JOURNAL OF VIROLOGY, June 1987, p. 2042–2045
0022-538X/87/062042-04$02.00/0

Vol. 61, No. 6

Coronavirus E1 Glycoprotein Expressed from Cloned cDNA Localizes in the Golgi Region

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Received 5 December 1986/Accepted 23 February 1987

Cloned cDNA encoding the membrane glycoprotein E1 of the coronavirus mouse hepatitis virus strain A59 was expressed transiently in a monkey fibroblast cell line (COS) by using a simian virus 40-based vector. As determined by indirect immunofluorescence microscopy, the E1 protein accumulated intracellularly in a perinuclear region coincident with a Golgi marker. The same three species of E1 that occur in virus-infected cells were also found in transfected cells. These are one unglycosylated form and two apparently O-glycosylated forms that could be labeled in a tunicamycin-resistant fashion with [^{3}H]glucosamine. Because O glycosylation occurs posttranslationally in the Golgi apparatus, we could show, by monitoring the rate of acquisition of oligosaccharides, that the transport of E1 from the rough endoplasmic reticulum to the Golgi apparatus had a half time of between 15 and 30 min.

Coronaviruses, a group of plus-stranded, enveloped RNA viruses, mature in intracellular membranes. In infected cells, viral particles form by the budding of viral nucleocapsids, assembled in the cytoplasm, into the lumen of the endoplasmic reticulum or into the Golgi apparatus, depending on the type of cell (2, 7, 21, 22). Infectious virions are shed by the cells through exocytosis of vesicles emanating from the Golgi region.

Intracellular virus budding is a relatively rare phenomenon. It has been described for coronaviruses, as well as for bunyaviruses (9, 10) and toroviruses (3, 24). For coronaviruses, the membrane glycoprotein E1 is thought to be the determinant of the budding site. This conjecture is based on two findings. First, in infected cells, E1 accumulates in internal membranes (2, 11; E. W. Doller and K. V. Holmes, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, T190, p. 267), while the other viral membrane protein E2 appears on the plasma membrane (Doller and Holmes, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980). Second, when infected cells are grown in the presence of the drug tunicamycin, which inhibits the addition of N-linked oligosaccharides to E2 but not the addition of O-linked oligosaccharides to E1, noninfectious virus particles are produced which contain E1 but lack E2 (7, 13, 16), suggesting that E2 is not essential for the budding process.

Because the signals that determine protein targeting to intracellular membranes are not well defined, the E1 protein is an ideal model for the study of such targeting. We have recently studied the insertion of E1 into membranes and the resulting topology of the protein (1, 14, 15, 18). By using in vitro translation in the presence of microsomes, we have found that the polypeptide is anchored in the lipid bilayer by three successive transmembrane helices and that only small parts are accessible to proteases on either side of the membrane; these accessible parts are regions of approximately 2,500 and 1,500 daltons from the NH_2 and COOH termini which are exposed to the lumenal and cytoplasmic compartments, respectively (15). Assembly of the protein into microsomes appeared not to involve cleavage of an N-terminal signal sequence. Instead, it was concluded that the insertion signal resides in an internal domain (18). Moreover, it was shown that integration of E1 into membranes requires interaction with the signal recognition particle (14), the cytoplasmic component of the protein translocation system.

To begin further studies of its biogenesis and role in coronavirus maturation, we wanted to express the E1 protein in cells in the absence of other mouse hepatitis virus (MHV) proteins. To accomplish this, the E1 gene was excised from the original cloning vector pHN42 (12; a generous gift from H. Niemann) by using the restriction enzymes *Aha*III and *Hin*dIII. After the insert DNA was purified by gel electrophoresis and filled in with DNA polymerase (Klenow fragment), *Xho*I linkers were added, and the fragment was subsequently digested with *Xho*I. The purified fragment was then ligated into the in vitro transcription vector pT3/T7-18 (Bethesda Research Laboratories, Inc.) in which an *Xho*I site had been inserted. The E1 gene was excised with *Xho*I and cloned into the *Xho*I site of the pBR322/SV40-based expression vector pJC119 (19). In this vector the E1 gene is under the control of the simian virus 40 late promotor that can be activated in COS-1 cells through the constitutive expression of large-T antigen (4). All manipulations were done by standard protocols. The resulting plasmid pJCE1 was transfected onto COS-1 cells, and E1 expression was monitored by indirect immunofluorescence. Cells were made permeable and E1 protein was detected by using a rabbit anti-MHV-A59 serum (dilution 1:200; 17) followed by affinity-purified fluorescein-conjugated goat anti-rabbit immunoglobulin G serum (1:20; Southern Biotechnology). Finally, the cells were treated with rhodamine-conjugated wheat germ agglutinin (1:100; a gift from Avi Kupfer). We only observed specific E1 fluorescence near the nuclei of cells expressing the protein (Fig. 1). By using rhodamine-coupled wheat germ agglutinin as a marker for the Golgi apparatus (5), it was concluded that in these COS cells the E1 protein accumulated in the Golgi region. We did not observe any fluorescence at the plasma membrane of permeabilized cells, nor could we detect surface labeling of nonpermeabilized cells (not shown). Apparently, E1 protein does not reach the plasma membrane. In contrast to many other genes expressed with this vector, the level of

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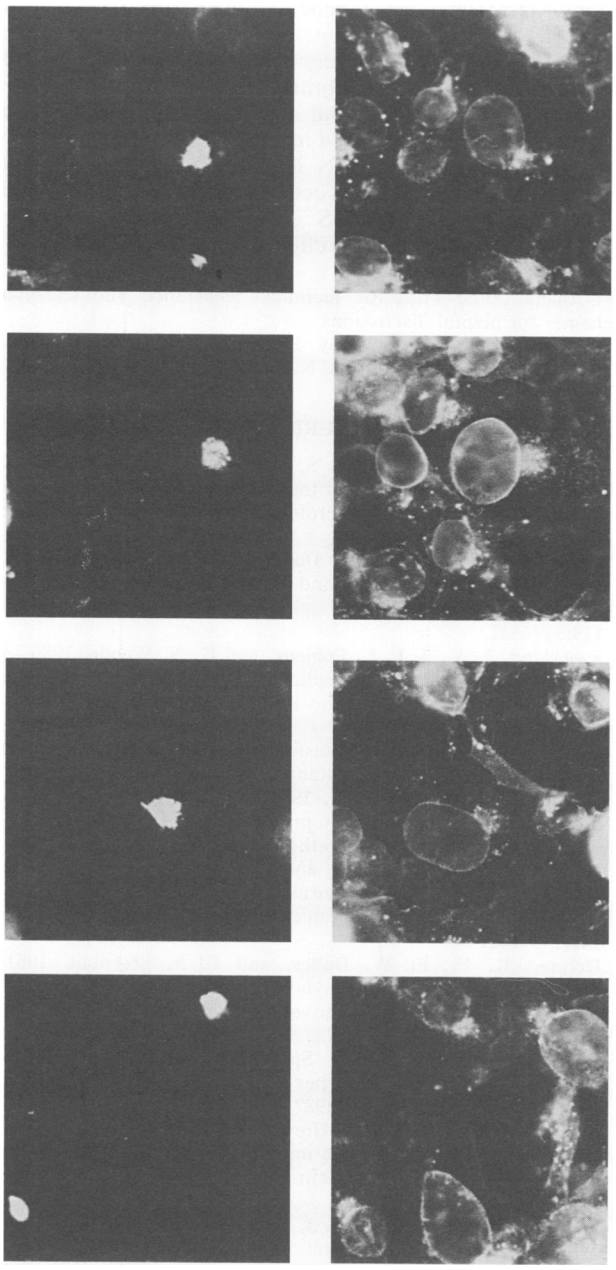

FIG. 1. Detection of the E1 protein by indirect immunofluorescence microscopy. COS-1 cells grown on cover slips were fixed 40 h after transfection with pJCE1. The cells were stained for E1 by using an antiviral antiserum, as described in the text. The micrographs on the left are of fluorescein isothiocyanate-labeled cells, and the pictures on the right are of the same fields after being stained with rhodamine-labeled wheat germ agglutinin.

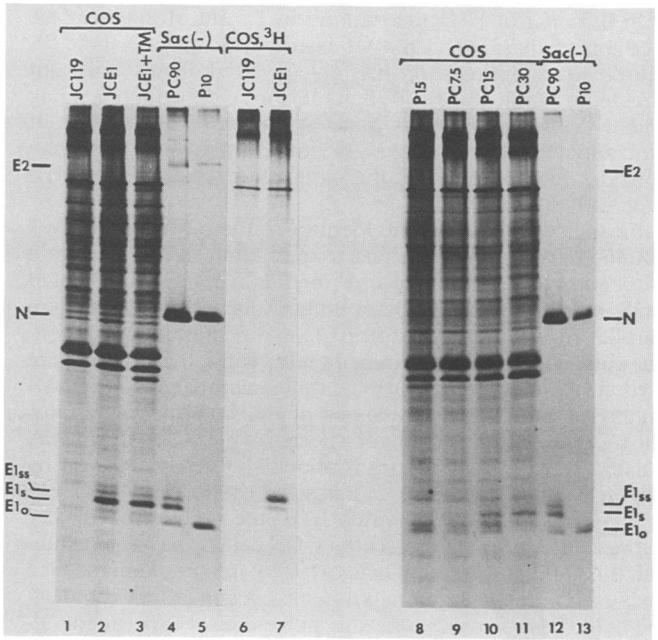

FIG. 2. Biochemical analysis of E1 in pJCE1-transfected COS-1 cells. Cells were transfected either with pJCE1 or with the parental vector pJC119. They were labeled with [^{35}S]methionine or [^{3}H]glucosamine, as described in the text. Immunoprecipitates were prepared and analyzed in a 15% polyacrylamide gel. Lanes: 1 through 3, immunoprecipitates from pJC119 (lane 1)- and pJCE1-transfected cells labeled with [^{35}S]methionine for 90 min. The material in lane 3 was obtained by labeling in the presence of tunicamycin (TM). Lanes 6 and 7, immunoprecipitates from pJC119 (lane 6)- and pJCE1-transfected cells labeled with [^{3}H]glucosamine; 8 through 11, pulse-chase experiment. pJCE1-transfected COS cells were labeled with [^{35}S]methionine for 15 min. The material in lane 8 was prepared immediately, whereas that of lanes 9 through 11 was harvested after a 7.5-, 15-, or 30-min chase, respectively. Lanes 4, 5, 12, and 13, immunoprecipitates of MHV-A59-infected Sac$^-$ cells either pulse-labeled with [^{35}S]methionine for 10 min (lanes 5 and 13) or pulse-labeled and chased with excess unlabeled methionine for 90 min (lanes 4 and 12). $E1_0$, unglycosylated form of E1; $E1_s$ and $E1_{ss}$, the two main glycosylated forms of E1; N, MHV-A59 nucleocapsid protein; E2, MHV-A59 membrane glycoprotein E2 precursor.

expression of E1 was low. Clear fluorescence was observed only in approximately 1% of the cells compared with 10 to 20% observed for other proteins, such as rat growth hormone (5). The reason for this low level of expression is unknown.

To further characterize the immunoreactive material in pJCE1-transfected COS-1 cells, biosynthetic labeling and analyses were done. Cells grown in 3.5-cm-diameter culture dishes were incubated for 90 min with 100 μCi of [^{35}S]methionine at 44 h after transfection. Lysates were prepared by the disruption of the cells on ice with 0.5 ml of 1% Triton X-114 in 50 mM Tris chloride (pH 8.0) containing 62.5 mM EDTA–2 mM phenylmethylsulfonyl fluoride–100 U of kallikrein inhibitor per ml. Nuclei were removed by centrifugation for 5 min at 10,000 × *g* and 4°C, and 1 ml of 0.4% sodium deoxycholate–1% Nonidet P-40 in the same buffer was added to the supernatants. Immunoprecipitates were then prepared as previously described (15) with 2 μl of rabbit anti-MHV-A59 serum and were analyzed by electrophoresis in 15% polyacrylamide gel. Three polypeptide species appeared in pJCE1-transfected cells that were not present in lysates from cells that had been transfected with the parental vector pJC119 (Fig. 2, lanes 1 and 2). Of these, the faster-migrating species comigrated with the unglycosylated form of E1 ($E1_0$) found in MHV-A59-infected Sac$^-$ cells after pulse-labeling with 150 μCi/ml for 10 min at 7 h postinfection (Fig. 2, lane 5) (16). The other two species had electrophoretic mobilities corresponding with those for the main glycosylated forms of E1 ($E1_s$ and $E1_{ss}$) which appeared in these infected Sac$^-$ cells during a 90-min chase (Fig. 2, lane 4). These two species, but not the faster-migrating form, could indeed be labeled when pJCE1-transfected COS cells were incubated

with 0.5 mCi of [^{3}H]glucosamine in 1.5 ml of glucose-free medium from 33 to 45 h after transfection (Fig. 2, lane 7). Moreover, synthesis of the glycosylated forms was not affected by tunicamycin. When the above-described labeling with [^{35}S]methionine was done in the presence of 3 μg of tunicamycin per ml, after a 2 h pretreatment with the same concentration of the drug, the same E1 species still appeared (Fig. 2, lane 3).

Further evidence for the identity of the polypeptides was obtained from a pulse-chase experiment. After a 15-min incubation in methionine-free medium, pJCE1-transfected COS cells in 3.5-cm-diameter culture dishes were labeled in parallel for 15 min with 250 μCi of [^{35}S]methionine. Then, one dish was harvested immediately, while the others were further incubated for various times with excess (2 mM) unlabeled methionine. Analysis of the immunoprecipitates showed that $E1_0$ was the main species synthesized during the pulse and that it was posttranslationally glycosylated, giving rise to $E1_s$ and $E1_{ss}$ (Fig. 2, lanes 8 through 11).

These results clearly identify E1 as the protein specifically expressed in pJCE1-transfected COS cells. They also establish the Golgi localization suggested by immunofluorescence because O glycosylation is known to occur in this compartment (8, 11). In addition, the pulse-chase experiment allowed us to estimate the transit time of the protein from endoplasmic reticulum to the Golgi membranes. The experiment showed that immediately after the 15-min chase E1 had become largely glycosylated. Thus, the half time for transit from the endoplasmic reticulum to Golgi membranes is between 15 and 30 min. This is similar to the kinetics of O glycosylation of E1 found in MHV-A59-infected cells (6, 16).

We conclude from these results that the E1 protein itself contains the necessary information for its specific targeting to Golgi membranes. E1 protein is therefore an ideal candidate for use in determining the site of intracellular budding. As mentioned above, coronaviruses have been demonstrated by electron microscopy to mature by budding from intracellular membranes. In certain cell types, such as Sac$^-$ cells (22, 23) and astrocytes (2), budding of MHV-A59 into reticular membranes was found to occur, whereas in the pituitary cell line AtT20, this budding appeared to occur exclusively into Golgi membranes (21). If the site of budding of coronaviruses is indeed determined by the properties of the E1 protein, it would be especially interesting to compare the localization of E1 expressed from cloned DNA in different cell types with the site of budding in those cells. Unfortunately, use of the simian virus 40-based vector pJC119 is restricted to monkey cells, and we found that MHV would not grow in these cells. Experiments are therefore in progress to try to express E1 from vectors with a broader host range, e.g., vaccinia virus.

In MHV-infected cells, E1 is synthesized from a bicistronic subgenomic messenger, mRNA6, in which the E1 gene is followed at its 3′ side by sequences encoding the viral nucleocapsid protein (20). The E1 gene is, however, silent in mRNA6, the nucleocapsid protein being synthesized from another subgenomic messenger, mRNA7 (20). The expression of E1 from pJCE1 in COS cells indicates that the presence of the nucleocapsid gene in mRNA6 is not a requirement for expression of E1 but is merely a consequence of the specific replication process of coronaviruses. The same conclusion could also be drawn from in vitro experiments which showed that E1 could be faithfully translated from mRNA that was synthesized from a transcription plasmid harboring the E1 gene only (data not shown).

Because of its unique features, the E1 glycoprotein of coronavirus MHV is an excellent tool to study aspects of membrane protein integration and targeting. By in vitro mutagenesis of the cDNA encoding E1, it may now be possible to identify the membrane insertion signal(s) of the protein, as well as additional topogenic domains. These mutant genes, when expressed in cells, will presumably also allow identification of signals involved in its sorting and transport. In this respect the accumulation of E1 in the Golgi apparatus, as found in COS cells, provides a sensitive phenotype for evaluating the effects of such mutations.

We thank Anne Pitta for technical assistance and Carolyn Machamer for helpful discussions.

This work was supported by Public Health Service grants AI-15481 and GM 33840 from the National Institutes of Health.

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