Transmembrane Topology of the Mammalian KDEL Receptor

PARAMJEET SINGH, BOR LUEN TANG, SIEW HENG WONG, AND WANJIN HONG*

Membrane Biology Laboratory, Institute of Molecular and Cell Biology, National University of Singapore, Singapore 0511, Singapore

Received 4 June 1993/Returned for modification 30 June 1993/Accepted 26 July 1993

The mammalian KDEL receptor is an integral membrane protein with seven hydrophobic regions. Fusion proteins comprising a 37-kDa N-glycosylation reporter fused downstream of amino-terminal fragments of the KDEL receptor with varying numbers of hydrophobic regions were synthesized in an in vitro translation system containing canine pancreatic microsomes. The luminal or cytosolic orientation of the reporter, and hence of the hydrophilic region to which it is fused, was inferred from the presence or absence of glycosylation, which occurs only in the lumen of the microsomes. The cytosolic orientation of the N and C termini was also confirmed immunocytologically. Our results suggest that the KDEL receptor is inserted into the membrane with only six transmembrane domains and that both the amino and carboxy termini are located in the cytoplasm.

Proteins resident in the lumen of the endoplasmic reticulum (ER) continually leave the compartment with the bulk flow of vesicular traffic to the Golgi complex (16). From the Golgi apparatus, they are specifically recovered by the retrograde pathway on the basis of their carboxy-terminal tetrapeptide sequence KDEL (or a closely related sequence), which is both necessary and sufficient for ER localization (17, 18). Recently, it was demonstrated that the KDEL (HDEL) sequence is also sufficient to confer ER localization on membrane proteins with their C terminus in the lumen (6, 23, 24). The receptor for this signal was initially identified in Saccharomyces cerevisiae as a 26-kDa membrane protein encoded by the ERD2 gene (21), and closely related mammalian homologues have been identified (10, 25). This receptor, which determines the specificity and capacity of the retention system (12, 21), has recently been shown to bind the KDEL ligand in vitro (26).

Although the exact mechanism of the retrieval process is not known, it is believed that soluble ER proteins bind the receptor in the cis-Golgi. The ligand-occupied receptor then enters the retrograde pathway which delivers the complex to the ER (11). Here, the soluble proteins are released, possibly through a pH-dependent change in the binding activity of the receptor (26). The regenerated receptor then returns to the Golgi complex by bulk vesicular transport to reenter the cycle. We have recently demonstrated that the receptor is indeed localized to the cis-Golgi and can recycle between the Golgi complex and the ER (25).

While much effort has been directed at elucidating the biochemical and molecular dynamics of the KDEL receptor, the transmembrane orientation of the protein has remained unaddressed. This knowledge is essential for detailed studies on the structure-function relationship of the receptor and could provide insights on the regions that might potentially interact with cytosolic factors involved in its intracellular migrations. We have therefore investigated the transmembrane topology of the KDEL receptor. The amino acid sequence deduced from its cDNA sequence exhibits seven hydrophobic regions (10, 25) (Fig. 1a) which have all been suggested to transverse the membrane (2, 10). We tagged the protein at its amino terminus with the 10-residue c-myc epitope and at various other points with an N-glycosylation reporter. The luminal or cytoplasmic placement of the reporter after insertion into the membrane reflected the topological position of its fusion point. A composite picture of the topology was formed from the various fusion proteins. We show that the KDEL receptor is inserted into the membrane with only six transmembrane domains and that both termini are exposed to the cytoplasm.

**MATERIALS AND METHODS**

**Materials.** DNA modification and restriction enzymes were from Bethesda Research Laboratories or Amersham plc. Oligonucleotides from Oligos Etc. Inc. were used without further purification. T7 and Sp6 polymerases, pGEM-11Zf(+) DNA, rabbit reticulocyte lysate, RQ1 DNase I, and dog pancreas microsomal membranes were from Promega. The transfection reagent DOTAP {N-[1-(2,3-dioleoyloxy) propyl]-N,N,N-trimethylammonium methylsulfate} and anti-immunoglobulin G antibodies coupled to fluorescein or rhodamine were from Boehringer Mannheim. Monoclonal antibodies (clone 9E10) to c-myc were from Oncogene Science while polyclonal antisera to p23 (KDEL receptor) and β-1,4-galactosyltransferase (GT) have been previously described (22, 25). The human breast carcinoma cell line BT-20 was from the American Type Culture Collection. All other reagents were from Sigma.

**Construction of recombinant mERD2.** Sequences coding for single or tandem N-glycosylation sites (N-X-S or N-X-S-N-X-S) were created at selected positions in the coding region of the mERD2 cDNA through polymerase chain reaction-mediated point or insertion mutagenesis (14), and the full-length recombinant cDNA was cloned between the EcoRI and XhoI sites of the in vitro expression vector pGEM-11Zf(+). The sequence for the 10-residue c-myc epitope, EQKLISEEDL (11), was similarly inserted between the second and third codons of mERD2, and the resulting cDNA was cloned between the EcoRI and XhoI sites of the mammalian expression vector pXJ41neo (27), under the control of the cytomegalovirus promoter. Fusions of p23 fragments to a glycosylation reporter domain were achieved by replacing the first 32 codons of the full-length dipetidyl peptidase IV (DPPIV) cDNA in pGEM-4Z with polymerase chain reaction-generated fragments coding for...
graded N-terminal stretches of p23. Restricting the plasmids with StuI before in vitro transcription yielded open reading frames beginning with p23 sequences and ending with 972 bp coding for the 36-kDa region of DPPIV with six potential N-glycosylation sites. All routine DNA experiments were performed according to standard protocols (1).

**In vitro transcription and translation.** About 5 μg of plasmid DNA that had been linearized with an appropriate restriction enzyme was transcribed in a 50-μl reaction with the Promega kit. The RNA transcripts from each reaction were purified and translated in rabbit reticulocyte lysate supplemented with dog pancreas microsomal membranes and [35S]methionine (50 μCi per reaction). Both procedures were done according to protocols provided by Promega. Immunoprecipitation, endoglycosidase H treatment, concaavalin A-Sepharose binding, separation of membrane-embedded proteins from nonmembrane proteins after Na2CO3 extraction at pH 11, and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) followed by fluorography were performed as described previously (13).

**Cell culture and transfection.** BT-20 cells were maintained in Eagle minimal essential medium supplemented with 10% fetal bovine serum. Liposome (DOTAP)-mediated transfection of cells was used for transient expression of transgenes. A total of 16 h after transfection, the cells were transferred to coverslips and incubated for another 24 to 60 h before immunofluorescence.

**Immunofluorescence microscopy.** Immunofluorescence was performed as previously described (13). Cells grown as a monolayer on glass coverslips were fixed with 2.7% paraformaldehyde and treated in phosphate-buffered saline with 20 μg of digitonin per ml to permeabilize only the plasma membrane or with 0.1% saponin to disrupt all membranes. A Zeiss microscope (Carl Zeiss Inc.) with epifluorescence optics was used to visualize cell labelling.

**RESULTS**

The hydrophilic regions flanking the hydrophobic domains are not accessible for N-linked glycosylation in vitro. To study the transmembrane topology of the KDEL receptor, we set out to map the orientation of each hydrophobic domain with respect to the membrane through inference from the luminal or cytoplasmic placement of its flanking hydrophilic regions. The experimental approach was to reenact the insertion of the KDEL receptor into the ER membrane through in vitro synthesis in the presence of microsomal membranes. The regions protruding into the lumen of the microsomes should then be exposed to the N-linked glycosylation-competent environment. Such regions, if possessing the appropriate N-glycosylation signals, would be glycosylated, and this would result in a lower mobility during electrophoresis in a denaturing polyacrylamide gel. Since the native KDEL receptor is totally devoid of any N-glycosylation signals, we introduced such signals into the hydrophilic regions one at a time. Through point and insertion mutagenesis of the p23 cDNA, N-glycosylation signals were engineered into the hydrophilic regions as single (Asn-X-Ser/Thr) or tandem (Asn-X-Ser-Ser-Asn-X-Ser/Thr) sites (Fig. 1c). These cDNAs were then transcribed in vitro, and the RNA transcripts were expressed in [35S]methionine-supplemented reticulocyte lysates in the presence or absence of microsomal membranes. The polypeptides synthesized from the wild-type and engineered p23 cDNAs were all of the expected size, approximately 23 kDa, but higher-molecular-

**FIG. 1.** (a) Hydropathy plot of the mammalian (bovine) KDEL receptor with the Kyte and Doolittle algorithm with a window of 11 amino acids. Regions above the horizontal line have greater-than-average hydrophobicity. (b) Amino acid sequence deduced from the mERD2 cDNA. The hydrophobic regions are boxed, and the arrows indicate the C-terminal end of fragments fused to the glycosylation reporter. (c) A schematic model of the KDEL receptor. The hydrophobic regions are marked as M1 to M7, and the intervening hydrophilic regions are marked as HR1 to HR6. The epitopes indicated are the c-myc sequence inserted between the second and third amino acid residues and the 21 C-terminal residues against which antipeptide rabbit polyclonal antiserum was raised. The arrows indicate the positions of the amino acid residues immediately preceding the N-glycosylation signals engineered into the primary sequence. (d) The graded amino-terminal fragments of p23 to which the glycosylation reporter was fused upstream at the C terminus.
weight forms exhibiting a lower mobility did not appear when translations were performed in the presence of microsomal membranes (data not shown). The newly synthesized polypeptides were obviously inserted into the membrane as evidenced by their association with the insoluble pellet after extraction with Na2CO3 at pH 11.0 and centrifugation through a sucrose cushion. We interpret this to mean that the hydrophilic regions that might be oriented into the lumen are not accessible to the N-glycosylation machinery. This is probably due to their short lengths, ranging from 6 to 16 residues, which, if lying between two transmembrane domains, would form very tight loops close to the surface of the membrane. This close proximity to the membrane might pose a steric obstruction to the oligosaccharyltransferase in accessing the N-glycosylation sites.

Hydrophilic regions HR1, -2, -4, and -6 are luminal while HR3 and -5 and the carboxy terminus are cytoplasmic. An alternative strategy was devised to determine the luminal or cytoplasmic placement of the intervening hydrophilic sequences. N-terminal portions of p23 extending to various points in the hydrophilic sequences were fused at their carboxy termini to a glycosylation reporter domain (Fig. 1b and d). This was chosen to be the 37-kDa extracellular portion of DPPIV (residues 33 to 356) which possesses a nest of six potential N-glycosylation sites but is devoid of any targeting information and does not stop translocation across the membrane or anchor in it (7). As such, during insertion into the membrane, the glycosylation reporter will orientate itself on the same side of the membrane as the p23 amino acid residue to which it is attached. In the presence of microsomal membranes, glycosylation of the reporter would indicate that the hydrophilic region of p23 to which it is attached is located in the lumen. The cDNAs for the fusion proteins were transcribed and translated in vitro in the presence or absence of microsomal membranes, and the synthesized polypeptides were analyzed by SDS-PAGE and fluorography. Glycosylation, as evidenced by a reduced mobility, was detected when the reporter was fused to residues Lys-25, Gly-31, Leu-49, Pro-115, His-171, and Phe-175 (Fig. 2a). Core glycosylation was also confirmed by selective recovery of the more slowly migrating forms of the fusion proteins by concanavalin A-Sepharose and their sensitivity to endoglycosidase H (Fig. 2b). The fusion points of
the glycosylated hybrid polypeptides are located in the hydrophilic sequences HR2, -4, and -6, and thus these regions of p23 are located on the luminal face of the microsomal membranes. On the other hand, fusions to Thr-85, Asp-91, Gly-142, Thr-148, and Ala-212 exhibited no glycosylation. These points, which are in the hydrophilic sequences HR3 and -5 and the carboxy terminus, must then be located on the cytoplasmic side of the membranes. Beginning with HR2, the hydrophilic regions alternate in their luminal or cytoplasmic placement, and thus the last five hydrophobic domains are each sandwiched between a luminally orientated region on one side and a cytoplasmic one on the other side. This indicates that these hydrophobic domains, M3, -4, -5, -6, and -7, each span the membrane. The short hydrophobic domain M2 lies between two luminally orientated regions, HR1 and HR2, and thus does not span the membrane.

The fusion proteins were examined for insertion into the membrane by extraction with sodium carbonate at pH 11.0 and pelleting the membrane-associated proteins through a sucrose cushion. Interestingly, the fusion protein KRD49, with the first two hydrophobic regions of p23, was not anchored in the membrane, as evidenced by its appearance in the supernatant (Fig. 3, lane 2). Only when at least three hydrophobic regions were attached did the fusion proteins remain attached to the membranes (Fig. 3, lanes 8 and 12). The lack of anchorage by domains M1 and M2 leaves open the question of whether M1 is indeed a transmembrane domain.

The amino terminus of p23 is exposed to the cytosol. In order to assess whether the first hydrophobic domain, M1, spans the membrane, it was necessary to determine whether the amino terminus of p23 was located on the luminal or cytoplasmic face of the membrane. We reasoned that if the N terminus was located in the lumen, it would not be accessible to membrane-impermeable agents, like antibodies, except when the membrane was permeabilized, whereas a cytoplasmic orientation would be accessible regardless of the integrity of the membrane. The experimental approach relied on indirect immunofluorescence to detect the interaction of an antibody with the N terminus of p23 in monolayer cells with either intact or permeabilized internal membranes. Specific labelling of the cells would indicate that the N terminus is accessible to the antisera.

Since no antisera to the N terminus were available, the sequence in this region was engineered to contain the 10-residue human c-myc epitope between the second and third amino acid residues. This epitope can be detected by the monoclonal antibody secreted by the hybridoma 9E10 (5). We first examined whether the insertion of the c-myc sequences affected the membrane-anchoring properties of the hydrophobic regions M1, M2, and M3. Fusions of the glycosylation reporter to amino-terminal fragments of p23, with the inserted c-myc epitope sequence, were transcribed

FIG. 4. The amino and carboxy termini of p23 are exposed to the cytosol. BT-20 cells, 60 h after transfection and transiently overexpressing the myc-tagged p23, were treated with digitonin to selectively permeabilize the plasma membrane (columns 1 and 2) or with saponin to disrupt all membranes (columns 3 and 4). The cells were then colabelled with mouse monoclonal antitubulin and rabbit polyclonal anti-GT antibodies (a to d) or monoclonal anti-c-myc and polyclonal anti-GT antibodies (e to h) before being labeled with fluorescein-anti-rabbit immunoglobulin G and rhodamine-anti-rabbit immunoglobulin G antibodies. Each pair of frames (a and b, c and d, e and f, and g and h) arises from the same section of cells photographed through a filter for fluorescein or rhodamine. Selective permeabilization of the plasma membrane was obtained with digitonin since the cytosolic tubulin (a) but not the luminal epitope of GT (b) was labelled (top row). Under these conditions, the myc-tagged N terminus was also labelled (e), indicating that it is cytosolic. Bar, 10 μm.
FIG. 5. BT-20 cells, 24 h after transfection and transiently expressing the myc-tagged p23 at a low level, were permeabilized with digitonin and processed for immunofluorescence as described for Fig. 4, except that they were treated with rabbit polyclonal anti-p23 antibodies specific for the carboxy terminus, labelled with rhodamine–anti-rabbit antibodies, and blocked with excess unlabelled anti-rabbit antibodies; blocking was followed by a fixation step. Then, the polyclonal anti-GT antibodies, followed by fluorescein–anti-rabbit antibodies, were introduced. The same section of the monolayer was photographed through a filter for rhodamine (myc) and another filter for fluorescein (GT). The p23 carboxy terminus, but not GT, was again labelled, indicating that it is cytosolic. Note that with the relatively low expression of the myc-tagged p23 observed soon after transfection, its location is predominantly in the Golgi complex as opposed to the ER localization associated with its overexpression at later times (Fig. 4). Bar, 10 μm.

and translated in vitro and tested for anchorage to the microsomal membranes in sodium carbonate at pH 11.0. The c-myc epitope tag inserted at the N terminus was found not to alter the N-glycosylation or membrane-anchoring properties of polypeptides containing M1, M2, and M3 (data not shown). BT-20 cells transiently expressing the epitope-tagged p23 were subjected to indirect immunofluorescence after treatment with either 0.1% saponin to permeabilize all membrane structures or 20 μg of digitonin per ml to permeabilize the plasma membrane while leaving the internal membranes intact. During colabelling experiments, cells permeabilized with digitonin were labelled only by monoclonal antisera to β-tubulin (Fig. 4a) but not by polyclonal antisera to GT (Fig. 4b), a Golgi membrane protein which is almost entirely in the lumen with only 24 residues exposed to the cytoplasm (3). On the other hand, cells treated with saponin were specifically labelled by antisera to the cytosolic β-tubulin (Fig. 4c) and GT (Fig. 4d). This established that the digitonin treatment selectively permeabilized the plasma membrane, enabling access to the cytosolic region, while the membranes of internal organelles like the Golgi apparatus remained intact and impermeable to antibodies. Thus, the luminal epitopes were not labelled.

When transfected cells were colabelled with antisera to the c-myc epitope and GT, those expressing the myc-tagged p23 generally exhibited the ER staining characteristic of p23 overexpression (9, 11), although a Golgi staining pattern was observed in cells with low levels of expression (Fig. 5). This confirmed that the ER staining of the myc-tagged p23 resulted from its overexpression and not from misfolding due to the epitope tag and its retention in the ER. The myc-tagged N terminus was labelled both when the cells were treated with digitonin (Fig. 4e) and when they were treated with saponin (Fig. 4g) whereas GT was detected in cells permeabilized with saponin (Fig. 4h) but not in those treated with digitonin (Fig. 4f). This clearly indicated that the amino terminus of p23 was exposed to the cytoplasm. In view of this, the first hydrophobic domain must span the membrane since its upstream flanking region, the N terminus, is in the cytoplasm and its downstream flanking regions, HR1 and HR2, are in the lumen.

The location of the p23 carboxy terminus was further confirmed by colabelling cells with polyclonal antisera specific to the C terminus and GT. When the plasma membrane was permeabilized with digitonin, the anti-p23 antibodies strongly labelled expressing cells against a background of nonexpressing cells staining less strongly for the endogenous KDEL receptor (Fig. 5), confirming that the carboxy terminus is orientated to the cytoplasm for both the endogenous p23 and the overexpressed myc-tagged p23. The expression of the myc-tagged p23 was significantly lower than that in Fig. 4, and it was localized predominantly to the Golgi complex with the endogenous wild-type KDEL receptor. This further served to illustrate that the N-terminal epitope tag did not cause p23 to misfold and be retained in the ER. Hence, the epitope-tagged N terminus reflects the cytosolic orientation of the N terminus in the wild-type KDEL receptor.

DISCUSSION

We have investigated the transmembrane topology of the mammalian (bovine) KDEL receptor (p23) by determining the membrane-spanning domains of the molecule. This was

Vol. 13, 1993

KDEL RECEPTOR TOPOLOGY

6439
achieved by mapping the luminal or cytoplasmic orientation of the hydrophilic regions flanking the hydrophobic segments which are potential transmembrane domains. Membrane-spanning segments would be sandwiched between a cytoplasmic region on one side and a luminal one on the other. The assay for luminal placement was exposure to its N-glycosylation system which could be detected through a reduced mobility on SDS-PAGE analysis when a glycosylation consensus was present. Since p23 contains no inherent glycosylation signals, a neat system existed through which the location of each region could be addressed individually by providing these signals. The initial approach was to insert single or tandem glycosylation sites into the hydrophilic regions of p23 and reenact its insertion into the membrane by expression in reticulocyte lysates with microsomal membranes. However, no glycosylation was detected in any of the various glycosylation signal-tagged forms even though the polypeptides were inserted into the membranes. In one construct, a tandem glycosylation signal was inserted after the residue Tyr-48, which is very close to the stretch of residues, Asp-50 to Ile-56, implicated in the direct interaction with the KDEL-bearing proteins (11) and is thus likely to be placed in the lumen. Its lack of glycosylation led us to conclude that the hydrophilic regions located in the lumen are not accessible to the N-glycosylation machinery. This could possibly be due to the short lengths of those stretches which would form tight loops close to the membrane, especially if they separate two transmembrane domains. Recently, it has been shown that effective glycosylation requires a minimum distance of about 14 residues between the N-glycosylation target site and a single transmembrane domain (15). Presumably, sites sandwiched between transmembrane domains would require this clearance on either side, dictating a minimum length of about 30 residues for effective glycosylation of intervening loops.

The second approach to resolve the locations of the intervening hydrophilic sequences was to replace all sequences downstream of the points in question with a glycosylation reporter fragment. Then this reporter, a fragment of the DPPIV polypeptide which carries a nest of six glycosylation sites and contains no membrane-targeting or translocation stop signals (7), would reflect the location of its fusion point after membrane insertion. Being a fairly large moiety, the reporter should be accessible to the glycosylation machinery. When the p23-reporter fusions were expressed in reticulocyte lysates with microsomal membranes, glycosylation was indeed observed. In fact, from the third hydrophilic region, HR3, to the carboxy terminus, the alternating presence or absence of glycosylation clearly indicated that the last five hydrophobic domains all traverse the membrane with the carboxy terminus located on the cytoplasmic face. It was also apparent that the second hydrophobic segment, M2, could not be membrane spanning since both its flanking sequences are luminaly placed. Instead, it is probably translocated across the membrane and exists in close association with it, possibly by being embedded within the membrane.

The amino terminus was assessed to be cytoplasmic since membrane-impermeable antibodies to the c-myc epitope, which was inserted into its sequence, were able to label cells expressing the tagged p23 when the internal membranes were intact. Under these conditions, the luminal epitopes of the Golgi membrane protein GT were not accessible to antisera. This placement of the amino terminus meant that the first hydrophobic domain also spanned the membrane. In the glycosylation reporter fusion experiments, this transmembrane domain could not by itself anchor the fusion protein in the membrane. However, the presence of the second transmembrane domain was sufficient to maintain it as a transmembrane domain, probably through direct inter-
action between these two membrane-spanning segments of the p23 polypeptide.

On the basis of the results of this study, we propose that the mammalian KDEL receptor is inserted into the membrane with only six membrane-spanning domains and that both its termini are located in the cytoplasm (Fig. 6). Although the transmembrane domains include charged residues, the sum total of these charges balances to neutrality. This model is consistent with earlier work that placed the KDEL binding site between Asp-50 and Ile-56 (11) since these residues are located at the luminal face of the membrane in our model. The KDEL receptor resembles the G protein-coupled receptors with seven transmembrane domains associated with signal transduction (4) and the membrane channel proteins with six transmembrane domains (19, 20), both of which are believed to interact with downstream effectors through their cytoplasmic domains. This structural similarity suggests a functional parallel in the interaction of the cytoplasmic domains of the KDEL receptor with various cytoplasmic factors. Some of these interactions are likely to be initiated by conformational changes induced by binding the KDEL ligand since the latter induces redistribution of the KDEL receptor from the Golgi complex to the ER (11).

The topological model of the KDEL receptor provides a logical basis for studying these interactions by highlighting the cytoplasmic domains for a focused investigation. Of particular significance is the placement of the C terminus, a region necessary for the function of the KDEL (HDEL) receptor since the loss of the C-terminal 12 residues accounts for a nonfunctional mutation of this protein in S. cerevisiae (21). In our model, the C terminus is located in the cytosol and is thus excluded from direct interaction with the KDEL ligand. Hence, its importance must arise from its involvement in a portion of the retrieval process other than ligand binding, possibly through interactions with a cytoplasmic factor(s) as previously suggested (8). In accordance with this, our recent in vitro experiments suggest that a cytosolic factor(s) indeed binds to the cytoplasmic tail of p23 (unpublished results) although it is not yet clear whether this interaction is dependent on ligand binding or necessary for its subsequent retrieval to the ER. The approach that we have reported here can also be exploited, with appropriate modifications, to determine the topology of other membrane proteins.

ACKNOWLEDGMENTS

We are grateful to Low Seng Hui for critical reading of the manuscript, Francis Leong and Oh Sock Yng for photographic work, and Y. H. Tan for his encouragement and support.

This work was supported by a research grant from the National University of Singapore to W. Hong.

REFERENCES


