Isolation of Coronavirus Envelope Glycoproteins and Interaction with the Viral Nucleocapsid

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The two envelope glycoproteins and the viral nucleocapsid of the coronavirus A59 were isolated by solubilization of the viral membrane with Nonidet P-40 at 4°C followed by sucrose density gradient sedimentation. Isolated E2 consisted of rosettes of peplomers, whereas E1, the membrane glycoprotein, was irregular and amorphous. Under certain conditions significant interactions occurred between components of Nonidet P-40-disrupted virions. Incubation of the Nonidet P-40-disrupted virus at 37°C resulted in formation of a complex between one of the viral glycoproteins, E1, and the viral nucleocapsid. This was caused by a temperature-dependent conformational change in E1, resulting in aggregation of E1 and interaction with the viral RNA in the nucleocapsid. E1 also bound rRNA. The E1-nucleocapsid complexes can be distinguished on sucrose and Renografin density gradients from native viral nucleocapsids. The separation of the membrane glycoprotein E1 from the peplomeric glycoprotein E2 permitted preparation of antisera against these isolated proteins. A model is proposed for the arrangement of the three major structural proteins in the coronavirus A59 virion in relation to the viral envelope and RNA.

Coronaviruses are a group of enveloped RNA-containing viruses which caused respiratory disease in humans and a variety of diseases in animals including mouse hepatitis, avian infectious bronchitis, transmissible gastroenteritis of swine, bovine calf diarrhea, and feline infectious peritonitis. Recent studies show that the approximately 5.5×10^6 -dalton virion RNA of coronaviruses is a single strand of positive polarity (27, 41, 47). Studies of the virion polypeptides of a number of different coronaviruses in several laboratories have led to reports of three to a dozen or more polypeptide species associated with virions (1, 3, 7, 11, 16, 19, 23, 24, 28-31, 33, 35, 44, 45, 48; O. W. Schmidt and G. E. Kenny, *Fed. Proc.* **38**:910, 1979). The reasons for the apparent diversity in the polypeptide patterns of coronaviruses are not fully understood. Differences in technique and imperfect discrimination of virion polypeptides from those of host origin may account for some of the disparities. However, even when the same gel systems and conditions are employed in the same laboratory, significant differences in the number and size of virion polypeptides of different coronavirus strains have been observed (30). We propose that some of this apparent dissimilarity in the polypeptide patterns of coronaviruses may be the result of unusual characteristics of their envelope glycoproteins.

This paper deals with the virion polypeptides of the coronavirus A59, which is a strain of mouse hepatitis virus. Previous studies with radioisotopic labeling and analyzing polypeptides by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and tryptic peptide analysis have shown that there are three major structural proteins in the A59 virion (44, 45). The designation, size, location, and radioisotopic labeling characteristics of these three major structural proteins of A59 virus are shown in Table 1. Two are envelope glycoproteins designated E1 and E2, and the third is a nucleocapsid protein labeled N. The smaller glycosylated polypeptide, E1, lies primarily within the lipoprotein envelope of the virus particle (44). The peplomers of the virion are composed of the larger glycoprotein, E2. E2 may be found on virions in two forms, as 180,000- and 90,000-dalton polypeptides, which show identical tryptic peptide maps. The 180,000-dalton form can be completely cleaved by trypsin treatment of virions to yield polypeptides of 90,000 daltons (45).

In this paper we report that it is possible to isolate each of the two envelope glycoproteins and the viral nucleocapsid by solubilization of the viral membrane with Nonidet P-40 (NP40) followed by sucrose density gradient sedimentation. This has permitted preparation of anti-

TABLE 1. *Characteristics of coronavirus A59 structural polypeptides*

Designation	Apparent mol wt on SDS-PAGE ^a ($\times 10^{-3}$)	Location in virion	Relative extent of labeling with:			
			Arginine	Methionine	Fucose	Glucosamine
E1	23	Envelope	+	++++	—	+
N	50	Nucleocapsid	+++	++	—	—
E2	180/90	Peplomer	+	+	++++	++++

^a SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

sera specific for each of the envelope glycoprotein components. In addition, we show that after solubilization of the viral membrane, incubation at 37°C results in the formation of a complex between the membrane glycoprotein E1 and the viral nucleocapsid. This occurs by a temperature-dependent interaction between the E1 glycoprotein and viral RNA in the nucleocapsid. These newly created E1-nucleocapsid complexes can be distinguished from purified native viral nucleocapsids. On the basis of these observations, we propose a model for the arrangement of components in the coronavirus virion.

MATERIALS AND METHODS

Cells and virus. A spontaneously transformed derivative of the BALB/c 3T3 cell line, designated 17 Cl 1, and the L2 derivation of the L929 cell line were grown in Dulbecco medium supplemented with 10% unheated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). The A59 strain of mouse hepatitis virus was produced in 17 Cl 1 cells and assayed by plaque titration in L2 cells as described previously (46).

Virus production and purification. 17 Cl 1 cell monolayers in glass roller bottles (120 by 260 mm; 690-cm² cell surface area) were inoculated with A59 virus at a multiplicity of 1 to 10 PFU/cell. After an adsorption period of 1 h at 37°C, 50 ml of Eagle minimal essential medium with 10% unheated fetal bovine serum was added to each roller bottle. Cells were incubated at 37°C.

Released virus was usually harvested 24 to 26 h after inoculation, after several cycles of infection, when high yields were obtained and well before significant amounts of cytopathic changes such as cell fusion or lysis had occurred. Released virus was centrifuged at 10,000 $\times g$ (average) in a Sorvall GSA rotor for 30 min at 4°C to remove debris. For optimal preservation of virus infectivity, freshly harvested virus was purified immediately at 4°C without freezing or storage.

The virus was precipitated by the addition of 5.0 g of NaCl per 150 ml of clarified supernatant followed by a half volume of 30% polyethylene glycol to give a final concentration of 10% polyethylene glycol and 2.2% NaCl. The precipitate was collected by centrifugation at 10,000 $\times g$ (average) for 30 min at 4°C, resuspended in TMEN 6 buffer (5 ml/3 roller bottles of original virus) containing 0.05 M Tris-maleate-0.001 M EDTA-0.1 ml NaCl (pH 6.0) at 4°C, and layered over a discontinuous gradient of 4 ml of each of 30 and 50% (wt/wt) sucrose in TMEN 6 buffer in a 17.5-ml

centrifuge tube or 3 ml of 30% and 2 ml of 50% (wt/wt) sucrose in TMEN 6 in a 12.5-ml centrifuge tube. After centrifugation for 4 h at 25,000 rpm in a Spinco SW 27.1 rotor (82,000 $\times g$, average) or 3 h at 30,000 rpm in a Spinco SW 41 rotor (110,000 $\times g$, average) at 4°C the narrow white virus band at the interface between 30 and 50% sucrose was collected, diluted 2.5-fold with TMEN 6 buffer, and layered over a 7.5- or 12-ml continuous gradient of 20 to 50% sucrose in TMEN 6 buffer. This gradient was centrifuged for 18 h at 25,000 rpm (82,000 $\times g$, average) at 4°C on a Spinco SW 27.1 rotor or 30,000 rpm in a Spinco SW 41 rotor (110,000 $\times g$, average), and the virus band at 1.17 to 1.19 g/ml was collected. The virus was then diluted with TMEN 6 buffer and either used directly, pelleted in an SW 50.1 Spinco rotor, or dialyzed against buffer or water. Data from a representative virus purification (Table 2) shows a 50- to 100-fold reduction in volume and greater than 100-fold decrease in total protein, with 60 to 100% recovery of virus infectivity. The final specific infectivity achieved was 5 $\times 10^{10}$ to 10 $\times 10^{10}$ PFU/ml of protein.

As a routine practice, up to 900 ml of virus was purified at one time. This is the volume obtained from 18 roller bottles of infected cells. Virus harvested at 24 h had a titer of 2 $\times 10^8$ to 5 $\times 10^8$ PFU/ml, and at 42 to 48 h the titer was 0.5 $\times 10^9$ to 2 $\times 10^9$ PFU/ml. Thus, depending on the time of harvest, yields of released virus were 10¹⁰ to 10¹¹ PFU/roller bottle.

Radiolabeling of virus. Radiolabeled compounds were added to the medium in final concentration of 2 to 4 µCi of L-³H-amino acid mixture, or [5-³H]uridine per ml, 3 µCi of [6-³H]fucose per ml, and 1 to 4 µCi of [³⁵S]methionine per ml. Infected cultures were incubated in the presence of these compounds from approximately 1 h after virus inoculation until virus was harvested. Labeled uridine and amino acid mixture were purchased from New England Nuclear Corp., Boston; labeled methionine and fucose were obtained from Amersham Corp., Arlington Heights, Ill. [³H]-uridine-labeled 28S HeLa cell rRNA was kindly provided by N. K. Chatterjee.

Disruption of virions with NP40. Purified virions from three or fewer roller bottles were usually pelleted and resuspended in 1 to 2 ml of TMEN 6 buffer at 4°C. NP40, kindly provided by Shell, Inc., was added to a final concentration of 0.25 to 1%, and the mixture was shaken vigorously by hand at least 20 times. In some experiments a portion of the detergent-treated virus was incubated at 37°C for 30 min at this stage. The detergent-treated virions were then layered at 4°C over 15 to 50% sucrose gradients in TMEN 6 buffer containing 0.1% NP40 or at 10°C over 30 to 75% sucrose gradients in TMEN 6 buffer containing 0.1%

TABLE 2. *Virus purification and recovery of virus infectivity*

Virus sample	Infectivity (PFU $\times 10^{10}$ / ml)	Volume (ml)	% Recovery of infectivity	Total protein (mg)	PFU $\times 10^{10}$ /mg of protein
Released virus in medium	0.055	150	100	78	0.11
Polyethyleneglycol precipitate	1.4	5	84	13	0.54
Interface from discontinuous sucrose gradient	3.8	2	80	1.32	5.0
Band from continuous sucrose gradient	2.5	2.2	66	0.61	9.0

NP40. A cushion of 76% Renografin or 65 to 75% sucrose was placed beneath the 15 to 50% sucrose gradient. Unless otherwise indicated, the gradients were sedimented at 38,000 rpm ($180,000 \times g$, average) for 16 to 20 h in an SW 41 rotor at 4 or 10°C. Gradient fractions were collected from the top of the gradient with an ISCO fractionating device or from the bottom by displacement with light paraffin oil delivered with a Cornwall syringe. Gradient fractions were collected into chilled tubes and held at 4°C. Samples for radioisotopic counting were prepared by transferring 50- to 450- μ l portions of each gradient fraction into 1 ml of water-10 ml of Aquasol (New England Nuclear). The refractive indexes of gradient fractions were determined with a Bausch and Lomb Abbé refractometer. Continuous 20 to 50% gradients of Renografin-76 (E. R. Squibb & Sons, Princeton, N.J.) containing 0.1% NP40 were employed for dissociation of nucleocapsid complexes. Sedimentation was carried out at 25,000 rpm ($82,000 \times g$, average) for 16 h in an SW 27.1 rotor at 4°C.

Electron microscopy. Samples of purified glycoprotein preparations were placed on carbon-coated Formvar-covered 400-mesh copper grids, negatively stained with 2% phosphotungstic acid at pH 7.2 and examined with a Philips 400 transmission electron microscope.

SDS-polyacrylamide gel electrophoresis. The method of high-pH discontinuous buffer SDS-polyacrylamide gel electrophoresis in cylindrical gels employed in this study has been described previously (44). Because the E1 polypeptide aggregates after boiling with SDS in the presence of mercaptoethanol (44), samples of viral polypeptides for this study were prepared for electrophoresis by heating at 37°C for 30 min in the absence of mercaptoethanol. Five to twenty percent polyacrylamide gradient slab gels 1.5 mm thick and 10 cm long were prepared by the method of Laemmli in a Hoeffer slab gel electrophoresis apparatus. Each well was loaded with approximately 40 μ l of a radiolabeled sample which had been heated to 37°C for 15 min with an equal volume of sample treatment mixture composed of 6 M urea, 4% SDS, 0.05% bromophenol blue in 0.0625 M Tris-chloride, pH 6.7. Gels were run at 125 V for about 4 h under constant voltage from a Savant power supply. Gels were impregnated with PPO (2,5-diphenyloxazole; Sigma) in dimethyl sulfoxide by the method of Bonner and Laskey (4), dried with a Savant gel drier onto Whatman no. 17 chromatography paper, exposed to Kodak XR-5 film at -70°C for 1 to 4 weeks, and developed with Kodak X-ray chemicals.

Preparation of antisera. Antiserum directed against A59 virion polypeptides were prepared by purifying released virus through discontinuous and continuous sucrose gradients as described above without prior precipitation with PEG. Purified, concentrated A59 virus was disrupted with 1% NP40 and frozen at -75°C in aliquots. A 0.1-ml amount of the virus preparation was inoculated with complete Freund adjuvant into rabbit footpads, followed after 2 weeks with a second footpad injection of an additional 0.1 ml with incomplete Freund adjuvant (Difco) and 1 week later by an intravenous injection of another 0.4 ml of detergent-treated virus. After the third injection, rabbits were bled from the ear at 1-week intervals for 1 month. This antibody specifically immunoprecipitated radiolabeled E1, N, and E2 and no cellular polypeptides from NP40 extracts of infected 17 Cl 1 cells.

Antisera against the isolated E1 and E2 proteins were prepared by a similar schedule of rabbit inoculations of E1 obtained from sucrose gradient sedimentation of A59 virions disrupted with NP40 at 4°C and of E2 from NP40-disrupted virus that had been incubated at 37°C before sucrose gradient sedimentation. These antisera were each passed through immunosorbent columns of Affigel 10 charged with the other viral glycoprotein. The specificity of the antisera was determined by immunoprecipitation of radiolabeled viral polypeptides from NP40 extracts of A59-infected cells.

Immunoprecipitation of viral polypeptides. Immunoprecipitates of radiolabeled viral polypeptides from extracts of infected cells, purified virions, or gradient-purified E1 or E2 were made by the method of Kessler (26). A 25- μ l amount of rabbit serum was incubated with 25 to 200 μ l of radiolabeled sample in phosphate-buffered saline containing 0.1% NP40 at 0°C for 1 h. Antigen-antibody complexes and antibody were precipitated with an excess of purified, Formalin-fixed staphylococci (Cowan 1 strain) for 10 min at 4°C, pelleted at 3,000 rpm for 10 min, washed three times in 1 ml of 0.05% NP40 with phosphate-buffered saline and then solubilized in sample treatment mixture by boiling for 1 min or treatment at 37°C for 15-30 min.

RESULTS

Separation of viral components on sucrose density gradients after NP40 treatment at 4°C. To isolate individual coronavirus structural proteins, purified virions were disrupted with the nonionic polyoxyethylene detergent NP40 at 4°C, and the solubilized envelope

glycoproteins were separated from the viral nucleocapsid by sucrose density gradient sedimentation. The sedimentation pattern of radioactivity in viral polypeptides labeled with a mixture of [^{35}S]methionine and [^3H]fucose is shown in Fig. 1. There are three peaks of radioactivity. Near the top of the gradient at a density less than 1.10 g/ml is a large peak of [^{35}S]methionine label. Below this, at a density of 1.12 to 1.13 g/ml, is a peak containing most of the [^3H]fucose label in the specimen as well as a small amount of methionine label. On the cushion of high density at the bottom of the gradient is a third peak containing a large amount of [^{35}S]methionine label and a small amount of [^3H]fucose.

Fractions from each of these three peaks were analyzed by SDS-polyacrylamide gel electrophoresis as shown in Fig. 2A to C. The upper peak consists of a single major polypeptide, the E1 membrane glycoprotein of 23,000 molecular weight, which is known to label particularly well with methionine (Table 1). The minor polypeptide bands seen in Fig. 2A are 38,000- and 60,000-molecular-weight aggregates frequently observed in preparations of E1 (44). The fucose-labeled peak from the middle of the sucrose gradient in Fig. 1 is shown in Fig. 2B to consist of three bands of E2, the native 180,000-molecular-weight form, the 90,000-molecular-weight form which results from proteolytic cleavage of the 180,000-molecular-weight species, and an aggregate near the top of the gel. The most rapidly

sedimenting peak of radiolabeled protein from Fig. 1, which sediments onto a high-density cushion, consists of the nonglycosylated nucleocapsid protein, N, which has a molecular weight of 50,000 (Fig. 2C). A small amount of aggregated E2 is also apparent in this fraction, as shown by the labeled fucose near the top of the gel. This may reflect contamination by incompletely solubilized envelope structures such as has been observed upon treatment of oncornavirus with NP40 (43). It will be shown that the N protein in this rapidly sedimenting peak is associated with viral RNA and thus represents viral nucleocapsid which has been released from virions by detergent treatment. Thus, sucrose density gradient ultracentrifugation of detergent-treated A59 virus resolves the three structural proteins into three clearly separated peaks.

Morphology of the isolated glycoproteins. Samples of E1 and E2 were prepared as shown in Fig. 1 above, dialyzed against TMEN 6 buffer with 0.1% NP40 for 3 to 5 days at 4°C, concentrated threefold with Aquacide II (Calbiochem), and examined in negative-stained preparations. An intact virion is shown for comparison (Fig. 3A). Figure 3B and C show that the morphology of the two viral glycoproteins clearly differs. Under these conditions E1 (Fig. 3B) forms rounded aggregates of various sizes ranging up to 12 nm in diameter. No spikelike projections were observed in the E1 preparations. Isolated E2, the peplomeric glycoprotein,

FIG. 1. Sucrose gradient distribution of A59 structural proteins after disruption of the viral envelope with 0.25% NP40 at 4°C. The virus was labeled with a mixture of [^{35}S]methionine and [^3H]fucose. A 1-ml amount of virus was sedimented onto a 15 to 50% sucrose gradient in TMEN 6 buffer with a cushion of 76% Renografin.

FIG. 2. SDS-polyacrylamide gel electrophoresis profiles of the [^{35}S]methionine- and [^3H]fucose-labeled major peaks of structural proteins shown in Fig. 1. (A) Upper peak (0.06 g/ml); (B) middle peak (1.12 g/ml); (C) lower peak (1.28 g/ml). Arrows indicate molecular weight designations of peaks ($\times 10^{-3}$).

aggregates to form rosettes (Fig. 3C). These rosettes consist of 12 ± 2 peplomers, each of which is 18 nm long by 7 nm wide at the tip.

The base of each peplomer is joined to a spherical center structure approximately 11.5 nm in diameter. The peplomers in the rosettes have the same size, club-shaped appearance, and orientation as the peplomers on the virion. Therefore, they probably have the same orientation in the rosettes as they do on the viral envelope. The diameter of the rosettes is from 45 to 54 nm depending on the size of the central structure. Individual peplomers and small aggregates were often observed on the grids. Whether these exist free in solution or were the result of disruption of rosettes on the grids is not known.

Thus, the two glycoproteins of this coronavirus are clearly distinguishable morphologically. On the basis of the electron microscopic and biochemical evidence, it is apparent that the peplomers on the coronavirus virion consist only of E2. From previous data on susceptibility of glycoproteins in the virion to proteolytic cleavage (45), E1 appears to lie for the most part within the viral membrane. This is further supportive evidence that E1 is not an essential component of the peplomer, although it may serve to anchor the peplomers within the viral envelope.

Aggregation of NP40-solubilized viral components by incubation at 37°C. When labeled virions disrupted with NP40 at 4°C were incubated at 37°C for 30 min before being layered over the sucrose density gradient, a strikingly different pattern of sedimentation of labeled viral components was obtained (Fig. 4). Although the peak of [^3H]fucose-labeled E2 was unchanged, the large peak of [^{35}S]methionine-labeled E1 was not found at the top of the gradient. Instead, a new large peak of ^{35}S label was observed above the high-density cushion, and the amount of radiolabel on the cushion was markedly decreased. Recovery of this new complex was incomplete due to its adherence to the centrifuge tube.

Thus, the sedimentation pattern of labeled viral components after detergent disruption was dependent upon temperature. When virions were disrupted with NP40 at room temperature or at 37°C before being placed over a sucrose density gradient, patterns like Fig. 4 were observed.

The SDS-gel electrophoretic profiles of the polypeptides found in the two peaks of 37°C-treated disrupted virus are illustrated in Fig. 5A and B. Figure 5A shows that the peak at a density of 1.12 g/ml, which labels well with [^3H]fucose and less well with [^{35}S]methionine, consists of E2 in 180,000- and 90,000-dalton forms plus aggregate. Figure 5B shows that the new, fast sedimenting peak formed by incubation of NP40-solubilized virus at 37°C contains

FIG. 3. *Electron microscopy of A59 virions and isolated E1 and E2 glycoproteins. (A) An individual A59 virion; (B) a concentrated preparation of E1 glycoprotein showing globular aggregates of varied sizes; (C) a concentrated preparation of isolated E2 glycoprotein. The E2 is in the form of peplomers like those on the virion. These peplomers appear singly or as aggregates or rosettes (arrows). Magnification, $\times 176,000$.*

the nucleocapsid protein N as well as the 23,000-dalton glycoprotein E1, and a small amount of the 38,000-dalton aggregate of E1. This complex containing N and E1 did not form when NP40-solubilized virus was held at 4°C (Fig. 1). Thus, during a brief incubation of the NP40-disrupted virus at 37°C, a complex is formed between the viral nucleocapsid and E1 envelope glycoprotein.

Similar experiments on 30 to 75% sucrose gradients with ^3H -amino acid- or [^3H]uridine-labeled nucleocapsids show that the buoyant density of the nucleocapsids released by NP40 at 4°C sediments at 1.28 g/ml, whereas the E1-nucleocapsid complex formed after incubation at 37°C sediments at 1.22 g/ml (data not shown).

Reconstruction of the E1-nucleocapsid complex from isolated components. It is possible to reconstruct the E1-N-RNA complex from E1 and nucleocapsid isolated at 4°C. [^{35}S]methionine-labeled E1 and [^3H]uridine-la-

beled nucleocapsid were isolated separately at 4°C, mixed, and incubated at 4 or 37°C. E1 and nucleocapsid showed no interaction at 4°C. They sedimented as two separate peaks as shown in Fig. 6A. Raising the temperature of the mixture to 37°C for 30 min resulted in formation of a complex containing E1-N-RNA (Fig. 6B). The same results were obtained when the N protein of the nucleocapsid was labeled with ^3H -amino acids. The apparent density of the E1-N-RNA complex (1.19 g/ml in Fig. 6B), like that of the complex formed during incubation of NP40-treated whole virions at 37°C (Fig. 4), was less than the density of native nucleocapsids (1.28 g/ml).

Thermosensitivity of E1. To determine which component(s) of the complex initiated the reaction between E1 and the nucleocapsid, the effect of a brief elevation of temperature to 37°C on isolated E1 or viral nucleocapsid was meas-

FIG. 4. Sucrose gradient sedimentation of A59 structural proteins after disruption of the viral envelope with 0.25% NP40 at 4°C and incubation at 37°C for 30 min of a portion of the sample shown in Fig. 1. Sedimentation was performed under the same conditions in a duplicate gradient to that in Fig. 1.

ured. As shown in Fig. 7, this treatment produced a dramatic change in the sedimentation behavior of E1. After incubation at 4°C, E1 sedimented as a broad peak with an average density of approximately 1.06 g/ml (Fig. 7A). E1 sedimented more rapidly in a narrower band to a density of 1.16 g/ml after incubation for 30 min at 37°C (Fig. 7B) or after incubation at 25°C for a longer interval. In contrast, the nucleocapsid exhibited no change in its sedimentation behavior after incubation alone under similar conditions (data not shown).

Interaction of E1 with viral RNA in the nucleocapsid. We observed that in Renografin density gradients N and the RNA from the nucleocapsid separated. We used this observation to determine whether the E1 glycoprotein binds to the nucleocapsid by associating with the N protein or with viral RNA. Viral nucleocapsid released by NP40 treatment at 4°C was purified by sucrose density gradient sedimentation as in Fig. 1 and then resedimented on a Renografin density gradient. Figure 8A shows that the RNA sediments at a density of 1.15 g/ml, whereas the N protein has a density of 1.13 g/ml. To determine which component of the nucleocapsid binds E1, the complex of E1-N-RNA formed by incubation at 37°C of NP40-solubilized virions was purified by sucrose density gradient sedimentation as in Fig. 4 and then analyzed on a Renografin density gradient (Fig. 8B). Two peaks of viral RNA were obtained: one

with a density of 1.15 g/ml like that shown in Fig. 8A, and a new RNA-containing peak at a density in Renografin of 1.21 g/ml which also contains most of the [³⁵S]methionine-labeled protein. The SDS-gel electrophoretic profile of labeled protein in this gradient reveals that almost all of the E1 protein is associated with the 1.21-g/ml peak of RNA (Fig. 8B inset, compare with Fig. 5B), whereas most of the N protein is found near the upper region of the gradient (data not shown).

Association of E1 with RNA. To determine whether E1 would bind to RNA from other sources, we analyzed the association of E1 with isolated 28S rRNA. The sucrose gradient sedimentation profiles of E1 and 28S rRNA obtained separately are shown superimposed in Fig. 9A. The E1 glycoprotein and the rRNA are found near the top of the gradients. The sedimentation pattern obtained after mixing E1 with 28S rRNA at 4°C is shown in Fig. 9B. Most of the rRNA now sediments as large heterogeneous complexes in a broad band in the lower region of the gradient. Thus, the association of the E1 with RNA does not appear to be selective for virion RNA. Additional experiments (Sturman, unpublished data) demonstrate that E1 can also bind to a variety of isolated virion RNAs as well as viral mRNA's.

Preparation of antisera against E1 and E2. The techniques for the separation of E1 from E2 which have been described above per-

FIG. 5. SDS-polyacrylamide gel electrophoresis profiles of [^{35}S]methionine- and [^3H]fucose-labeled major peaks of structural proteins shown in Fig. 4. (A) Upper peak (1.12 g/ml); (B) lower peak (1.22 g/ml). Arrows indicate molecular weight designation of peaks ($\times 10^{-3}$).

mit purification of sufficient quantities of these glycoproteins for antisera to be developed to each. To obtain highly purified E2, we made use of the observation that virtually all E1 could be removed from an NP40-solubilized preparation by allowing it to bind to the ribonucleoprotein cores at 37°C and sedimenting the complex in a sucrose gradient. The specificity of these antisera is demonstrated by immunoprecipitation of labeled purified E1 or E2 as shown in Fig. 10. Purified E1 labeled with ^3H -amino acids appears in the 23,000- and 38,000-dalton forms (Fig. 10A) and is not immunoprecipitated by normal preimmune rabbit serum (Fig. 10B) or against the N protein (Fig. 10D, to be discussed elsewhere). Both forms of E1 are precipitated by antibody directed against all three virion polypeptides and by antibody against E1 (Fig. 10C and E). A

small amount of E1 is seen in the immunoprecipitate of gradient-purified E1 reacted with antibody prepared against purified E2 (Fig. 10F). Thus, the anti-E2 is apparently contaminated with a small amount of anti-E1 antibody.

The purified E2 preparation appears as a mixture of the 90,000- and 180,000-dalton forms of E2 (Fig. 10G). It is not immunoprecipitated by normal rabbit serum or anti-N antibody (Fig. 10H and J). In this gel E2 is weakly precipitated by antibody directed against the three virion polypeptides (Fig. 10I). A small amount of contaminating E1 is also immunoprecipitated from the E2 preparation with anti-E1 antibody (Fig. 10K). Antibody against E2 strongly immunoprecipitates E2 in 90,000- and 180,000-dalton and aggregate forms and does not precipitate the small amount of E1 in the purified E2 preparation (Fig. 10L), although it can precipitate a

FIG. 6. Temperature-dependent association of isolated E1 and nucleocapsid. E1 labeled with [^{35}S]methionine and nucleocapsid labeled with [^3H]uridine were isolated from virions by treatment with NP40 at 4°C , mixed, incubated at (A) 4°C or (B) 37°C for 30 min, and then layered onto 15 to 50% sucrose gradients containing 0.1% NP40 with a 75% sucrose cushion. The gradients were sedimented at 35,000 rpm for 16 h in an SW 41 rotor at 4°C .

FIG. 7. Effect of temperature on the sedimentation of E1. $[^{35}\text{S}]$ methionine-labeled E1 isolated from virions by treatment with NP40 at 4°C and sucrose density gradient sedimentation was diluted twofold in TMEN 6 with 0.1% NP40 and incubated at (A) 4°C or (B) 37°C for 30 min and then layered onto 15 to 50% sucrose gradients containing 0.1% NP40 with a 75% sucrose cushion. The gradients were sedimented at 35,000 rpm for 16 h in an SW 41 rotor at 4°C.

small amount of E1 in the concentrated E1 preparation (Fig. 10F). This shows that the overwhelming specificity of this antisera is against E2. In addition, the anti-E1 antibody which strongly precipitates E1 (Fig. 10E) contains no anti-E2 specificity.

DISCUSSION

In this manuscript we have shown that following NP40 treatment of mouse hepatitis virus A59 virions at 4°C, it is possible to obtain purified preparations of the membrane glycoprotein E1, the peplomeric glycoprotein E2, and viral nucleocapsid by separation on sucrose density gradients containing NP40. This type of separation of envelope glycoproteins from viral nu-

cleocapsids or cores by solubilization of the viral membrane at high detergent-membrane ratios with polyoxyethylene detergents such as NP40, Triton X-100, and Sterox SL has been obtained with many viruses including togaviruses (12, 21, 42), paramyxoviruses (38, 39), rhabdoviruses (8, 18), and oncornaviruses (43). These surfactants bind to the hydrophobic domains of amphiphilic membrane proteins (20, 32, 36, 37) forming soluble protein-detergent complexes which can be separated readily from the hydrophilic nucleocapsid by density gradient sedimentation. In most instances protein-detergent complexes containing more than one species of protein are not found and homogeneous preparations of individual soluble envelope proteins have been obtained from mixtures of complexes by rate zonal sedimentation (38, 39), isoelectric focusing (13), or column chromatography (5, 40). In the case of Semliki Forest virus, where protein-Triton X-100 complexes contain all three envelope proteins (15, 42), the spike structure itself is a trimer of the three species (49).

Electron microscopic studies of viral envelope proteins solubilized by polyoxyethylene detergents have shown that peplomers become aggregated into multimeric structures or "rosettes" upon removal of detergent (22, 38, 42). This paper shows that rosettes of the peplomeric protein E2 of mouse hepatitis virus A59 can be obtained after detergent disruption of virions with NP40, sucrose gradient sedimentation, and dialysis (Fig. 3C). These rosettes are composed solely of the E2 glycoprotein (Fig. 2B) and are morphologically distinct from E1 aggregates (Fig. 3B). The rosettes are composed of approximately 12 peplomers like those seen on the virion. We do not yet know how many molecules of E2 constitute a single peplomer.

The data show that protein-detergent micelles containing both E1 and E2 are decidedly uncommon in our preparations and that the bulk of E1 and E2 are clearly separable (Fig. 1). Although in the sucrose density gradient of virus disrupted at 4°C there is a slight overlap of E1 and E2 (Fig. 1), this could be due either to a mixture of two distinct types of micelles or to a small number of micelles containing both polypeptide species. At present these possibilities have not been resolved. The formation of distinct protein-detergent micelles may be related to differences in the location and size of the hydrophobic domains of the E1 and E2 glycoproteins. Based on data from proteolytic cleavage studies, E2 is a peripheral glycoprotein attached at the base to the viral membrane. In contrast most of the E1 molecule is concealed within the viral membrane except for a small glycosylated portion which extends outside the lipid envelope (45).

FIG. 8. Sedimentation of nucleocapsid and E1-nucleocapsid complex on Renografin. (A) Dissociation of viral RNA from the N protein of the nucleocapsid by sedimentation in a Renografin density gradient. The nucleocapsid band from a 15 to 50% sucrose gradient of virus disrupted with NP40 at 4°C (as in Fig. 1) was layered over a 20 to 50% Renografin density gradient. (B) Renografin density gradient sedimentation of the complex of E1-viral nucleocapsid. The E1-N-RNA complex from a 15 to 50% sucrose density gradient with a 76% Renografin cushion (as in Fig. 3) was layered over a 20 to 50% Renografin density gradient. Renografin gradients were sedimented at 25,000 rpm in an SW 27.1 rotor for 16 h at 4°C. (B, Inset) SDS-polyacrylamide gel electrophoretic profile of the 1.21-g/ml peak in Fig. 9B.

The structure of the coronavirus nucleocapsid has not yet been elucidated completely. Electron microscopy of budding coronavirus virions (34) suggests that the nucleocapsid is helical. Negative staining of disrupted virions has sometimes shown release of helical or tubular internal components (9, 25). However, the conformation of

the coronavirus nucleocapsid appears to be considerably more susceptible to distortion than the nucleocapsids of paramyxoviruses or myxoviruses. Several groups of investigators have isolated a "ribonucleoprotein core" or "subviral particle" after treatment of coronaviruses with NP40. The ribonucleoprotein cores have been

FIG. 9. Interaction of E1 with 28S rRNA. [³⁵S]-methionine-labeled E1 prepared by sucrose gradient sedimentation of A59 virions disrupted with NP40 at 4°C and [³H]uridine-labeled 28S rRNA were incubated separately (A) or together (B) at 4°C for 30 min in 20 mM NaCl-10 mM Tris-hydrochloride-1 mM EDTA (pH 7.5) (NTE) and analyzed by sucrose gradient sedimentation in gradients containing 15 to 30% sucrose in NTE with 0.1% NP40 at 40,000 rpm for 3 h at 4°C.

isolated from two porcine coronaviruses (17, 35), the JHM strain of mouse hepatitis virus (47), and the human coronavirus 229E (9, 25). Analysis of the polypeptides in these ribonucleoprotein cores by polyacrylamide gel electrophoresis has demonstrated large amounts of the nucleocapsid protein of 50,000 to 60,000 molecular weight which we have called N as well as one or more small envelope polypeptides which may be analogous to E1. The effects of temperature or other conditions on the polypeptide composition of the ribonucleoprotein cores were not previously recognized. In this report we demonstrate the importance of these conditions on the binding of E1 to nucleocapsids and RNA and show that at elevated temperature the E1 glycoprotein binds to the viral nucleocapsid and specifically to the RNA of the viral nucleocapsid.

The composition of the subviral components which can be separated on sucrose or Renografin gradients from virions solubilized with NP40 at different temperatures are summarized in Table 3. The buoyant densities in Renografin of free A59 viral RNA and the E1-RNA complex are similar to the values which have been reported for other nucleic acids and ribonucleoprotein complexes in iodinated density gradient media (2, 6, 10).

The interactions of E1 with RNA depend upon the condition of E1. We have observed

that removal of the peripheral 5,000-molecular-weight glycosylated portion of E1 by protease treatment of virions has a pronounced effect on the interaction of the 18,000-molecular-weight remainder of the cleaved E1 with RNA. NP40 treatment of protease-treated virions results in the formation of a complex of cleaved E1 with nucleocapsid even at 4°C (Sturman, unpublished data) under conditions in which the intact E1 molecule remains soluble. Additional experiments have demonstrated that isolated E1 can bind RNA from a variety of sources including rRNA (Fig. 10 and Sturman, unpublished data); thus, the E1 glycoprotein may act under certain conditions like an RNA binding protein.

Our observations on the anomalous behavior of the glycoprotein E1 with regard to aggregation and binding to RNA demonstrate again the unusual nature of this glycoprotein which was previously shown (44) to aggregate when boiled

FIG. 10. Specific immunoprecipitation of E1 and E2. Aliquots of purified E1 or E2 (100 μ l) labeled with 3 H-amino acids were suspended in 0.1 ml of 0.5% NP40–0.5% sodium deoxycholate in phosphate-buffered saline at 0°C. The indicated sera (25 to 75 μ l) were added, and the samples were incubated for 60 min at 0°C. Immune complexes were then precipitated with 200 μ l of 10% (vol/vol) Cowan reagent at 0°C for 10 min. The immunoprecipitates were pelleted and washed three times with 0.05% NP40 in phosphate-buffered saline. The labeled proteins were removed from the immunoprecipitate by treatment for 15 min at 37°C with 6 M urea, 4% SDS in 0.0675 M Tris-chloride buffer pH 6.7. The samples were analyzed by electrophoresis on a 5 to 20% gradient SDS polyacrylamide gel. Samples applied to the gel were as follows: purified E1 (A); immunoprecipitates of E1 with normal rabbit serum (B), anti-mouse hepatitis virus (C), anti-N (D), anti-E1 (E), and anti-E2 (F); purified E2 (G), immunoprecipitates of E2 with normal rabbit serum (H), anti-mouse hepatitis virus (I), anti-N (J), anti-E1 (K), and anti-E2 (L); gradient MHV as a marker (M).

in the presence of SDS and β -mercaptoethanol. The present results suggest that conformational changes induced in E1 by heating at 37°C after solubilization with NP40 cause aggregation of E1 and association of E1 with RNA. Thus, in separations of viral RNA or viral nucleocapsids from coronavirus virions, it is essential to examine and control the parameters which may influence interactions between subviral components such as E1 and viral RNA.

Although such an effect of temperature of solubilization with nonionic detergents has not been reported with other viruses, the ionic strength of the medium has been shown to influence the solubility of the matrix protein and its association with the nucleocapsid of the paramyxoviruses simian virus 5 (38), Newcastle disease virus (39), and Sendai virus (40) and with vesicular stomatitis virus (14). It has also been observed for Sindbis virus that ionic strength influences the interaction between the envelope glycoprotein E2 and the nucleocapsid (5).

The experimental evidence obtained here and in previous studies (44, 45) suggests a model for the arrangement of components in the coronavirus A59 virion (Fig. 11). The single long strand of RNA is associated with the nucleocapsid protein N to form the viral nucleocapsid that lies within the envelope. The membrane glycoprotein E1 appears to possess three domains: a glycosylated hydrophilic region which projects outside the viral envelope, a hydrophobic portion which extends across the membrane, and a third domain which may be associated with the viral RNA on the inner side of the viral envelope. E2 is the glycoprotein which forms the peplomers that are peripherally associated with the viral membrane. To estimate the relative

FIG. 11. Schematic model of the location of E2, E1, and N proteins in relation to the viral envelope and RNA.

amounts of the three polypeptides in the virion, we labeled A59 virus with [^3H]valine. Approximately 40% of the label was incorporated into E1, 40% was incorporated into N, and 20% was incorporated into E2. If each of the three polypeptides contained valine in the same proportion, then the A59 virion would contain a molar ratio of approximately 16 E1–8 N–1 E2 (assuming that E2 was in the 180,000-dalton form).

We suggest that the current controversy over the number of coronavirus polypeptides and their location within the virion may be attributed to certain distinctive features of the two coronavirus envelope glycoproteins. First, the peplomeric glycoprotein which we have called E2 undergoes limited proteolytic cleavage, yielding glycosylated polypeptides approximately half the size. Second, the membrane glycoprotein designated E1 readily forms aggregates of various sizes under a variety of conditions and can bind to the viral RNA in the nucleocapsid.

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TABLE 3. Subviral components of NP40-solubilized coronavirus virions separated on sucrose or Renografin density gradients

Density gradient	Incubation temp (°C, 30 min)	Approx buoyant density of major peaks (g/ml)	Components
Sucrose	4	1.28	N-RNA
		1.12	E2
		1.06	E1
	37	1.22	E1-N-RNA
		1.12	E2
Renografin	4	1.15	RNA
		1.13	N
	37	1.21	E1-RNA
		1.15	RNA
		≤1.13	N

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