp Met Leu Arg Arg Ile Lys Glu Asp Glu Asp Asp Lys Thr Val Met

                                            700                                      710                                      720
GAT CTT GCT GTA GTT TTG TTT GAA ACG GCA ACA CTT CGG TCA GGA TAT CTT CTA CCA GAC ACC AAG GCG TAT GGA GAT AGA ATA GAA AGA 2312
Asp Leu Ala Val Val Leu Phe Glu Thr Ala Thr Leu Arg Ser Gly Tyr Leu Leu Pro Asp Thr Lys Ala Tyr Gly Asp Arg Ile Glu Arg

                                            730                                      740                                      750
ATG CTT CGC CTC AGT TTA AAC ATT GAC CCT GAA GCA CAG GTG GAG GAA GAA CCA GAA GAA GAG CCT GAA GAC ACC TCA GAA GAC GCA GAA 2402
Met Leu Arg Leu Ser Leu Asn Ile Asp Pro Glu Ala Gln Val Glu Glu Glu Pro Glu Glu Glu Pro Glu Asp Thr Ser Glu Asp Ala Glu

                                            760                                      770                                      780
GAC TCA GAG CAG GAT GAG GGA GAA GAG ATG GAT GCA GGG ACA GAA GAA GAA GAG GAG GAA ACA GAA AAG GAA TCT ACA GAG AAG GAT GAA 2492
Asp Ser Glu Gln Asp Glu Gly Glu Glu Met Asp Ala Gly Thr Glu Glu Glu Glu Glu Glu Thr Glu Lys Glu Ser Thr Glu Lys Asp Glu

781
TTG TAA  ATTATACTCTCGCTATGAATCCCGTGTGGAGAGGGAATGTGAAGTTTTGAAGTCATTTCTTTTGAGAGACTTGTTTTGGATGCTTCCCCAAGCCTCCTTCTCCCCTGCAC 2608
Leu Stop

TGTAAAATGTTGGGATTATGGGTCACAGGAAGAAGTGGTTTTTTTAGTTGAATTTTTTTTAACATTCCTCCTGAATGTAAATTTGTACTATTTAACTGACTATTGGTGTAAAATCTTGT 2727

CATGTGTATAAAAATAAAAAAAGATCCCAAAT                                                                                          2759
```

signal peptide cleavage was between the Ala–Asp residues (positions −1 and 1, respectively), removing the first 21 amino acids. The ERp99 signal peptide has the typical features of a leader peptide. It possesses a hydrophobic central section preceded by a positively charged N-terminus and followed by two small hydrophobic residues separated by one amino acid (Watson, 1984). The calculated molecular weight of mature ERp99, 90,096, is in good agreement with the size of the polypeptide moiety of ERp99 estimated by SDS–PAGE. There are six potential N-glycosylation sites in the ERp99 sequence. Our earlier results have shown that only one of these sites is used under normal circumstances (Lewis *et al.*, 1985b). We have used this information to develop a possible model for the orientation of ERp99 in the membrane.

Hydropathy analysis of the protein sequence (Fig. 13) reveals, in addition to the expected hydrophobic signal peptide, a strongly hydrophobic 21–23 residue region, beginning at Leu 170, which has the characteristics of a membrane-spanning region. There are other regions of the polypeptide that have moderate hydrophobicity and that might also be membrane-spanning segments, but analysis of these regions using the algorithm of Eisenberg *et al.* (1984) showed that only the segment beginning at position 170 possessed the necessary characteristics of a membrane-spanning region. ERp99 also possesses two long, highly charged regions from positions 265 to 347 and from 731 to the C terminus.

A large data base (Barker *et al.*, 1987) was used to search for homology between ERp99 and other protein sequences. Extensive homology was observed between ERp99 and hsp90 of *Saccharomyces cerevisiae* and hsp83 of *Drosophila melanogaster*. A comparison of the sequences of ERp99 and hsp90 is shown in Fig. 14. The two sequences show 46.5% identity over a 719 amino acid overlap with the indicated gaps introduced for optimal alignment. Similarly, ERp99 showed 47.4% identity over a 382 amino acid overlap with hsp83. A search of the literature revealed that the

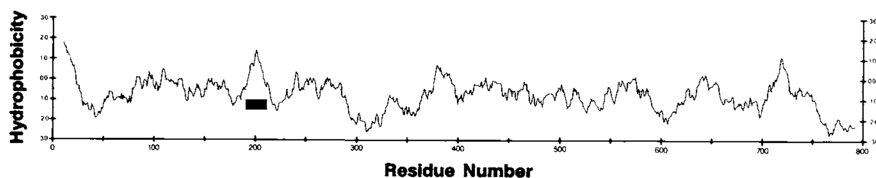

Fig. 13. Hydropathy plot of the predicted amino acid sequence of murine ERp99. The hydropathy plot was obtained using the algorithm and hydropathy values of Kyte and Doolittle (1982) for an amino acid segment length of 21. Hydrophobic regions are above the line and drophilic regions are below. The position of the putative membrane-spanning region is indicated by the dark box. Taken from Mazzarella and Green (1987).

```
               10        20        30        40        50        60        70        80        90       100       110       120
ERp99 MRVLWVLGLCCVLLTFGFVRADDEVDVDGTVEEDLGKSREGSRTDDEVVQREEEAIQLDGLNASQIRELREKSEKFAFQAEVNRMMKLIINSLYKNKEIFLRELISNASDALDKIRLISL
                                                                             :: : ::::    : ::::  : ::::::::::::::::::::  ::
hsp90                                                                       MASETFEFQAEITQLMSLIINTVYSNKEIFLRELISNASDALDKIRYKSL
                                                                                     10        20        30        40        50

               130       140       150       160       170       180       190       200       210       220       230
      TDENALAGNEELTVKIKCDKEKNLLHVTDTGVGMTREELVKNLGTIAKSGTSEFLNKMTEAQEDGQSTSELIGQFGVGFYSAFLVADKVIVTSKHNNDTQHIWESD-SNEFSVIADPRGN
       :   :     :   :     :    :    : : :::  ::  :::::::::  :                :::::::::: ::::: : : :: : : : ::::      : :  :
      SDPKQLETEPDLFIRITPKPEQKVLEIRDSGIGMTKAELINNLGTIAKSGTKAFMEAL-----SAGADVSMIGQFGVGFYSLFLVADRVQVISKSNDDEQYIWESNAGGSFTVTLDEVNE
               60        70        80        90        100            110       120       130       140       150       160

      240       250       260       270       280       290           300       310          320       330       340       350
      TLGRGTTITLVLKEEASDYLELDTIKNLVRKYSQFINFPIYVWSSKTETVEEPL------EEDEAAKEEKEESDDEAAVEE--EEEEKKPKTKKVEKTVWDWELMNDIKPIWQRPSKEVE
      ::::  : ::       :::  ::        : :   ::     :     : :           :     : : : :     ::  :::::::::::     :    :   :  :: : :
      RIGRGTILRLFLKDDQLEYLEEKRIKEVIKRHSEFVAYPIQLVVTKEVEKEVPIPEEEKKDEEKKDEEKKDEDDKKPKLEEVDEEEEKKPKTKKVKEEVQEIEELNKTKPLWTRNPSDIT
          170       180       190       200       210       220       230       240       250       260       270       280

               360       370       380       390       400       410       420       430       440       450       460       470
      EDEYKAFYKSFSKESDDPMAYIHFTAEGEVTFKSILFVPTSAPRGLFDEYGSKKSDYIKLYVRRVFITDDFHDMMPKYLNFVKGVVDSDDLPLNVSRETLQQHKLLKVIRKKLVRKTLDM
       :: ::::: :     ::     ::   ::    :  ::: :  :: ::   ::         :::::::::::    :   :  :::::::: ::::: :::: ::: :   :::::  : :
      QEEYNAFYKSISNDWEDPLYVKHFSVEGQLEFRAILFIPKRAPFDLFES--KKKKNNIKLYVRRVFITDEAEDLIPEWLSFVKGVVDSEDLPLNLSREMLQQNKIMKVIRKNIVKKLIEA
          290       300       310       320       330        340       350       360       370       380       390       400

               480       490       500       510       520       530       540       550       560       570       580       590
      IKKIADEKYN-DTFWKEFGTNIKLGVIEDHSNRTRLAKLLRFQSSHHSTDITSLDQYVERMKEKQDKIYFMAGSSRKEAESSPFVERLLKKGYEVIYLTEPVDEYCIQALPEFDGKRFQN
        ::          :    :  :::::: ::  ::  ::::::  :            :::  :: :: : :  ::      : : :   : :::       :    :  ::  :: : :::     : :: ::
      FNEIAEDSEQFEKFYSAFSKNIKLGVHEDTQNRAALAKLLRYNSTKSVDELTSLTDYVTRMPEHQKNIYYITGESLKAVEKSPFLDALKAKNFEVLFLTDPIDEYAFTQLKEFEGKTLVD
          410       420       430       440       450       460       470       480       490       500       510       520

               600       610       620       630       640       650       660       670       680       690       700
      VAKEGVKFDESEKTKESREATEKEFEPLLNWMKDKALKDKIEKAVVSQRLTESPCALVASQYGWSGNMERIMKAQAYQTGKDISTNYYASQKKTFEINPRHPLIRDMLRRIKE-DEDDKT
        :      :     :  ::    :: :::      :     : :   :: :::  :     : :      : ::: ::::::::::        : :     : : :::::: :    : :          :   :      :::
      ITKD-FELEETDEEKAEREKEIKEYEPLTKALKE-ILGDQVEKVVVSYKLLDAPAAIRTGQFGWSANMERIMKAQAL---RDSSMSSYMSSKKTFEISPKSPIIKELKKRVDEGGAQDKT
             530       540       550       560       570       580       590            600       610       620       630

      710       720       730       740       750       760          770       780       790       800
      VMDLAVVLFETATLRSGYLLPDTKAYGDRIERMLRLSLNIDPEAQVEEEPEEEPEDTSED-AEDSEQDEGEEMDAGTEEEEEETEKESTEKDEL
      : ::    : ::: : :: :          :: :    : ::::        :  ::            :        :  :  :
      VKDLTKLLYETALLTSGFSLDEPTSFASRINRLISLGLNIDEDEETETAPEASTAAPVEEVPADTEMEEVD
       640       650       660       670       680       690       700
```

Fig. 14. Comparison of the sequence of murine ERp99 and hsp90 of *Saccharomyces cerevisiae*. The sequences have been aligned for maximum homology. The identical amino acids are indicated by the dots. Taken from Mazzarella and Green (1987).

first 14 amino acids of mature ERp99 are identical to the N-terminal sequence reported for GRP94 (Lee *et al.*, 1984) and that the C-terminal half of ERp99 is 96% identical to the sequence derived from a cDNA clone encoding the C-terminal half of GRP94 (Lee *et al.*, 1983; Munro and Pelham, 1986). Other similarities which strengthen this finding are that, like ERp99, GRP94 is a dimeric glycoprotein (Lee *et al.*, 1984) and is encoded by an abundant mRNA of approximately 3000 nucleotides (Lee *et al.*, 1983). Furthermore, a comparison of the ERp99 cDNA sequence with the cDNA sequence determined for "hsp108" by Kulomaa (1986) indicates that these proteins are 91% identical. An alignment of these sequences is given in Fig. 15. Thus, the relationship between the ER protein ERp99, and hsp90, hsp83, and GRP94 is analogous to that reported for the rat liver hsp70-related protein, p72 and *D. melanogaster* hsp70, GRP78 and heavy chain binding protein (BiP) (Munro and Pelham, 1986).

III. CONCLUSIONS AND FUTURE DIRECTIONS

The elucidation of the specific sorting signal(s) that define a membrane protein as an ER protein will require further investigation. The studies that have been reported so far help to focus our attention on protein domains which could contain sorting signals dictating ER localization. The results described by Kabcenell and Atkinson (1985) in their investigation of the sorting of the rotavirus VP7 protein emphasized the role of an amino-terminal transmembrane domain as a positive signal for ER localization. In addition, the observations that the incorporation of a charged amino acid in the middle of the transmembrane region (Adams and Rose, 1985a), or the deletion of a substantial part of the transmembrane region (Adams and Rose, 1985b) blocked transport of VSV G protein to the cell surface, but not membrane insertion, are consistent with the view that the transmembrane domains of membrane proteins can contain important information for protein sorting as the proteins traverse the transport pathway. On the other hand, the observation that many ER proteins have a significant portion of their protein chain exposed on the cytoplasmic side of the ER membrane has been interpreted to support the possibility that the cytoplasmic domain of ER proteins may contain positive or negative sorting signals which result in ER localization of the particular protein (Kreibich *et al.*, 1983b; Paabo *et al.*, 1987). Recent observations by Munro and Pelham (1987) and Medda *et al.* (1987) implicate the importance of luminal domains resulting in the ER localization of ER proteins. As more ER proteins are purified, cloned, sequenced, and analyzed in

```
                 10        20        30        40        50        60        70        80        90       100       110       120
MOUSE    MRVLWVLGLCCVLLTFGFVRADDEVDVDGTVEEDLGKSREGSRTDDEVVQREEEAIQLDGLNASQIRELREKSEKFAFQAEVNRMMKLIINSLYKNKEIFLRELISNASDALDKIRLISL
CHICKEN  .KSA.A.A.A.T..LAAS.T.-E.....A.....................................K.I...................................................

                130       140       150       160       170       180       190       200       210       220       230       240
MOUSE    TDENALAGNEELTVKIKCDKEKNLLHVTDTGVGMTREELVKNLGTIAKSGTSEFLNKMTEAQEDGQSTSELIGQFGVGFYSAFLVADKVIVTSKHNNDTQHIWESDSNEFSVIADPRGNT
CHICKEN  .................G.....M.......I...K...I....................M.D.S......................R.........................D......

                250       260       270       280       290       300       310       320       330       340       350       360
MOUSE    LGRGTTITLVLKEEASDYLELDTIKNLVRKYSQFINFPIYVWSSKTETVEEPLEEDEAAKEEKEESDD-EAAVEEEEEEKKPKTKKVEKTVWDWELMNDIKPIWQRPSKEVEEDEYKAFYK
CHICKEN  .......................V....K.......................V..E..-......T..N....................................................

                370       380       390       400       410       420       430       440       450       460       470       480
MOUSE    SFSKESDDPMAYIHFTAEGEVTFKSILFVPTSAPRGLFDEYGSKKSDYIKLYVRRVFITDDFHDMMPKYLNFVKGVVDSDDLPLNVSRETLQQHKLLKVIRKKLVRKTLDMIKKIADEKY
CHICKEN  T....H........................N................F....................................................................E...
HAMSTER                                                     .............................................................................

                490       500       510       520       530       540       550       560       570       580       590       600
MOUSE    NDTFWKEFGTNIKLGVIEDHSNRTRLAKLLRFQSSHHSTDITSLDQYVERMKEKQDKIYFMAGSSRKEAESSPFVERLLKKGYEVIYLTEPVDEYCIQALPEFDGKRFQNVAKEGVKFDE
CHICKEN  ...........V.........................ESNL......................A......................................................E.
HAMSTER  ........................................................................................................................

                610       620       630       640       650       660       670       680       690       700       710       720
MOUSE    SEKTKESREATEKEFEPLLNWMKDKALKDKIEKAVVSQRLTESPCALVASQYGWSGNMERIMKAQAYQTGKDISTNYYASQKKTFEINPRHPLIRDMLRRIKEDEDDKTVMDLAVVLFET
CHICKEN  ...S......L........................L.....Q....................................................K.....V..N......S.........
HAMSTER  ......N.............R..................................................A...............A............V.........L.........

                730       740       750       760       770       780       790       800
MOUSE    ATLRSGYLLPDTKAYGDRIERMLRLSLNIDPEAQVEEEPEEEPEDTSEDAEDSEQDEGEEMDAGTEEEEEE--TEKEST-EKDEL
CHICKEN  .......M.....E................LD.D.....-.....AA.E..---...-..V..DA.DS.----.Q....DV....
HAMSTER  ...............A..............................T..---T....E..V..........EQ.A....A.....
```

Fig. 15. Amino acid conservation of ERp99 (GRP94). A comparison of the amino acid sequences of ERp99 from mouse (Mazzarella and Green, 1987), chicken (Kulomaa *et al.*, 1986), and hamster (Sorger and Pelham, 1987) is shown. Identity to murine ERp99 is indicated by a dot. In cases where the sequences differ, the particular amino acid found at that position is listed. A dash is used to indicate where gaps were introduced to produce optimal alignment. A comparison of mouse ERp99 and chicken ERp99 reveals that they have 91% homology; mouse ERp99 and hamster ERp99 have 96% identity.

sorting studies, a description of which signal or mixture of signals is operating to localize a particular protein to the ER membrane should emerge. Our studies of ERp99 should provide significant contributions to our understanding of the ER protein sorting problem.

We have formulated a simple model for the way in which ERp99 is oriented in the membrane (Fig. 16). This model is based on data from our earlier work (Lewis *et al.*, 1985b) as well as from the results described in this paper. ERp99 is synthesized as a precursor with a cleavable signal peptide (Figure 7). We have placed the N-terminus within the lumen to account for this fact. ERp99 is a glycoprotein with, in all likelihood, only one N-linked oligosaccharide. Analysis of the sequence indicates that ERp99 has six potential sites for N-linked glycoslation. Our model places only two of these sites in the lumen where they have the potential to be acceptors for the core oligosaccharide. The presence of two sites in the lumen, one utilized and the other cryptic, could explain our observations concerning ERp99 expression in COS cells. An overabundance of ERp99 in the ER membrane may allow detection of an abnormal N-glycosylation reaction at the second site. Analysis of the hydrophobicity of the ERp99 sequence indicates that the best candidate for a transmembrane region is the 21–23 residue hydrophobic segment beginning at position 170. Our model uses this segment as the membrane-spanning, stop transfer region allowing ERp99 to pass through the membrane only once with 75% of the protein remaining on the cytoplasmic side of the membrane. Analysis of the hydrophobicity and hydrophobic moment of this segment using the algorithm of Eisenberg *et al.* (1984) indicated that the values for this region are characteristic of transmembrane regions of multimeric proteins

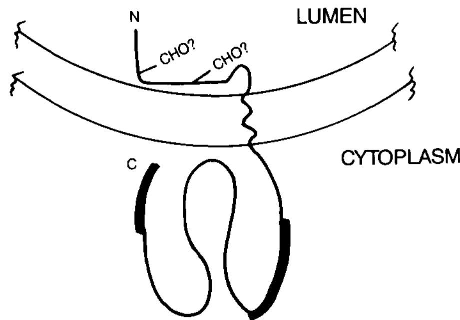

Fig. 16. Model for the membrane orientation of murine ERp99. The model employs one membrane-spanning region (residues 170 to 192). Two potential glycosylation sites (CHO) are within the lumen. It is not known which is utilized as the actual acceptor. The positions of the highly hydrophilic regions are indicated by the thick lines. Taken from Mazzarella and Green (1987).

(e.g., membrane IgM). In this regard, it is interesting to note that when ERp99 is analyzed under nonreducing conditions, approximately 50% of the protein is found in a homodimer (R. Mazzarella, unpublished observation). The proposed orientation places all the hydrophilic regions of ERp99 on the cytoplasmic side of the membrane. This is similar to the model proposed for the orientation of the signal recognition particle (SRP) receptor (Lauffer *et al.*, 1985). It is also consistent with our earlier observation that ERp99 is completely susceptible to proteases in intact membrane preparations (Lewis *et al.*, 1985b). Under the conditions used in these experiments, known luminal proteins were protected from degradation. The newly synthesized IgA produced by the MOPC-315 plasmacytoma cell used in these studies was resistant to papain digestion, and another ERp, ERp59, which we have recently shown by immunological criteria (R. B. Freedman, personal communication) and DNA sequence analysis (R. Mazzarella and M. Green, unpublished results) to be the enzyme protein disulfide isomerase, a known luminal enzyme, was resistant to both papain and trypsin digestion (Lewis *et al.*, 1986). From our model, a peptide of approximately 20,000 Da would be expected to survive protease treatment. We have not observed such a fragment. It is possible that the anti-ERp99 polyclonal antibody used to analyze the protease protection experiments cannot recognize, or binds only weakly to, this fragment of ERp99. It should be possible to test our model. Using the deduced amino acid sequence, we can prepare antipeptide antibodies against different regions of ERp99. These antibodies can be assayed for their ability to react with ERp99 in intact microsomes. Antibodies against putative luminal regions should bind to the protein only after the disruption of microsomes or the solubilization of the protein. This approach was used successfully to analyze the orientation of HMG-CoA reductase, another ER protein (Liscum *et al.*, 1985).

A comparison of the ERp99 sequence with the sequences of other proteins has shown that ERp99 is homologous to hsp90 of *S. cerevisiae* and hsp83 of *D. melanogaster*. In addition, ERp99 is identical to GRP94. This observation is analogous to the finding by Munro and Pelham (1986) that the heat shock-related protein designated p72 is homologous to hsp70 from *D. melanogaster*, GRP78 and the ER protein BiP, the immunoglobulin heavy chain binding protein. It is interesting to note that, similar to the relationship of p72 and hsp70, the overlap of ERp99 with hsp90 is not observed until approximately 70 amino acids into the ERp99 sequence. This extra N-terminal sequence contains the signal peptide which is responsible for mediating the initial sorting of ERp99 to the ER membrane in addition to one of the proposed N-glycosylation sites. The other potential site (ERp99 position 86) is conserved between ERp99 and hsp90. In

contrast to the overlap between p72 and hsp70, however, ERp99 also has extra amino acids at its C-terminal end. Our model of ERp99 places most of the protein on the cytoplasmic side of the ER membrane, unlike p72 which is in the lumen of the ER. Thus, our hypothesis differs from that of Pelham who has indicated that GRP94 may be a luminal ER protein by virtue of the fact that it, like GRP78 and protein disulfide isomerase (Pelham, 1986), possesses the common C-terminal sequence Lys-Asp-Glu-Leu-COOH (KDEL) (Edman *et al.*, 1985). It does not necessarily follow, however, that because ERp99 possess this sequence, it is a luminal protein. Indeed, if this sequence fulfills the same function for ERp99 that it does for GRP78, it would only be necessary that this part of the protein be in the lumen of the ER. In this case, our model (Fig. 16) would have to be modified by postulating that the ERp99 protein chain passes through the membrane twice, once at the indicated region and again near the C terminus. Analysis of the protein sequence, using the algorithm of Eisenberg *et al.* (1984), indicates that there is another potential membrane-spanning region, albeit a relatively poor one, from residues 687 to 707 that could serve this function. It is our aim to test our working model directly by using methods designed to produce specific truncations, internal deletions, and point mutations within the ERp99 coding sequence and to investigate the effect of these alterations on the sorting and membrane orientation of the mutated proteins expressed from these clones.

In addition to using ERp99 as a model system to study ER protein sorting, it is interesting to determine the function of ERp99 in the ER membrane. No enzymatic activity has been assigned to hsp90, GRP94, or related proteins. Hsp90, however, is abundant in many cells and has been reported to have several activities. Hsp90 forms a complex with pp60^{src} and a 50,000 Da phosphoprotein in cultured avian cells (Opperman *et al.*, 1981; Brugge *et al.*, 1981; Adkins *et al.*, 1982). Other viral transforming proteins containing tyrosine specific kinase activity, pp140^{fps} and pp94^{yes}, have also been shown to be transiently associated with hsp90 (Lipsich *et al.*, 1982). Recently, hsp90 has been shown to bind to the steroid hormone receptor (Sanchez *et al.*, 1985; Schuh *et al.*, 1985; Catelli *et al.*, 1985) and to actin (Catelli *et al.*, 1985). It has been postulated that all of these findings point to the involvement of hsp90 as a carrier for the transportation of important biological molecules within the cell (Koyasu, 1986). In contrast to our finding that ERp99 is localized to the ER, other laboratories have reported that the larger glucose regulated protein is localized to the Golgi (Lin and Quelly, 1982; Welch *et al.*, 1983) or to the plasma membrane (Pouyssegur and Yamado, 1978; McCormick *et al.*, 1982). Given the fact that these proteins are identified merely by their molecular weight on SDS–PAGE and, in some cases, their isoelectric point and that

there is a paucity of sequence information available about them, it is possible that these other, larger glucose-regulated proteins are not identical to ERp99. When more sequence data are obtained, it should be possible to reconcile these findings.

We have described what appears to be a novel relative in the hsp90 family of proteins. Future work will focus on trying to understand if and how the ER localization of ERp99 is involved in its function. It is possible, for example, that ERp99 has a function analogous to the one proposed for p72 (BiP) but performs this function on the cytoplasmic side of the ER membrane. It is also possible, however, that although there is extensive sequence homology among the hsp90 family, they fulfill different functions within the cell. Given the relatively strict localization of ERp99 to the RER and its transmembrane orientation in the ER membrane, we are currently investigating the possibility that ERp99 has a role in the translocation of nascent proteins through the ER membrane. Approaches being used include the expression of cloned ERp99 and derivatives in yeast to determine if ERp99 can restore viability to mutants in which the HSP90 genes have been inactivated. In addition, the use of antisense RNA strategies (Izant and Weintraub, 1984; Kim and Wold, 1985) could provide insights into the role of ERp99 in cells under normal and "stressful" growth conditions. Our study of ERp99 should provide valuable information concerning the mechanism of protein transport and sorting, as well as the possible functions of heat shock and "stress" proteins.

In this way, it should be possible to construct a sorting fate map of a protein, such as ERp99, in which alterations producing changes in the protein localization could be superimposed on the ERp99 amino acid sequence and structure. This map will be useful in several ways. By determining which alterations in the ERp99 sequence produce the positive effect of movement out of the ER compartment, we hope to gain insight into the manner in which ERp99 is initially localized in the ER membrane. In addition, depending upon how far along the normal transport pathway the altered ERp99 molecules travel, we may also gain insights into features of the protein that are necessary for the complete transport of the protein to the plasma membrane or into the medium as a secreted protein. Furthermore, if specific features of the ERp99 molecule (i.e., the transmembrane regions or the cytoplasmic domain) can be shown to be responsible for its ER localization, it should be possible to confirm this observation by constructing suitable chimeric molecules between ERp99 and either secretory protein sequences, or plasma membrane protein sequences. These types of experiments have the drawback that the construction of these chimeras result in "new" proteins that may

not behave in simple, predictable ways. They should prove useful, however, to confirm the results of mutagenesis studies and to determine if any ERp99 domains contain ER localization information when excised from the molecule and fused to foreign proteins.

In the future, it should be possible to use the results obtained from sorting studies of proteins such as ERp99 to gain further insight into the mechanism of protein transport in eukaryotic cells. It would be worthwhile, for example, to determine if there are specific proteins which interact with ERp99 to either keep it in the ER normally or allow it to be transported out of the ER, if it is altered in a suitable way. In addition, it may be possible to select mutants of eukaryotic cells with altered components of the transport pathway by using the recombinant DNA constructions to create a situation in which such mutants can be selected. For example, if it was observed that a VSV-G–ERp99 chimera was sorted to the ER, it should be possible to construct a stable expression vector containing this chimera, transform a suitable host cell with it, and select a mutant cell in which this chimera was transported to the surface and could be detected by the expression of the VSV domain on the plasma membrane. This type of study should help us to begin to elucidate the cellular machinery involved in protein transport. Finally, it should be possible to accomplish the expression of ERp99 in yeast in order to take advantage of both the availability of secretion mutants in that system (for review, see Schekman, 1985) and the possibility of producing large amounts of altered ERp99 molecules for chemical and physical studies which could complement our studies of the effects of protein sequence alterations on ERp99 sorting. We believe that the study of the sorting of proteins such as ERp99 will provide a rich source of experimental results and scientific insights that will make a significant contribution to our knowledge of protein transport and sorting in general.

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