Identification of Membrane Insertion Sequences of the Rabbit Gastric Cholecystokinin-A Receptor by in Vitro Translation*

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To determine which amino acid sequences account for transmembrane folding of G7 receptors, the membrane domain of the rabbit cholecystokinin-A (CCK-A) G-protein-coupled receptor has been investigated by in vitro transcription/translation of two types of fusion vectors containing sequences that include putative transmembrane segments. First, the seven putative transmembrane domains of the CCK-A receptor were inserted individually into pGEM vectors beginning with the cDNA encoding the first 101 (HK-M0) or 139 (HK-M1) amino acids of the α subunit of the gastric H,K-ATPase. These were separated by the cDNA for the inserted transmembrane domains from the cDNA encoding the last 177 amino acids of the β subunit of the H,K-ATPase containing five N-linked glycosylation consensus sequences (Bamberg, K., and Sachs, G. (1994) J. Biol. Chem. 269, 16909–16919). Transcription/translation of these fusion vectors in rabbit reticulocyte lysate ± dog pancreatic microsomes followed by SDS-polyacrylamide gel electrophoresis defined the presence of signal anchor sequences in HK-M0 by glycosylation and stop transfer sequences in HK-M1 by inhibition of glycosylation. Six out of the seven putative transmembrane domains had membrane insertion signals, but no membrane insertion activity was found for the H3 segment in these vectors. To test the effect of specific upstream and downstream sequences on membrane insertion, vectors were also made starting with the cDNA encoding the N terminus of the CCK-A receptor separated from the last 177 amino acids of the H,K-ATPase β subunit by cDNA encoding CCK-A receptor sequences of different lengths. In addition to transcription/translation, endoglycosidase H treatment was used to verify glycosylation when multiple bands were found in the presence of microsomes. The four positive charges in the loop between H1 and H2 were required for the correct orientation of the first transmembrane domain. The H3 segment acted as a stop transfer sequence only when the whole N terminus and H3 were followed by the positive charges in the cytoplasmic loop between H3 and H4. The activity of H6 as a signal anchor sequence depended on preceding positive charges. These translation data using two types of fusion vectors establish a seven-transmembrane folding model using only in vitro translation for the CCK-A receptor beginning with two signal anchor sequences and then alternating stop transfer and signal anchor insertions. Positive charges between H1 and H2, H3 and H4, and H5 and H6 function as cytoplasmic anchors in the membrane folding of this receptor.

G-protein-coupled receptors (GPCRs)1 are a family of integral polytopic membrane proteins involved in the transmission of signals across the plasma membrane. Found in a wide range of organisms, all GPCRs are believed to have seven transmembrane helices based on their hydrophathy profile and some corroborating experimental evidence. The mechanism of assembly of this type of protein is thought to be an alternative presence of signal anchor and stop transfer sequences. A signal anchor is defined as a hydrophobic sequence that has the ability to insert independently into the membrane of the ER. After translation of the cytoplasmic N-terminal domain of a protein, the presence of a signal anchor sequence will result in membrane insertion of that sequence due to interaction with the translocon complex (1). The sequence following the signal anchor will then appear in the lumen of the ER. This translocation process is stopped by the translation of a second hydrophobic region, a stop transfer sequence, that binds to the translated protein-translocon complex and is retained in the membrane, resulting in a cytoplasmic location of the following sequence. In the case of opsin, in vitro translation of various regions was interpreted as showing the presence of at least two signal anchor sequences. The presence of stop transfer sequences was not evaluated (1). Data from mammalian cell expression and functional complementation of truncated constructs of the muscarinic subtype 3 receptor, where successive pairs of transmembrane sequences were obtained by cutting the cDNA sequence in the cellular or extracellular loops, have also been interpreted as showing the presence of successive independent folding units (2). These data support a folding model for this class of protein in which signal anchor sequences are followed by stop transfer sequences, resulting in sequential insertion of topogenic signals (3).

It has been widely assumed that GPCRs have the same general structure as bacteriorhodopsin, an integral membrane protein from Halobacterium halobium whose seven transmembrane segments have been defined at 3.4-Å resolution by electron diffraction (4). Similarly, rhodopsin, in two-dimensional crystallization, contains at least four transmembrane helices, differing from bacteriorhodopsin in the tilt between its helices (5). Bacteriorhodopsin is predicted by hydrophathy analysis to contain seven independent signal anchor sequences. The hydrophathy plots of the muscarinic subtype 3 and CCK-A receptor sequences give less clear-cut predictions, suggesting that the third and seventh hydrophobic sequences may not be independent.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) U88601 (for the rabbit CCK-A receptor) and X64694 (for the H,K-ATPase).

1 The abbreviations used are: GPCRs, G-protein-coupled receptors; ER, endoplasmic reticulum; CCK-A, cholecystokinin-A; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.
signal anchor sequences, but merely stop transfer sequences (see Fig. 1A).

Although the generality of the predicted seven membrane domains for these receptors is implied by their hydrophobic plots, apart from crystallization, no single experimental method has been developed that is able to confirm this structure. Rather, results from a multiplicity of methods applied to various GPCRs have converged to provide the necessary evidence for seven transmembrane segments, and analysis of the adjacent sequences necessary for proper membrane insertion of the transmembrane segments of these receptors has not been performed.

The N-terminal region of most GPCRs contains one or more N-linked glycosylation consensus sequences that are glycosylated in the expressed receptor, thereby confirming an extra-cytoplasmic location of the N termini of these receptors (6). Epitope insertion studies applied to bovine rhodopsin identified three intracellular and extracellular loops and confirmed the location of the N and C termini (7). Anti-peptide antibodies have shown that the β2 adrenergic receptor N-terminal region is extracytoplasmic and that the C terminus is cytoplasmic (8, 9), thereby establishing the presence of an odd number of membrane segments. Additionally, an epitope in the loop between H1 and H2 was found to be cytoplasmic (10). By using in vitro translation and protease protection of glucatame receptor β1, the loop between H5 and H6 containing the G-protein-binding site has been assigned to the cytoplasmic surface (11).

Part of the third intracellular loop of the luteinizing hormone/chorionic gonadotropin receptor has also been suggested to be involved in the rate of internalization of the receptor due to interaction with a small region located just after H7. This indicates that those two regions are most likely on the cytoplasmic side (12).

The cDNA sequence for the rabbit stomach CCK-A receptor predicts a protein of 427 amino acids (13). The hydropathy profile and amino acid sequence of the rabbit CCK-A receptor (see Fig. 1A) show the presence of seven hydrophobic sequences with three N-linked glycosylation consensus sequences in the predicted extracytoplasmic domain. In this study, using an in vitro translation technique, each of these sequences was assayed for its membrane insertion capacity either as a signal anchor or stop transfer sequence in two types of vectors as illustrated in Fig. 1B. These vectors are designed to be able either to determine the presence of independent signal anchor and stop transfer sequences (HK-M0 and HK-M1, respectively) or to determine sequential insertion of topogenic signals (CCK-A vectors) by in vitro translation in the absence and presence of microsomes.

**EXPERIMENTAL PROCEDURES**

**Construction of HK-M0 and HK-M1 Vectors**

Two expression plasmids, HK-M0 and HK-M1, were constructed in pGEM7zf + (A/HindIII) as described previously in detail (14). These vectors enable analysis of signal anchor sequences (HK-M0) and stop transfer sequences (HK-M1) of the CCK-A receptor (see Table I).

**HK-M0 Vector**—The HK-M0 vector begins with 314 nucleotides (12 to 302) coding for the first 101 amino acids of the rabbit gastric H,K-ATPase β subunit, which contains a putative cytoplasmic anchor sequence consisting of a positively charged sequence containing 8 positively charged amino acids between positions 25 and 39. Then, a linker sequence is present that has two restriction sites, BglII and HindIII, to allow insertion of variable sequences. The end of the vector is made up of the sequence coding for the last 177 amino acids of the β subunit. This contains five of the original consensus sequences for the N-linked glycosylation of the β subunit, but does not contain the transmembrane segment (17).

**HK-M1 Vector**—The HK-M1 fusion protein vector is identical to the HK-M0 vector except for the N-terminal region, which has 425 nucleotides of the rabbit gastric H,K-ATPase α subunit cDNA (12 to 413) coding for the first 139 amino acids, therefore including the first transmembrane segment of the gastric H,K-ATPase.

After translation in the absence or presence of microsomes, the mobility shift on SDS-PAGE due to the glycosylation of the five N-linked glycosylation consensus sequences was used to determine the location of the C terminus of the fusion proteins. This suggests that causes HK-M0 to be glycosylated is characterized as a signal anchor sequence. Likewise, any sequence that prevents the entry of the C terminus of HK-M1 into the interior of the microsomes, thereby preventing the glycosylation of the β region, is characterized as a stop transfer sequence. These constructs have been previously used to characterize the membranes of three P-type ATPases, which have their N-terminal region on the cytoplasmic surface (14–16).

**Variable Segment**—The sequences coding for the putative transmembrane segments of the CCK-A receptor were synthesized by PCR and ligated into the BglII and HindIII sites of the HK-M0 and HK-M1 vectors. Sense primers contained the BglII site, and antisense primers contained the HindIII site. The appropriate sequence for the primers and conditions for the amplification were determined using Oligo Version 4.0 primer analysis software (National Biosciences, Plymouth, MN). The different amino acid sequences analyzed for competence as membrane insertion signals are presented in Table I.

**Construction of CCK-A Vectors**

Since the N-terminal region of the β subunit of the H,K-ATPase contains a cytoplasmic anchor sequence, in contrast to the CCK-A receptor, vectors were constructed encoding different C-terminal deletions of the CCK-A receptor fused to the final 177 amino acids of the H,K-ATPase β subunit. This approach also determines whether interaction between the sequences of the receptor itself can influence membrane insertion by native protein-protein interactions (see Table II).

The HK-M0 vector was cut by the restriction enzymes XbaI and HindIII, thereby eliminating the fragment coding for the first 101 amino acids of the H,K-ATPase and the linker sequence. The linear plasmid obtained, which contains the T7 promoter upstream from the XbaI site and the sequence coding for the last 177 amino acids of the β subunit of the H,K-ATPase downstream from the HindIII restriction site, was prepared for the insertion of the CCK-A receptor-coding fragments of different lengths. These fragments all started at nucleotide −32 in the coding sequence of the rabbit CCK-A receptor and ended after each putative transmembrane domain. The DNA fragments were synthesized using PCR. The sense primer contained an XbaI site, whereas the antisense primers were the ones used for the HK-M0 and HK-M1 constructs. The sequences of the resulting fusion proteins are described in Table II. All these constructs encode two N-terminal glycosylation consensus sequences as found in the native N-terminal CCK-A sequence and five N-terminal glycosylation consensus sequences due to the last 177 amino acids of the β subunit of the H,K-ATPase. SDS-PAGE mobility observed in the presence of no translocation, translocation of the C terminus only, or translocation of both N- and C-terminal regions, respectively.

**Vector Amplification and Purification**

The expression plasmids containing the different protein sequences were replicated in Escherichia coli XL-1 blue or E. coli Top10 (Stratagen) and isolated using anion-exchange columns (Qiagen Inc., Chatsworth, CA). Inserts were screened either by PCR with the T7 sense primer and the downstream PCR2 primer (17) or by restriction analysis of DNA minipreps (Promega). The inserts in all plasmids were verified by dideoxy sequencing. Sequence analysis was done with the help of Geneworks software (Version 2.0, Intelligenetics, Inc./Betagen).

**PCR and Cloning**

A typical 100-μl reaction contained 1 ng of the template, a 0.3 μM concentration of each primer, 200 μM deoxynucleotides (Pharmacia Biotech Inc.) in the buffer supplied by the manufacturer, and 1 unit of Vent polymerase (New England Biolabs Inc.). For the longer fragments, the ExTaqara enzyme (Oncor) was used according to the manufacturer’s instructions. The PCR products were digested with the appropriate restriction enzymes (BglII and HindIII) for the fragments coding for individual domains and pairs and XbaI and HindIII for the fragments containing the whole N-terminal region and then gel-purified for ligations according to standard protocols (18).
Presented are the name, the amino acid sequence of the CCK-A insert, its amino acids positions, the presence or absence of glycosylation in the HK-M0 vector, and the signal anchor or stop transfer activity.

### Table I

| Insert | Sequence | Position | Glycosylation in HK-M0 | Glycosylation in HK-M1 | Activity |
|--------|----------|----------|------------------------|------------------------|----------|
| CCKIS  | -ILLYSLIFLLSVLGLTIVLIRNK- | 45–70     | +<sup>a</sup>          | −<sup>a</sup>          | SA/ST    |
| CCKIL  | -ILLYSLIFLLSVLGLTIVLIRKRMR- | 45–73     | +<sup>a</sup>          | −<sup>a</sup>          | SA/ST    |
| CCK2   | -RTVINPISLAISIISMLCLFMCMPFNLIP- | 73–101    | +<sup>a</sup>          | −<sup>a</sup>          | SA/ST    |
| CCK3   | -CTKTIGLYMSVSTLVNLVAILGQK- | 114–136   | +<sup>a</sup>          | −<sup>a</sup>          | SA/ST    |
| CCK3LN | -NLKDFIFGSLACLKTTTLMTGVSVSSTVVLNVAIS- | 102–136   | −                  | +            | −        |
| CCK3LC | -CKTTLYMGTSVSSTVNLVAILGESGAI- | 114–143   | −                  | +            | −        |
| CCK3LL | -NLKDFIFGSLACLKTTTLMTGVSVSSTVVLNVAISLGYAI- | 102–143   | −                  | +            | −        |
| CCK3LN-KR | -NLKDFIFGSLACLKTTTLMTGVSVSSTVVLNVAISLGYAIACKPLQSR- | 102–150   | −                  | +            | −        |
| CCK4K  | -CKTTLYMGTSVSTVLVAILGESGAIACKPLQSR- | 114–150   | −                  | +            | −        |
| CCK3LN-KK | -NLKDFIFGSLACLKTTTLMTGVSVSSTVVLNVAISLGYAIACKPLQSRVQWTK- | 102–160   | −                  | +            | −        |
| CCK3KK | -CTKTYYLMGTSVSTVLVAILGESGAIACKPLQSRVQWTKSHALK- | 114–160   | −<sup>b</sup>          | +<sup>b</sup>          | −        |
| CCK4   | -HALKVIATACLFSVAFMTPIYSNL- | 157–182   | −                  | −<sup>a</sup>          | ST       |
| CCKlp4 | -ERYGAICKPLQSRVQWTKSHALKVIALAATCLFSVAFMTPIYSNL- | 138–182   | +                  | ND           | SA/ND    |
| CCKlp4LC | -ERYGAICKPLQSRVQWTPVKTNQ- | 157–192   | +<sup>a</sup>          | −<sup>a</sup>          | SA/ST    |
| CCKIp4LC | -ERYGAICKPLQSRVQWTPVKTNQ- | 138–192   | +                  | ND           | SA/ND    |
| CCK5   | -QQAWHTLLLILFLIPGVMVAYAG- | 206–230   | +                  | −<sup>a</sup>          | SA/ST    |
| CCK6   | -RMLMVIVLFLCWMPIFSAAN- | 321–332   | −<sup>a</sup>          | −            | −        |
| CCK6LN | -RMLMVIVLFLCWMPIFSAAN- | 321–332   | −<sup>a</sup>          | −            | −        |
| CCK6LC | -RMLMVIVLFLCWMPIFSAAN- | 321–332   | −<sup>a</sup>          | −            | −        |
| CCK6LN-KK | -RMLMVIVLFLCWMPIFSAAN- | 321–332   | −<sup>a</sup>          | −            | −        |
| CCK7   | -ISIFILLLYSSSCVNPYICVFMR- | 351–374   | +<sup>a</sup>          | −            | −        |
| CCK7LC | -ISIFILLLYSSSCVNPYICVFMR- | 351–420   | −<sup>a</sup>          | −            | −        |

**β region:** SFLAGYSPAAQVDNICTKSTYFFQESFGAPNHTKFSCKFTADMLENCGLTSGLPSGFEGKPKCIFIKMNRRVPFLPSNSSTPRPVSDCTFDLMPHQLATLPQVEYYYPNFTSFLHYFYPKDYKQPSNLAALKLNVPTNTEVVLKCIgLADHVTFDNPFDHYEGKEVFKLIQK

<sup>a</sup> presence of glycosylation; <sup>b</sup> absence of glycosylation; SA, signal anchor; ST, stop transfer; ND, not determined.

### Table II

**CCK vectors**

In the fusion constructs, different lengths of the cDNA encoding the CCK-A sequence starting at amino acid 1 are followed by the cDNA encoding the CCK receptor and in the β region.

#### Construct

| N-terminal region | Position |
|-------------------|----------|
| CCK N             | MDAVASSLGNASGIPPCESPCELGLDNETLFCLDQPPPSKEWQPAVQ- | 1–44 |
| CCK N-1S          | MDAVASSLVITLIRNK- | 1–70 |
| CCK N-1L          | MDAVASS-LVITLIRKRMR- | 1–73 |
| CCK N-2           | MDAVASS-LFCMPFNLIP- | 1–101 |
| CCK N-3           | MDAVASS-VSTLNVAIS- | 1–136 |
| CCK N-5LC         | MDAVASS-ISLERYGAI- | 1–143 |
| CCK N-3KR         | MDAVASS-GAICKPLQSR- | 1–150 |
| CCK N-3KK         | MDAVASS-VWQTSHALK- | 1–160 |
| CCK N-4           | MDAVASS-MTPPIYSNL- | 1–182 |
| CCK N-4LC         | MDAVASS-VFPTKTNQ- | 1–192 |
| CCK N-5           | MDAVASS-PGIVVMVAYG- | 1–230 |
| CCK N-6           | MDAVASS-CWMPFSAANA- | 1–332 |
| CCK N-6LC         | MDAVASS-CWMPFSAANA- | 1–350 |
| CCK N-7           | MDAVASS-CWMPFSAANA- | 1–350 |

**H-K-ATPase C-terminal β region**

SFLAGYSPAAQVDNICTKSTYFFQESFGAPNHTKFSCKFTADMLENCGLTSGLPSGFEGKPKCIFIKMNRRVPFLPSNSSTPRPVSDCTFDLMPHQLATLPQVEYYYPNFTSFLHYFYPKDYKQPSNLAALKLNVPTNTEVVLKCIgLADHVTFDNPFDHYEGKEVFKLIQK

**β region:** SFLAGYSPAAQVDNICTKSTYFFQESFGAPNHTKFSCKFTADMLENCGLTSGLPSGFEGKPKCIFIKMNRRVPFLPSNSSTPRPVSDCTFDLMPHQLATLPQVEYYYPNFTSFLHYFYPKDYKQPSNLAALKLNVPTNTEVVLKCIgLADHVTFDNPFDHYEGKEVFKLIQK

### In Vitro Translation

The protein was synthesized from isolated plasmid cDNA using the TNT™ in vitro transcription/translation system (Promega) in the presence of [35S]methionine (Amersham Corp.) according to the manufacturer’s suggestions. This method allows transcription and translation to be performed in a single step. The translation products were separated on 10% Laemmli gels that were subsequently fixed and washed overnight in 45% methanol and 10% acetic acid and then dried. The products formed in the presence of microsomes were run in lanes next to lanes containing the translation products formed in their absence. Molecular mass standards were obtained from Bio-Rad.

### Radioactivity Measurement

Gels were placed into a cassette containing Kodak X-Omat AR x-ray film and were exposed for 12–96 h at room temperature. Alternatively, the radioactivity of the gels was detected using a PhosphorImager and AMBIS software (image acquisition and analysis). The molecular masses of the different translated constructs were calculated using RFLP scan software (Version 2.01).

### Deglycosylation

After the transcription/translation reaction, the microsomes were solubilized in SDS and treated with endoglycosidase H (endo-β-N-acetylglucosaminidase H from Boehringer Mannheim) according to the manufacturer’s protocol. The products of the reaction were separated by SDS-PAGE, and the radioactivity was measured using the PhosphorImager system as described above.

### Materials

All chemicals were analytical grade or better. Molecular biology reagents were obtained from Promega, Pharmacia, Oncor, and New England Biolabs Inc. Other reagents were obtained as stated in above.
RESULTS

The translation product of the HK-M0 vector showed no shift in apparent molecular mass in the presence of microsomal membranes because the β region of the HK-M0 fusion protein did not access the lumen of the ER (Fig. 2, lanes 1 and 2). The HK-M1 vector product showed a shift in molecular mass of 12.5 kDa in the presence of microsomes. The shift was removed by treatment with endoglycosidase, indicating that the HK-M1 segment is able to insert into the membrane and that each core glycosylation contributes 2.5 kDa to the molecular mass of the product (data not shown). The hydrophobic sequence in the HK-M1 vector thus acts as a signal anchor.
The transmembrane segments of the CCK-A receptor were predicted by a variety of algorithms used for selecting the hydrophobic sequences for the transcription/translation studies.

| Segment | Eisenberg et al. (21) | Rao-Argos (19) | Kyte-Doolittle (20) | GES scale (22) |
|---------|-----------------------|----------------|-------------------|---------------|
| H1      | Leu-47 -> Ile-67 mb  | Ala-42 -> Ile-67 | Ile-65 Pro-65     | Leu-47 -> Ile-67 |
| H2      | Asn-77 -> Phe-97 mb  | Thr-74 -> Ile-100 | Asn-77 Leu-99     | Asn-77 -> Phe-97 |
| H3      | Thr-17 -> Leu-137 gl | Asp-106 -> Leu-137 | Thr-18 -> Thr-133 | Thr-17 -> Leu-137 |
| H4      | Ala-158 -> Ile-178 mb| Ala-158 -> Pro-175 | Ala-162 Met-173   | Ala-158 -> Ile-178 |
| H5      | Phe-212 -> Ile-233 mb| Ala-208 -> Leu-236 | Phe-212 Ile-232   | Thr-211 Met-231 |
| H6      | Leu-311 -> Trp-334 mb| Val-310 -> Ala-331 | Met-313 Ile-328   | Leu-346 Pro-366 |
| H7      | Ile-351 -> Phe-371 gl| Gly-349 Met-372   | Pro-350 Thr-360   | Phe-376 Arg-396 |

- mb, transmembrane multimeric prediction; gl, globular prediction in the Eisenberg analysis.

sequence (Fig. 2, lanes 3 and 4). Previous experience with this method has shown that all signal anchor sequences in P-type ATPases also show stop transfer properties (14–16), but some sequences show only stop transfer ability. In general, those sequences with only stop transfer properties have lower hydrophobicities. The hydrophobicity plot and various hydropathy algorithms for the CCK-A receptor (Fig. 1 (A and B) and Table III) were used as a guide for the construction of the different fusion vectors.

HK-M0 and HK-M1 Vector Analysis of CCK-A Receptor Hydrophobic Sequences

H1 Segment—The putative transmembrane segment H1 (amino acids 45–70) promoted the glycosylation of the β region when inserted into the HK-M0 vector and prevented glycosylation of the β region when inserted into the HK-M1 vector, although here in the presence of microsomes, translation was inefficient (Fig. 2, lanes 8–12). The band produced was not removed by an alkaline wash, showing that it was membrane-inserted. When the insert terminated at position 73, which had two additional positive charges at its C-terminal end (data not shown), similar signal anchor and stop transfer properties were found. H1 therefore acts as both a signal anchor and a stop transfer sequence, in agreement with its position as the first transmembrane sequence in the seven-transmembrane segment model. In this model, its predicted orientation is the opposite of its signal anchor orientation in the HK-M0 vector.

H2 Segment—The cDNA coding for the putative transmembrane domain H2 (amino acids 73–101) promoted glycosylation of the β region when inserted into the HK-M0 vector and prevented glycosylation when inserted into the HK-M1 vector. Hence, this segment can also perform as either a signal anchor or stop transfer sequence (Fig. 2, lanes 9–12). The efficiency of this sequence as a signal anchor sequence was lower than that of the H1 sequence, but is in its predicted natural orientation in the HK-M0 vector.

H3 Segment—cDNA sequences of different lengths coding for amino acids between positions 102 and 160, containing the H3 domain, were inserted into the HK-M0 and HK-M1 vectors (Table I). Neither signal anchor nor stop transfer properties were observed. Only the results obtained with the CCK3 and CCK3LN-KK segments are shown (Fig. 3, lanes 1–8). Hence, translation of this segment of the receptor in the HK-M0 and HK-M1 vectors does not provide evidence for its presence in the membrane domain of the receptor.

H4 Segment—The cDNA encoding the putative transmembrane domain H4 (amino acids 157–182, CCK4; Table I) did not promote the glycosylation of the β region in the HK-M0 vector, but almost completely prevented the glycosylation of the same β region when inserted into the HK-M1 vector (Fig. 3, lanes 9–12). This sequence therefore was able to act only as a stop transfer signal. When the sequence was extended at its C-terminus to amino acid 192 (CCK4LC; Table I), some glycosylation of the β glycosylation consensus sites was present in the HK-M0 vector, indicating that this sequence containing H4 can act as a weak signal anchor (Fig. 3, lanes 13 and 14). Expression of this C-terminally elongated domain in the HK-M1 vector still resulted in some glycosylation of the β region, suggesting that its action as a stop transfer sequence is partial (Fig. 3, lanes 15 and 16). This is the first segment we have found that acts as a signal anchor in HK-M0, albeit weakly, and is not able to fully block the glycosylation of the β region when inserted in the HK-M1 construct.

When the cytoplasmic loop preceding H4 was also present in the HK-M0 vector (CCK4LC; Table I), a stronger glycosylation of the β region was obtained. The importance of this preceding loop in the membrane insertion process of this segment (Fig. 3, lanes 17–20). This region thus contains a signal anchor sequence, as expected of its position in the receptor sequence, but requires sequences adjacent to the hydrophobic region for this signal anchor property to be displayed.

H5 Segment—The cDNA sequence encoding the putative transmembrane domain H5 (amino acids 206–230) acted as both a signal anchor and a stop transfer sequence. The sequence promoted glycosylation when inserted in the HK-M0 vector and prevented glycosylation when inserted in the HK-M1 vector. It was as effective a signal anchor sequence as the HK-M1 sequence of the gastric H,K-ATPase (Fig. 4, lanes 1–4), although its position in the sequence requires only stop transfer properties.

H6 Segment—Insertion of the cDNA sequence coding for the putative transmembrane domain H6 (amino acids 312–333, CCK6; Table I) did not promote glycosylation of the β region.
when inserted into the HK-M0 vector. In the HK-M1 vector, it prevented glycosylation, indicating that it can act as a stop transfer signal (Fig. 4, lanes 5–8). It can be seen that there was weak translation of this sequence in the HK-M0 vector in the absence of microsomes and in the HK-M1 vector in the presence of microsomes. This was not the case when H6 was extended either on the N-terminal side to position 302 (CCK6LN) or on the C-terminal side to position 350 (CCK6LC; Table I), or on both sides, this sequence effectively promoted glycosylation of the β region in the HK-M0 vector (Fig. 4, lanes 9–14). H6 performed as a signal anchor, as expected of its position in the receptor sequence. Again, it appears that adjacent sequences are necessary for this signal anchor property to be discerned.

**H7 Segment**—The putative transmembrane domain H7 (amino acids 351–374) promoted a very weak glycosylation of the β region when inserted in the HK-M0 vector, but fully prevented glycosylation when inserted into the HK-M1 vector (Fig. 4, lanes 5–14). Moreover, when H7 was extended C-terminally to include all but the final 7 amino acids (CCK7LC; Table I), the weak signal anchor activity disappeared, but the stop transfer activity remained. Hence, the natural C-terminal sequence provides only stop transfer ability. The results from analysis of the sequence using the HK-M0 and HK-M1 vectors show that the hydrophobic regions H1, H2, and H5 can act as independent signal anchor sequences. Addition of natural preceding or succeeding sequences modifies the membrane insertion properties of H4, H6, and H7, both by stimulating (H4 and H6) and by inhibiting (H7) their signal anchor activities. H3 does not insert by itself in these vectors even when surrounded by the loops between H2 and H3 and between H3 and H4.

A summary of the results of in vitro transcription/translation of the HK-M0 and HK-M1 fusion vectors containing single hydrophobic sequences is presented in Table I. This set of vectors provides evidence for most, but not all, of the predicted transmembrane sequences.

**CCK Vector Analysis of Receptor Folding**

**CCK N-0 Vector**—This vector contains the N-terminal region of CCK-A, excluding the first transmembrane domain (amino acids 351–374).
acids 1–44), fused to the C-terminal β sequence. Neither N- nor C-terminal glycosylation was observed for this construct (data not shown), as expected from a lack of hydrophobic domains.

**CCK N-1 Vectors**—Two vectors, CCK N-1S and CCK N-1L, (Table II) were constructed to analyze the membrane insertion of H1 in more detail since its orientation is opposite to that predicted by the seven-transmembrane segment model when translated in the HK-M0 vector. One vector contained the cDNA coding for the hydrophobic sequence and two positive charges on the C-terminal side (CCK N-1S). The longer vector (CCK N-1L) contained two additional positive charges on the C-terminal side, which are predicted to be in the cytoplasmic loop. This H1/H2 loop contains four positive charges, with no negatively charged amino acids. The results are presented in Fig. 5.

Translation of the vector CCK N-1S (amino acids 1–70) in the presence of microsomes produced two glycosylated products (Fig. 5, lanes 3 and 4). The first had a molecular mass shift of 4–5 kDa, which corresponds to the presence of two core glycosylation sites. This suggests that the N-terminal region of this product is in the lumen. The second and predominant glycosylated product had a 12-kDa shift, similar to that found with the HK-M1 vector (Fig. 5, lanes 1 and 2), indicating the presence of five core glycosylation sites. The C-terminal β region of this alternative translation product is therefore in the interior of the microsomes. Thus, the translation of this vector results in two populations of translated protein with the membrane segments oppositely oriented. The intensity of the bands indicates that the cytoplasmic localization of the N terminus is favored in this vector, which is opposite to the natural orientation.

The translation product of the vector CCK N-1L (amino acids 1–73) contained two extra positive charges (RMR) following H1 as compared with CCK N-1S (Table II). The translation in the presence of microsomes produced one predominant band corresponding to only two core glycosylation sites. Hence, the addition of all four positive charges following the H1 transmembrane segment favors the natural orientation of the first transmembrane segment.

**CCK N-2 Vector**—In the absence of microsomes, the apparent molecular mass of the CCK N-2 product (amino acids 1–101) (Table II) was smaller than that of the CCK N-1 translation product, suggesting a tight association between the first two transmembrane helices of the receptor, which may prevent full denaturation by SDS. This phenomenon was observed previously for some of the vectors used for translation of the H,K-ATPase (14).

The translation of the CCK N-2 vector in the presence of microsomes resulted in a molecular mass shift of –25 kDa, implying that the seven potential core glycosylation sites present in the translated sequence are glycosylated (Fig. 6A, lanes 1 and 2). Therefore, both the N-terminal region and the H,K-ATPase β region are located in the lumen, and hence, both H1 and H2 are membrane-inserted. It is also seen that there is only one major core glycosylated product, indicating that the complete sequence in this region orients appropriately in this in vitro translation system. This result is consistent with the signal anchor properties of H1 and H2 observed in the HK-M0 vector. The efficiency of H2 as a signal anchor sequence is improved by the presence of its natural H1 partner as compared with its presence alone in the HK-M0 vector.
**CCK N-3 Vectors**—The translation products of the vectors CCK N-3 (amino acids 1–136) and CCK N-3LC (amino acids 1–143; Table II) in the absence of microsomes had an approximate molecular mass of 31 kDa. There are seven potential glycosylation sites on its sequence. The presence of microsomes during translation produced three additional bands. The most dominant of these had the same shift in apparent molecular mass as the CCK N-2 vector, indicating that both the N and C termini are on the luminal side. Of the two other weaker bands, one (lower) has a molecular mass shift consistent with two glycosylation sites utilized. However, the multiple bands present in this part of the gel make it difficult to distinguish these translation artifacts using only the translation product as an indicator. In the presence of its natural preceding sequence, the preponderance of the N- and C-terminal glycosylated products with seven core sites utilized shows that the H3 hydrophilic loop (a, lanes 1–4) and extending to beyond the fifth hydrophobic segment (b, lanes 5 and 6).

![Fig. 7](SDS-PAGE analysis of products resulting from in vitro transcription/translation, in the absence and presence of microsomes (Mic), of the CCK vector extending from the N-terminal amino acid to beyond the fourth hydrophobic segment into the succeeding hydrophilic loop (a, lanes 1–4) and extending to beyond the fifth hydrophobic segment (b, lanes 5 and 6).)

**CCK N-4 Vectors**—The translation products of the vectors CCK N-4 (amino acids 1–182) and CCK N-4LC (amino acids 1–192) had an approximate molecular mass of 35 kDa in the absence of microsomes. The latter construct includes the native glycosylation sequence following H4 (190NQT) for a total of two N-terminal and six C-terminal glycosylation sites. Translation of either construct with microsomes gave a shift in apparent molecular mass of −20 kDa, suggesting that all the glycosylation sites are present on the luminal side (Fig. 7, lanes 1–4). When combined with the previous translation data, this result provides evidence for the presence of four transmembrane segments in the translation product of this vector.

The CCK4 and CCK4LC segments had different activities when expressed in HK-M0 (see above). Only CCK4LC acted as a signal anchor in the HK-M0 vector; but the gel pattern of the translation of the two constructs CCK N-4 and CCK N-4LC was similar, with H4 acting as a signal anchor in both constructs. These results suggest that the 10 amino acids from positions 182 to 192 following the hydrophobic region are necessary for independent membrane insertion of this domain, but are not necessary in the presence of the native upstream sequence.

**CCK N-5 Vector**—The translation product of the vector CCK N-5 (amino acids 1–230) carries eight potential consensus glycosylation sites (two in the N-terminal region, one in the loop between H4 and H5, and five in the H,K-ATPase C-terminal β region) and had a molecular mass of −40 kDa in the absence of microsomes. In the presence of microsomes, the band shifted to −48 kDa. This molecular mass shift shows that the three natural N-linked glycosylation consensus sequences are glycosylated, but that the β region, with its five glycosylation consensus sites, is not (Fig. 7, lanes 5 and 6). This result is consistent with H5 acting as a stop transfer sequence following H4, as predicted by the seven-transmembrane segment model.

![Fig. 8](SDS-PAGE analysis of products resulting from in vitro transcription/translation, in the absence and presence of microsomes (Mic), of the CCK vector extending from the N-terminal amino acid to beyond the sixth hydrophobic region (a, lanes 1 and 2) and to beyond the seventh hydrophobic region (b, lanes 3 and 4) and the intact CCK receptor in pcDNAI (c, lanes 5 and 6).)
**Rabbit Gastric CCK-A Receptor Membrane Insertion Sequences**

In *in vitro* glycosylation of CCK vectors

Shown is the glycosylation of fusion proteins resulting from transcription/translation of cDNA fusion vectors containing an increasing number of hydrophobic sequences inserted between the N-terminal sequence of the CCK-A receptor and the H,K-ATPase C-terminal β region.

| Vector   | Position | No. of sites |
|----------|----------|--------------|
| CCK N    | 1–44     | None         |
| CCK N-1S | 1–70     | 2 or 5       |
| CCK N-1L | 1–73     | Mostly 2     |
| CCK N-2  | 1–101    | 7            |
| CCK N-3  | 1–136    | 7            |
| CCK N-3LC| 1–143    | 7            |
| CCK N-3KR| 1–150    | 2 and 7      |
| CCK N-3KK| 1–160    | 2 and 7      |
| CCK N-4  | 1–182    | 8            |
| CCK N-4LC| 1–192    | 8            |
| CCK N-5  | 1–230    | 3            |
| CCK N-6  | 1–333    | 9            |
| CCK N-6LC| 1–350    | 8            |
| CCK N-7  | 1–374    | 3 and 9      |

**CCK N-6 Vector**—The translation product of the vector CCK N-6 (amino acids 1–333) had a molecular mass of 49 kDa. The sequence of this fusion protein contains nine potential N-linked glycosylation consensus sites (two in the N-terminal region, one in the loop between H4 and H5, one created by the restriction site *HindIII* at the end of H6, and five present in the C-terminal β region). According to the model, all sites must be localized on the luminal side. The presence of microsomes produced a main band shifted 30 kDa above the unglycosylated product (Fig. 8, *lanes 1* and 2), demonstrating that the N terminus, C terminus, and extracytoplasmic loop between H4 and H5 are within the microsomes. This result is consistent with H6 acting as a signal anchor and with the presence of six transmembrane segments.

**CCK N-7 Vector**—The CCK N-7 vector translation product (amino acids 1–374) has nine glycosylation consensus sites (two in the N-terminal region, one in the loop between H4 and H5, one at the end of H7 created by the restriction site *HindIII*, and five present in the β subunit). Only the first three sites are expected to be glycosylated. The translation with microsomes showed two major bands at 67 and 80 kDa. The band at 67 kDa represents an apparent molecular mass shift of 13 kDa compared with the unglycosylated product, most likely resulting from core glycosylation of the three natural N-glycosylation consensus sequences (Fig. 8, *lanes 3* and 4). The higher apparent molecular mass band at 80 kDa could be explained by the presence of the H,K-ATPase β sequence in the lumen, indicating that H7 did not act fully as a stop transfer sequence in this vector, contrary to its behavior in the HK-M1 vector. The two arginines (positions 375 and 377), which are not included in the fusion protein, could act as a cytoplasmic anchor for the C-terminal region of H7, preventing transit of this segment through the translocon.

The *in vitro* transcription/translation results of the CCK vectors are summarized in Table IV. In combination with the analysis of the receptor using the HK-M0 and HK-M1 vectors, explicit evidence was obtained for the presence of seven transmembrane segments in the CCK-A receptor.

**Full-length Sequence**

The *in vitro* translation product of the wild-type CCK-A receptor in the pcDNAI vector (13) (Fig. 8, *lanes 5* and 6) obtained in the absence of microsomes had a molecular mass of 37 kDa, smaller than that predicted from the amino acid sequence (47 kDa). In the presence of microsomes, the molecular mass of the translation product shifted to 53 kDa, which is larger than expected for the addition of three core glycosylation sites to a 37-kDa protein, but would correspond to the addition of these carbohydrates to the calculated molecular mass of 45 kDa.

**DISCUSSION**

Cell-free *in vitro* translation has been used to characterize the membrane domain of several polytopic proteins. The insertion of individual membrane segments into fusion proteins with well characterized membrane topologies and glycosylation sites has allowed inference about insertion properties of these membrane sequences. Although this *in vitro* system can only partially reflect the intracellular environment, the *in vitro* behavior of the constructs used in a study of the H,K-ATPase (14) were later determined to perform in an identical manner when expressed in oocytes and HEK 293 cells.2

The two systems we employed use the presence or inhibition of glycosylation as an indicator of membrane insertion. From all our experiments, the addition of a core glycosylation site results in an increase of ~2.5 kDa to the relative molecular mass in the SDS-PAGE system used. In the case of the HK-M0 vector, the five glycosylation sites, giving a relative molecular mass increase of 12.5 kDa, have been established when used for determining the topology of the P-type ATPases we have studied (14–16). There are no endogenous glycosylation sites in the catalytic subunits of these ATPases; hence, the results of glycosylation are produced as the presence or absence of a single additional band of radioactivity in the presence of microsomes. In the case of the CCK-A vector system, there are three possible endogenous glycosylation consensus sequences, and these, as well as the five C-terminal glycosylation sites, may be utilized, depending on the vector. Furthermore, if translocation is imperfect, a mixture of bands can be obtained, as illustrated, for example, in the translation product of the H1 sequence without the full complement of positive charges following the hydrophobic sequence itself. We therefore used digestion by endoglycosidase H to distinguish between glycosylated bands and background translation products. This is illustrated for the H3-containing vectors, where two major bands are seen, with the ratio changing as the insertion sequence is elongated to include positive charges. Since deglycosylation reduces these to the single band obtained in the absence of microsomes, the interpretation of their molecular mass as reflecting either seven or two sites of glycosylation is verified.

In this study, we first assessed the ability of each putative transmembrane domain to insert independently using a fusion vector technique (HK-M0 and HK-M1) previously applied to three different P-type ATPases (14–16). A signal anchor sequence able to insert into the microsomal membrane *in vitro* is likely to be in the membrane in the native protein. However, a stop transfer sequence must follow a signal anchor sequence to have relevance to membrane folding of the protein (15). We found that some putative membrane segments had signal anchor properties (H1, H2, and H4–H6), whereas H7 had only stop transfer properties, and H3 had no membrane insertion activity. We then evaluated the assembly of the natural sequence of the receptor in a vector containing cDNA coding for the N-terminal sequence of the receptor. Combining the data from both types of vectors resulted in confirmation of seven transmembrane segments for this integral membrane protein, but the data also illustrate that correct membrane insertion depends on factors other than simple hydrophobicity of the hydrophobic sequences, such as the nature of the upstream and downstream sequences.

Predictive algorithms (Table III), such as those of Rao and Argos (19), Kyte and Doolittle (20), and Eisenberg *et al.* (21)

2 K. Geering, personal communication and unpublished data.
and TOP Pred using the GES scale (22), show seven peaks of hydrophobicity, which we used to define putative transmembrane domains for the fusion vectors. The algorithms of Klein et al. (23) and Eisenberg et al. predict only five transmembrane domains. The two transmembrane domains that are excluded (H3 and H7) have lower hydrophobicities. The Eisenberg analysis predicts these sequences as globular. Various models of these receptors (24, 25) have positioned these segments more toward the center of the membrane domain of the protein, and therefore, these have larger protein-protein interactions.

The translation of an almost full-length sequence (CCK N-7) confirms the presence of an odd number of transmembrane domains. Most of the predicted transmembrane domains (H1, H2, and H4–H7) have membrane insertion properties in the HK-M0 and HK-M1 vectors that enable them to act as either signal anchor or stop transfer sequences. H4 acts as a signal anchor in the HK-M0 vector only when the loop between H3 and H4 is present (HK-M0/CCKlp4). The putative transmembrane domain H7, despite its weak hydrophobicity, acts as a weak signal anchor in HK-M0 and a strong stop transfer sequence in HK-M1. Following H6, acting as a signal anchor, the stop transfer activity of H7 is sufficient to keep the C terminus on the cytoplasmic side of the ER. The H3 domain, which has a hydrophobicity similar to that of H4 and H7, does not act as either a signal anchor or stop transfer sequence in the HK-M0 or HK-M1 vector. If this information on its own were incorporated uncritically into a model with an odd number of transmembrane sequences, a model with only five membrane segments would result. Such limited information has been used in proposing a model for the structure of the Na,K-ATPase based on in vitro translation alone (26). These data may have reflected problems in membrane folding rather than defining membrane topology.

The coordinate use of CCK-A receptor and HK-M0/HK-M1 vector constructs provided proof for the seven-transmembrane domain postulate using only in vitro translation and demonstrated a stepwise, cotranslational mechanism of assembly of the transmembrane segments of this G7 receptor. In the presence of microsomes, the N-terminal region of CCK N-1 is translocated into the lumen, whereas the presence of positive charges following H1 anchors the subsequent loop within the cytoplasm. Successively elongated constructs move the C-terminal region into or back out of the membrane following each additional transmembrane domain, indicating that a series of signal anchor/stop transfer pairs directs insertion into the membrane. The transcription/translation product of the CCK-A receptor construct containing the first two hydrophobic sequences (CCK N-2) had two transmembrane sequences with both N and C termini present in the microsomal lumen. Translation of the N-terminal sequence up to the end of the H3/H4 loop, which contains one negative and four positive charges, results in a product with N-terminal but no C-terminal glycosylation, showing that H3 is now able to act as a stop transfer sequence provided that positive charges in the loop following this hydrophobic sequence are present (CCK N-3KK). This C-terminal loop is probably required for cytoplasmic anchoring to prevent translocation of the H3 segment across the membrane. In addition to depending on the loop between H3 and H4 for insertion, H3 seems to require the native N-terminal sequence as shown by the failure of H3 to insert if the N terminus of the HK-M1 vector (HK-M1/CCK3KK) is substituted for the native N terminus. Consequently, interaction of the insert CCK3KK with the natural sequence of the receptor is apparently required to stabilize this segment in the membrane.

The addition of H4 (CCK N-4) results in both the N- and C-terminal regions being localized to the lumen of the microsomes, as expected from two pairs of membrane segments with the N-terminal domain on the extracytoplasmic surface. No product with only two glycosylation sites is observed, showing that the natural sequence containing successive transmembrane segments integrates accurately into the microsomal membrane. For the rest of the molecule, the sequential addition of the succeeding hydrophobic domains shown in the constructs CCK N-5, CCK N-6, and CCK N-7 follows the expected mechanism of sequential insertion by putting the β region outside, inside, and then again outside of the microsomal lumen, respectively.
The use of these CCK-A receptor vectors showed that the membrane insertion of the H3 sequence depends on the upstream sequence and also on the downstream sequence between H3 and H4. The results obtained with the two types of vectors (Tables I and II) taken together experimentally establish the seven-transmembrane domain model and suggest a cotranslational sequential assembly mechanism for this protein.

The translations of these constructs allowed investigation of the factors governing membrane insertion such as hydrophobicity and charge distribution. The positive inside rule states that positive charges tend to stay in the cytoplasm, as found in bacteria (27–31). This rule also applies to eukaryotic proteins (32), although there is no transmembrane potential across the ER membrane. Moreover, negative charges are thought to promote translocation to the lumen of the ER (33). More recently, a charge difference rule has been proposed (34).

Our results show that the presence of only two positive charges at the C terminus of the H1 segment still mainly results in an orientation with the C terminus in the lumen. The addition of two more positive charges produces a translated peptide with the C-terminal region entirely in the cytoplasm, which is its natural orientation. These results show that all the positive charges in the loop between H1 and H2 are essential for the correct orientation of the first transmembrane domain of this receptor with the native N-terminal sequence. All known GPCRs have a cluster of positive charges in the cytoplasmic loop between the first and second hydrophobic sequences and lack cytoplasmic anchoring prior to the first sequence (6). P-type ATPases, on the other hand, have positive charge sequences prior to their first hydrophobic sequence and have a cluster of negative charges between the first and second sequences. Hence, the orientation of the first two transmembrane sequences is opposite in these integral membrane proteins, determined by the presence or absence of cytoplasmic anchoring sequences prior to or following the first transmembrane domain.

A similar cytoplasmic anchoring effect of positive charges subsequent to H3 was found, permitting H3 to act as a stop transfer sequence in the CCK vectors. However, the positive charges in this loop are not clustered, which might indicate the involvement of other factors influencing folding, thereby allowing the positive charges to be seen by the translocon or to interact with the upstream sequence of the protein. For instance, the loop between H2 and H3 is easily translocated, as observed in the construct CCK N-3, even though it has three positive charges and one negative charge.

The effect of positive charges is also found in the H6 region. The sequential insertion model would require H6 to be a signal anchor, and it behaves in this way in the CCK-A vector (CCK N-6). By itself, it does not act as a signal anchor sequence in HK-M0, but the addition of four of the positive charges immediately prior to H6 (HK-M0/CCK6LN) promotes a strong glycosylation of the β region. These charges within the sequence of the CCK-A receptor therefore promote membrane insertion, whereas the charges in the N-terminal region of the α chain of the HK-ATPase present in the HK-M0 vector do not. This result suggests that the positively charged cytoplasmic anchor and the hydrophobic sequence may also have to be in proximity to affect membrane insertion or orientation (35). Furthermore, when the H6 putative transmembrane domain is extended on the C-terminal side, including two positive and one negative charge (HK-M0/CCK6LC), this sequence also promotes a strong glycosylation of the β region, therefore acting as a signal anchor. This suggests that the sequence on the C-terminal side can independently facilitate the membrane insertion of H6.

In the full sequence of the CCK-A receptor, the loops between H1 and H2 and between H3 and H4 have only positive charges and are on the cytoplasmic side, whereas the loops between H2 and H3, H4 and H5, and H6 and H7 include negative charges and are translocated. For the larger H5/H6 loop, the cluster of positive (and some negative) charges might be responsible for anchoring this region on the cytoplasmic side.

Some of these data relating to the effect of adjacent positive charges are illustrated in the models of Fig. 9. This figure illustrates that there are regions of the translocon complex that interact with clusters of positive charges, preventing transfer of these clusters across the membrane as an alternative to the charge difference rule. From the work described above, an analytical in vitro translation method using a combination of vectors able to analyze individual or consecutive segments is able to identify the transmembrane sequences of a typical GPCR, such as the CCK-A receptor, and may have general applicability to most polytopic integral membrane proteins. The glycine transporter, for instance, was also investigated recently using in vitro translation. Here also the C-terminal sequence of the β subunit of the H,K-ATPase followed different length constructs of this transporter, and a new model was proposed for the first four of its transmembrane sequences (36), further illustrating the effectiveness of this approach in defining membrane topology of polytopic integral membrane proteins.