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Intracellular Processing of the Porcine Coronavirus Transmissible Gastroenteritis Virus Spike Protein Expressed by Recombinant Vaccinia Virus

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The Spike (S) protein from a virulent British field isolate of porcine transmissible gastroenteritis virus (TGEV) FS772/70 was constructed from cDNA and inserted into the vaccinia virus (VV) thymidine kinase gene locus under the control of the VV early/late gene P7.5K promoter. Recombinant S protein was synthesized as an endo-P-N-acetylglucosaminidase H (Endo H)-sensitive glycoprotein with high mannose simple oligosaccharides (gpl90) that underwent post-translational modification to an Endo H-resistant glycoprotein with complex oligosaccharides (gp210). Immunofluorescence analysis demonstrated that the majority of recombinant S protein was retained at the Golgi but some S protein was expressed on the plasma membrane. Monoclonal antibodies (mAbs) raised against native S protein reacted with this recombinant S protein; also, mice infected with the recombinant vaccinia virus (rVV) expressing the S protein induced TGEV neutralizing antibodies. A truncated S protein (SΔ) was also expressed in rVV-infected cells by introducing a deletion into the S protein cDNA that removed 292 amino acids from the C-terminus. The SΔ protein (gpl70) was shown to be antigenically similar to TGEV S protein by immunofluorescence and immunoprecipitation tests but was retained in the endoplasmic reticulum and not expressed on the cell surface.

INTRODUCTION

Transmissible gastroenteritis virus (TGEV) belongs to the family Coronaviridae, a large group of enveloped viruses with a positive-stranded RNA genome. The virus causes gastroenteritis in neonatal pigs, resulting in a high mortality and morbidity. TGEV virions are composed of three structural proteins: a basic phosphorylated nucleoprotein (N) M, 47,000 was shown to associate with the viral genomic RNA to form the nucleocapsid and interact with a glycosylated membrane protein (M) observed as a series of polypeptides M, 28–31,000, and the peplomer or spike (S), a surface h/l, 200,000 glycoprotein (Garwes and Pocock, 1975). The TGEV S protein has been shown to elicit a neutralizing antibody response (Lauze et al., 1986; Jimenez et al., 1986; Garwes et al., 1987) capable of conferring some protection to suckling pigs (Garwes et al., 1978/79). By analogy to the S protein of mouse hepatitis virus (MHV), the TGEV S protein may possess the cell receptor binding components (Collins et al., 1982) and virulence determinants of the virus (Fleming et al., 1986). The S protein of TGEV and coronaviruses antigenically related to TGEV such as feline infectious peritonitis (FIPV), canine coronavirus, and porcine respiratory coronavirus, are not cleaved (for a review see Spaan et al., 1988). However, the S proteins from MHV, infectious bronchitis virus, bovine coronavirus, human coronavirus (OC43 and 229E), and porcine hemagglutinating encephalomyelitis virus which are not antigenically related to TGEV are proteolytically cleaved into two subunits (Spaan et al., 1988). Some coronavirus S proteins have been demonstrated to induce cell fusion (Collins et al., 1982; Sturman et al., 1985; de Groot et al., 1989), generating multinucleated syncytia.

The complete amino acid sequences for the S proteins of the virulent FS772/70 strain (Britton and Page, 1990) and the avirulent Purdue strain (Jacobs et al., 1987; Rasschaert and Laude, 1987) have been published and compared with other coronaviruses. The FS772/70 strain S protein has a 1449-amino acid precursor polypeptide with 33 potential N-linked glycosylation sites and 97% sequence homology at the nucleotide and the amino acid level with the Purdue strain. From the deduced amino acid sequence the coronavirus S protein was shown to contain characteristic features: a 16-amino acid cleavable secretory signal; a heptad repeat sequence that forms α-helical structures which may interact with other subunits to form a coiled-coil oligomeric structure (de Groot et al., 1987); a hydrophobic sequence near the C-terminus that is probably responsible for anchoring the S protein to the virion envelope. This membrane anchor region is imme-
immediately followed by a cysteine-rich domain, a feature common to all other coronaviruses, that may stabilize protein–lipid interactions.

In this paper we report the construction of a FS772/70 cDNA S gene and its expression by a recombinant vaccinia virus (rVV) to study the antigenicity and cellular localization of the S protein. The role of the membrane anchor domain was investigated by the introduction of a C-terminal gene deletion downstream of the heptad repeat sequence.

**MATERIALS AND METHODS**

**Cells and viruses**

TGEV, strain FS772/70, was cultivated in a porcine continuous cell line (LLC-PK1) maintained with medium containing 10 µg ml⁻¹ trypsin (Hofmann and Wyler, 1988). CV-1 and human 143 thymidine kinase negative (HTK-) cells were grown in Eagles MEM medium (Flow Labs) containing 10% heat-inactivated fetal calf serum. Transfections were performed on subconfluent monolayers of CV-1 cells previously infected with wild-type vaccinia virus (VV) (WR strain) using plasmid DNA calcium phosphate precipitates (Mackett et al., 1985). Recombinant vaccinia viruses were cultivated in HTK- cells in the presence of 25 µg ml⁻¹ 5-bromodeoxyuridine (BUDR; Sigma) as described by Mackett et al. (1985).

**Construction of the TGEV S and SΔ genes from cDNA**

Cloning procedures were as described by Maniatis et al. (1982). Enzymes were used according to manufacturers’ instructions (New England Biolabs, Bethesda Research Labs). Plasmids pTG47, pTG25, and pTG30 (Britton and Page, 1990) were used to reconstruct a DNA copy of the TGEV S gene; the procedure is outlined in Fig. 1. Essentially BamHl linkers (pCGGATCCG, No. 1021 Biolabs) were added to the HpaI site known to be 11 bp upstream of the S gene initiation codon (Britton and Page, 1990), and the 1.29-kb HindIII fragment was cloned into pUC9 and excised as a 0.85-kb XbaI–BamHl fragment. The BamHl–Styl fragment derived from pTG47, the Styl–KpnI fragment derived from pTG25, the KpnI–XbaI fragment derived from pTG30, the 0.85-kb XbaI–BamHl fragment, and BamHl dephosphorylated pBR322 were mixed, ligated in a five-way reaction mixture, and transformed into DH1 *Escherichia coli* cells. As a result of the different cohesive ends an insert of 4.6 kb would correspond only to the complete S gene. An Ap⁹Tc³ transformant was found to contain a plasmid, pPBP1, with a 4.6-kbp insert in pBR322, corresponding to the TGEV S BamHl gene cassette. The reconstructed gene was verified by sequencing the junction regions. The TGEV cDNA S gene was subcloned from pPBP1 and inserted into the BamHl cloning site of the VV plasmid insertion vector pGS20 (Mackett et al., 1984), downstream of the VV early/late P₇.₅K promoter. The recombinant plasmid, pGSP-1, containing a correctly orientated S gene, was identified by restriction endonuclease digestion with SalI.

The S protein with a C-terminal deletion was generated by cloning a 3.7-kbp SalI fragment from pGSP-1 into the SalI site of pUC12. Restriction endonuclease digestion of recombinant plasmids produced either a 0.25- or 3.45-kbp BamHl fragment and the latter was recloned back into BamHl-digested pGS20. The recombinant plasmid insertion vector pGSPΔ-38 was found to contain a correctly orientated SΔ gene by Styl restriction endonuclease digestion of plasmid DNA. A synthetic oligonucleotide primer 5’-GTGTGCGGCTAC-TATAACTA-3’, that binds to the complementary DNA strand 43 bp downstream of the BamHl cloning site in pGS20, verified the position of the SΔ protein stop codon in pGSPΔ-38 by sequencing (Fig. 2A).

**Construction of recombinant viruses**

The rVV's, vTS-1 and vTSΔ-1, were generated with pGSP-1 and pGSPΔ-38 using methods described by Mackett et al. (1984). Thymidine kinase negative viruses were screened by DNA dot blot and DNA from vTS-1 and vTSΔ-1 infected cells was analyzed by Southern blot using a [³²P]-labeled S gene cDNA probe as described in Pulford and Britton (1990).

**Animal inoculations**

Groups of three female Balb/C mice were immunized by intraperitoneal (ip) inoculation with 1–2 × 10⁶ PFU/mouse of partially purified recombinant or wildtype VV as described by Pulford and Britton (1990). After 4 weeks, one animal from each group was sacrificed to obtain convalescent serum, the remaining animals were hyperimmunized with homologous virus by ip inoculation and bled after a further 4 weeks. Mice initially inoculated with either phosphate-buffered saline (PBS) or WR strain VV were inoculated after 4 weeks with a dose of vTS-1 to establish if age or a previous VV infection affected the stimulation of TGEV neutralizing antibody. TGEV neutralizing antibody was assessed by plaque reduction assay as described by Garwes et al. (1987).

**Radiolabeling of viral proteins**

Viral proteins were routinely radiolabeled by incubating TGEV-infected LLC-PK1 cells or rVV-infected HTK⁻
cells, at 8 hr p.i. or 17 hr p.i., respectively, in methionine-free medium for 1 hr and then in the presence of 50–100 \( \mu \text{Ci ml}^{-1} \text{L}^{[35\text{S}]} \) methionine (Amersham International; see figure legends for further details). After pulse labeling, cells were washed in PBS (10 mM potassium phosphate, 150 mM NaCl, pH 7.2) and chased in medium supplemented with 2 mM L-methionine. Cells were washed in PBS before treating with TEST lysis buffer (20 mM Tris-HCl, pH 7.6, 2 mM EDTA, 100 mM NaCl, 1% Triton X-100, 0.1% aprotablet).

Radioimmunoprecipitation

Nuclei and cell debris were pelleted from infected cell lysates by centrifugation at 100,000 g using a Beckman TLA-100 ultracentrifuge. Tissue culture medium was clarified by low speed centrifugation. Immunoprecipitations were performed by mixing 1 vol of cell lysate with 1/100th vol of porcine TGEV hyperimmune antiserum at 4°C for 1 hr and the resulting immune complexes were incubated overnight with formalin-fixed Staphylococcus aureus cells (SAC; BRL) previously washed three times in TEST buffer. Immune complexes were washed three times with TEST buffer and resuspended in Laemmli sample buffer, and proteins were analyzed on 6% SDS-polyacrylamide gels and detected by fluorography after immersing gels in 0.8 M sodium salicylate for 30 min.

Endoglycosidase H analysis

Pelleted immune complexes were washed as described above, resuspended in 40 \( \mu \text{l} \) 50 mM Tris-\( \cdot \)HCl, pH 6.8, containing 0.25% SDS, and boiled for 2 min. The solubilized proteins were incubated in the presence or absence of 1 \( \mu \text{M} \) Endo H (Boehringer-Mannheim) in 90 mM sodium citrate, pH 5.5, at 37°C for 18 hr. The proteins were analyzed on 6% polyacrylamide gels and detected by fluorography.

Indirect immunofluorescence

Glass coverslips with subconfluent monolayer cultures of HTK- or LLC-PK1 cells were infected with TGEV and wild-type or rVVes. At 20 hr p.i. cells were either fixed with cold 80% acetone and air dried or washed in cold PBS and maintained at 4°C for surface staining. Cells were incubated for 1 hr with porcine TGEV hyperimmune antiserum diluted 1:100 in PBS and then extensively washed with PBS. Cells were then incubated for 30 min with fluorescein isothiocyanate-conjugated rabbit anti-pig immunoglobulin G (Nor-dic Immunology) diluted 1:40 in PBS. Coverslips were washed, air dried, and mounted on glass slides with 80% glycerol. Fluorescent cells were observed and photographed with a Leitz Wetzlar UV microscope.

RESULTS

Construction of S and S\( \Delta \) cDNA genes

A 4.6-kbp BamHI S gene cassette was constructed from TGEV FS772/70 cDNA and used to generate pPBP1 (Fig. 1). The 4350-bp S gene was capable of encoding a precursor polypeptide of 1449 amino acids with a \( M, 159,811 \). After cleavage of the N-terminal signal peptide, shown to be absent in virion-associated Purdue strain S protein (Rasschaert and Laude, 1987), the protein would consist of 1433 amino acid residues with a \( M, 157,891 \). The S gene BamHI gene cassette was subcloned into the VV insertion vector pGS20 to give pGSP-1 as described under Materials and Methods. The S\( \Delta \) gene, contained in pGS\( \Delta \)A-38, was 3474 bp long, capable of encoding a 1157-amino acid polypeptide, corresponding to a deletion of 292 residues from the complete S protein and also included eight amino acid residues derived from the pUC12 polylinker and pGS20 sequences. The S\( \Delta \) gene terminated in a new TGA stop codon contained within the VV TK sequences (Fig. 2). The predicted size of the precursor S\( \Delta \) polypeptide was \( M, 126,748 \), which when modified by the removal of the 16-amino acid N-terminal signal peptide was reduced to \( M, 124,916 \). The C-terminal deletion also resulted in removal of eight potential N-linked glycosylation sites predicted for the S protein. The S gene initiation codon was 54 bp away from the VV early promoter RNA start site for both pGSP-1 and pGS\( \Delta \)A-38 insertion vectors. The TGEV S genes were inserted into the VV genome by homologous recombination into the TK locus, and Southern blot analysis was used to confirm that the resulting rVVes vTS-1 and vTS\( \Delta \)-1 contained the 4.6- and 3.45-kbp TGEV BamHI gene fragment, respectively.

Animal inoculations with vTS

Two plaque-purified rVV clones, vTS-1 and vTS-2, were used to immunize Balb/C mice, and the level of TGEV neutralizing antibodies was measured by a plaque reduction assay (Table 1). Both recombinant virus clones induced TGEV neutralizing antibodies that were boosted with a second inoculation. Mice previously inoculated with the wild-type VV and inoculated with vTS-1 produced a 22-fold lower level of TGEV neutralizing antibody compared to the convalescent serum of vTS-1. A primary infection with wild-type VV could have stimulated the mouse immune system.
into producing a rapid clearance of a subsequent rVV infection and prevented the expression of large amounts of S protein. Mice given a single inoculation with vTS-1 4 weeks after the other mouse group had a fourfold reduction in the level of TGEV neutralizing antibody. This suggested that age may have a significant effect on the induction of TGEV neutralizing antibodies, but further animal studies are needed to make any firm conclusions.

Expression of the recombinant spike antigens

TGEV gene products synthesized by vTS-1 and vTSΔ-1 were analyzed by pulse labeling rVV-infected HTK" cells with L-[^35S]methionine in the presence or absence of 10 μg ml^-1 tunicamycin for 6 hr. TGEV S protein was immunoprecipitated from cell lysates or tissue culture fluids using porcine TGEV hyperimmune antiserum. This resulted in S polypeptides of Mᵣ 190,000 (gp190) and Mᵣ 210,000 (gp210) being immunoprecipitated from vTS-1-infected cell lysates in the absence of tunicamycin (Fig. 3, lane 2), while in the presence of tunicamycin, a Mᵣ 160,000 (p160) precursor S polypeptide was synthesized (Fig. 3, lane 3). Cells infected with vTSΔ-1 expressed a Mᵣ 170,000 glycosylated polypeptide (gp170) only (Fig. 3, lane 4) that was expressed as a Mᵣ 130,000 (p130) precursor protein (Fig. 3, lane 5) in the presence of tunicamycin. It was noted that the levels of S protein expressed were appreciably less in the presence of tunicamycin (Fig. 3). Comparison of the nonspecifically precipitated proteins in the presence or absence of tunicamycin (Fig. 3) indicated that the reduction in S protein synthesis was not due to inhibition of viral replication in the presence of tunicamycin. These observations suggested therefore that either glycosylation was required for efficient synthesis of the S protein or the majority of the anti-S antibodies were directed against the glycosylated form.
of the protein resulting in less efficient immunoprecipitation of the nonglycosylated form. Although gp210 was immunoprecipitated from the vTS-1-infected cell culture medium in the absence of tunicamycin (Fig. 4, lane 3), no detectable extracellular SΔ protein (gp170) was recovered from medium of vTSAΔ-1-infected cells (Fig. 4, lane 6), suggesting that the membrane anchor is required for export out of cells. The lack of detectable p160 or p130 in the culture medium of vTS-1- and vTSAΔ-1-infected cells, respectively (Fig. 4 lanes 4 and 7), could result from the levels being too low to be detected by the antibodies due to reduction in synthesis or the lack of glycosylation of the S protein in the presence of tunicamycin.

Intracellular transport of TGEV and recombinant S protein

The rate of S protein intracellular transport was compared by measuring the acquisition of Endo H resistance in TGEV-, vTS-1-, and vTSAΔ-1-infected cells. After a 1-hr pulse, the TGEV S protein consisted of a major gp190 component and a minor gp210 component (Fig. 5A). After a 1-hr chase, gp190 and gp210 were present in approximately equal proportions but after a 2-hr chase, gp210 was the dominant S protein component and the majority was Endo H resistant. TGEV S protein steadily became Endo H resistant with time such that after a 4-hr chase virtually all S protein had been modified in the Golgi. S protein was also detected in the tissue culture medium of TGEV-infected cells after a 1-hr chase and accumulated steadily with

### TABLE 1

TITRATION OF COMPLEMENT-INDEPENDENT TGEV NEUTRALIZING ANTIBODY RAISED IN MICE INFECTED WITH VIRUSES

| Vaccinia virus | First bleed | Vaccinia virus | Boost |
|---------------|-------------|---------------|-------|
| WR            | <10         | WT            | <10   |
| vTS-1         | 466         | vTS-1         | 1122  |
| vTS-2         | 162         | vTS-2         | 433   |
| WR            | <10         | vTS-1         | 22    |
| PBS           | <10         | vTS-1         | 114   |
The recombinant S protein was completely Endo H sensitive after a 1-hr pulse and after chasing became partially Endo H resistant with approximately half of the protein observed as unresolved components of high molecular weight (Fig. 5B). Extracellular S protein was not detected in the culture medium after chasing for 4 hr because S protein transport from the RER through the Golgi stack and out of the cell was significantly disrupted in the absence of coronaviral morphogenesis. Infection of LLC-PK1 cells with vTS-1 produced a discrete $M_r$ 210,000 Endo H-resistant form of S protein (data not shown). This was in contrast to the partial Endo H-resistant forms observed in vTS-1-infected HTK cells, implying that the TGEV S protein may be post-translationally modified to a different extent depending on the origin of the cell line. The SΔ protein remained completely Endo H sensitive, even after a 4-hr chase, demonstrating that the truncated S protein was glycosylated with high mannosé oligosaccharides only and was not subject to Golgi-specific modifications (Fig. 5B).

**Cellular location of recombinant S proteins**

The cellular location of the S and SΔ protein in rVV-infected cells was analyzed by indirect immunofluorescence microscopy (Fig. 6). Acetone-fixed TGEV-infected LLC-PK1 cells, probed with anti-S-specific serum, had a granular appearance with occasional bright accumulations in localized parts of the cell (data not shown). In vTS-1-infected HTK cells (Fig. 6A) and LLC-PK1 (Fig. 6C) cells the S protein was concentrated into...
an intracellular compartment in all infected cells (represented with arrows), while vTSΔ-1-infected cells had a uniform distribution of SA protein throughout the cytoplasm (Fig. 6B and 6D). Recombinant S protein was also observed on the cell surface of unfixed infected HTK− cells (Fig. 6E), unlike SA protein (Fig. 6F), demonstrating that the full-length protein has all the intrinsic properties for cell surface transport and does not require the cooperative effect of other TGEV proteins. The possibility that soluble S protein released from human cells may bind back to cell surface receptors and give the appearance of cell surface fluorescence can be disregarded as TGEV S protein only binds porcine cell surface receptors.

DISCUSSION

Studies described in this paper with TGEV recombinant S protein have shown that it is an N-linked glycoprotein with a $M_r$ 190–210,000. Mice inoculated with two separate clones of vTS demonstrated that the recombinant S protein was immunogenic and elicited TGEV neutralizing antibodies. This was in contrast to previous studies with TGEV N and M proteins expressed by rVV that induced an immune response but no neutralizing antibodies (Pulford and Britton, 1990).

The TGEV S protein neutralizing epitopes and major antigenic sites were retained by the SA protein but this protein was not transported to the plasma membrane or through the Golgi stack and must therefore be accumulated in the endoplasmic reticulum. Hu et al. (1984) constructed an incomplete TGEV S protein gene that initiated 8 bp downstream of a Hpal site but ended 80 bp upstream of a XbaI site. This gene construct expressed an Endo H sensitive $M_r$ 180,000 polypeptide that was not transported to the cell surface in rVV-infected cells (Hu et al., 1985). Sequence analysis of the TGEV Purdue-115 (Jacobs et al., 1987; Rasschaert et
Coronavirus S proteins are known to be highly glycosylated with N-linked oligosaccharides and undergo modification with complex sugars at the medial compartment of the Golgi stack during virus maturation (Niemann et al., 1982). Simple high mannose and hybrid structures can be removed from glycoproteins by digestion with Endo H but glycoproteins modified with complex sugar structures are resistant to cleavage with Endo H (Hubbard and Ivatt, 1981; Dunphy and Rothman, 1985). The rVV vTS-1 produced two S protein species in human cells with different oligosaccharide composition, assigned gp190 and gp210, while the SΔ protein was expressed in human cells as a single glycoprotein species designated gp170. The gp210 was partially resistant to Endo H, suggesting that gp190 was modified to gp210 by the addition of complex oligosaccharides at the Golgi. The SΔ protein remained Endo H sensitive even after extensive chasing, implying that it was not transported to the Golgi stack.

Pulse-chase analysis of TGEV- or vTS-1-infected cells demonstrated that S protein was initially synthesized as gp190 but gradually accumulated as gp210 due to post-translational modifications. Endo H digestion of recombinant S protein expressed in HTK'-produced a range (M, 160–210,000) of partially resistant polypeptides. Both of these observations were made for the FIPV (79-1146) S protein expressed in a bovine papilloma virus transformed mouse cell line (de Groot et al., 1989). However, the TGEV S gene expressed by a rVV in porcine LLC-PK1 cells produced an Endo H-resistant gp210 product with no partially resistant Endo H intermediates. A similar observation was also made for the FIPV S gene expressed by a rVV in feline cells (Vennema et al., 1990), suggesting that expression of coronavirus S proteins in a cell line compatible with their origin may have profound effects upon the extent of post-translational processing of these proteins.

The acquisition of Endo H resistance was significantly retarded for recombinant S protein compared to S protein expressed during a TGEV infection. Retardation of coronavirus S protein intracellular transport has also been observed for the FIPV and MHV S proteins expressed by rVVs (Vennema et al., 1990). In this study, retardation between the RER and the Golgi in the absence of virus budding was interpreted as a transient accumulation of the S protein at or near the site of virus budding and suggested that the S protein may have a role in defining the site of virus budding, a function previously ascribed to the M proteins of coronaviruses (Tooze et al., 1985). Immuno-affinity precipitation studies with vTS-infected cells demonstrated that recombinant S protein accumulated in a polar perinuclear compartment of the cell, a similar observation was seen for the intracellular localization of rVV expressed TGEV M protein (Pulford and Britton, 1990), and at the cell surface. The presence of TGEV gp210 in the culture medium of vTS-infected cells suggested that gp210 may be released from the cell.

Proteins can be transported from the RER to the Golgi by interacting with carrier proteins or following membrane incorporation, by the flow of membrane from the ER to the cell surface. This membrane flow may be responsible for the cell surface presence of coronavirus S proteins. The SΔ protein was not processed in the Golgi apparatus, nor found on the cell surface nor found to be exported into the extracellular medium, indicating that the C-terminal membrane anchor domain of TGEV S protein may be required for all of these functions. Puddington et al. (1986) used a mutant VSV G protein to establish that the cytoplasmic tail of the G protein was essential for its transport through the Golgi stack. In addition, Bray et al. (1989) used rVVs to demonstrate that a Dengue virus envelope protein truncated at the C-terminus, unlike the wild-type protein, was not secreted into the extracellular medium. However, Sveda et al. (1982) found that C-terminal sequences of the influenza virus hemagglutinin (HA) were essential for cell surface expression and that mutants with a disrupted anchor domain were secreted, suggesting that influenza virus HA, unlike TGEV, VSV, and Dengue virus envelope proteins, underwent a different protein maturation pathway. Evidence suggests that unless a membrane glycoprotein folds into a native or near-native conformation it will not be exported from the RER (Lodish, 1988). However, the general conformation and antigenicity of the SΔ protein appeared to be retained as it reacted with both polyclonal and monoclonal TGEV S antisera in contrast to denatured S protein or S protein expressed in E. coli cells (Correa et al., 1990; Delmas et al., 1990; Pulford, unpublished observations). The results presented in this paper suggest that the SΔ protein was retained in the RER possibly due to the removal of essential C-terminal transport signals.

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