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ISOLATION OF THE SUBUNITS OF THE CORONAVIRUS ENVELOPE GLYCOPROTEIN E2 BY HYDROXYAPATITE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The coronavirus glycoprotein E2, which is responsible for virus attachment to cell receptors and virus-induced cell fusion, was purified by solubilization of virions with Triton X-114 and phase fractionation. Native E2 and tryptic subunits of the glycoprotein were separated by size-exclusion high-performance liquid chromatography (HPLC). Two distinct 90 kD E2 subunits, which had identical electrophoretic mobilities when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, were separated by hydroxyapatite HPLC in the presence of sodium dodecyl sulfate.

INTRODUCTION

Coronaviruses are large, enveloped viruses, which possess a distinctive appearance and cause several important diseases in man and domestic and laboratory animals[1]. These viruses have attracted considerable interest also because of their unique replication strategy and distinctive biological characteristics[2]. The coronavirus virion contains three major structural proteins, designated E1, E2, and N. E1 is a highly hydrophobic membrane glycoprotein (23 kD), which undergoes self-aggregation upon heating in sodium dodecyl sulfate (SDS) and interacts with the viral nucleocapsid. The second glycoprotein E2 is larger (180 kD) and forms the distinctive viral spikes that bind to host cell receptors. The third protein N is an internal phosphoprotein (50 kD) that forms the helical nucleocapsid, together with the $5.4 \cdot 10^6$ mol.wt. single (+)-strand viral RNA genome.

Trypsin treatment of virions cleaves the 180 kD E2 into 90 kD species and activates coronavirus-induced cell fusion[3,4]. Host-dependent cleavage of E2 may be an important determinant of coronavirus virulence, mediating virus penetration into cells and permitting spread of the virus from cell to cell without exposure to host immune responses. We are interested in analyzing the structural domain of E2 that is responsible for virus-induced cell fusion. Attempts to separate the two tryptic subunits of E2 by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing were unsuccessful. We therefore utilized Triton X-114 solubilization and

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phase fractionation, followed by size-exclusion and hydroxyapatite high-performance liquid chromatography (HPLC) in the presence of SDS to isolate the two 90 kD subunits of E2.

EXPERIMENTAL

Virus

The studies were carried out with murine coronavirus MHV-A59, which was propagated in 17 clone 1 cells and purified by sucrose density gradient ultracentrifugation, as described[5], with the following modification: Virions, precipitated with polyethylene glycol, were resuspended at 4°C in 0.05 *M*, HEPES buffer, pH 7.1, containing 0.85% sodium chloride (HEPES–saline). The 30–50% (w/w) discontinuous sucrose gradient also contained HEPES–saline. For protease treatment, purified virions, harvested from a continuous sucrose gradient, were treated with 10 μg/ml trypsin–TPCK (Worthington) for 30 min at 37°C in TMEN 6.5 (50 m*M* Tris–maleate, 1 m*M* EDTA, 100 m*M* sodium chloride, pH 6.5), followed by incubation for 30 min at 4°C with 50 μg/ml soybean trypsin inhibitor (Worthington). The virus was then resedimented through 1.0 ml 20% sucrose, containing TMEN 6.5, in an SW41 rotor at 25000 rpm for 2.5 h at 4°C, and the pellet was resuspended in ice-cold 10 m*M* Tris–HCl, 150 m*M* NaCl, pH 7.4. No non-viral proteins were detected in such preparations by Coomassie Blue or silver stain techniques. Radiolabeled virus was obtained by addition of one or more of the following to the medium, between 1 and 28 h postinfection: 2.5 μCi/ml [^{3}H]fucose (American Radiolabeled Chemicals); 3 μCi/ml [^{35}S]methionine (New England Nuclear); or 20 μCi/ml [^{3}H]palmitic acid (Amersham).

SDS-PAGE

Viral proteins were analyzed on 10% cylindrical polyacrylamide gels in a Laemmli buffer system and fractionated with a Gilson gel fractionator[6].

Triton X-114 solubilization and phase fractionation

Triton X-114 was prepared according to the method of Bordier[7]. Resuspended viral pellets, containing 0.6–4.0 mg protein/ml, were allowed to react for 20 min at 4°C with 1% (w/w) Triton X-114. The sample was incubated at 30°C for 3 min and centrifuged in an Eppendorf microfuge at room temperature for 2 min at 15600 *g*. The upper (aqueous) phase was removed, SDS (Pierce Sequanol grade) was added to a final concentration of 1%, and the mixture was incubated at 100°C for 1.5 min. To remove particulates, the sample was centrifuged at 15600 *g* for 2 min prior to chromatography.

Chromatographic methods

The HPLC system consisted of two Waters M45 pumps, a U6K injector, a Model 441 detector at 229 nm, and a Model 680 gradient controller. Absorbance was recorded with a Houston chart recorder, and fractions were collected with a LKB Ultrorac fraction collector.

For size-exclusion chromatography a BioSil TSK (BioRad) guard column and BioSil TSK 400, 300 × 7.5 mm, and Spherogel TSK 4000 (Altex), 300 × 7.5 mm,

columns were connected in series, creating a TSK G 4000 SW column of uniform composition, 600 × 7.5 mm. Approximately 500 μg of protein were injected, with 50–60% recovery. Elution was performed at a flow-rate of 0.5 ml/min with 0.05 *M* sodium phosphate buffer, pH 6.8, containing 0.1% SDS. Fractions were collected and aliquots were analyzed for radioactivity with a Beckman LS250 liquid scintillation spectrometer after addition of Aquasol (New England Nuclear). The columns were washed with HPLC-grade water and stored in 0.05% sodium azide.

For separation of 90A and 90B subunits of E2, peak fractions of the 90kD species, recovered by size-exclusion chromatography, were pooled and introduced onto a HPHT (BioRad) column, 100 × 7.5 mm, in 0.05 *M* phosphate buffer, pH 6.8, containing 0.1% SDS. The HPHT guard column was not used, as it produced irreversible adsorption of proteins and a rapid increase in back pressure. An in-line precolumn filter (Waters) was substituted and also, in some cases, a TSK guard column (BioRad). Protein (100–200 μg) was injected, with 65–70% recovery. The protein was eluted at a flow-rate of 1 ml/min by a linear gradient of 0.15 to 0.5 *M* sodium phosphate, pH 6.8, containing 0.1% SDS. Fractions (1–5 ml) were collected and analyzed for radioactivity as above. After use, columns were washed with 0.05 *M* sodium phosphate buffer, pH 6.8, (without SDS) and stored in 0.05 *M* phosphate buffer containing 0.05% sodium azide.

Conventional hydroxyapatite chromatography was performed on BioGel HT (BioRad), and Ultrogel (LKB) columns, as described by Moss and Rosenblum[8]. The flow-rate with Ultrogel was controlled by a peristaltic pump at 0.2 ml/min, as separation was not achieved at higher flow-rates.

RESULTS AND DISCUSSION

Triton X-114 phase fractionation of coronavirus

Serious limitations have been noted in the use of SDS-PAGE for the study of coronavirus proteins[6]. As illustrated in Fig. 1, multiple forms of E1 were produced

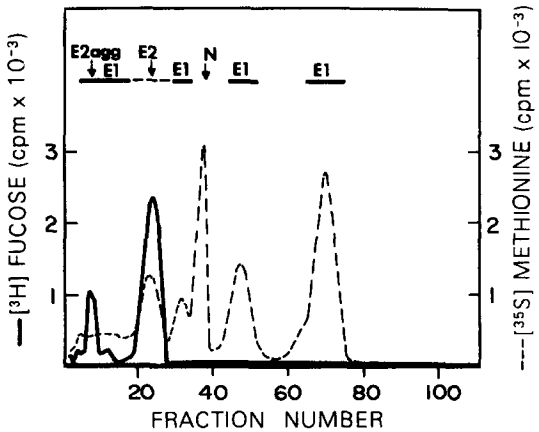

Fig. 1. SDS-PAGE analysis of coronavirus proteins from trypsin-treated coronavirus, labeled with [^{3}H]fucose and [^{35}S]methionine. E2 can be identified readily by radiolabeling with fucose, which is not present in E1. The sample containing 2% SDS was heated at 100°C for 2 min before analysis. The distribution of E1 is indicated by bars, E2 and N are denoted by arrows.

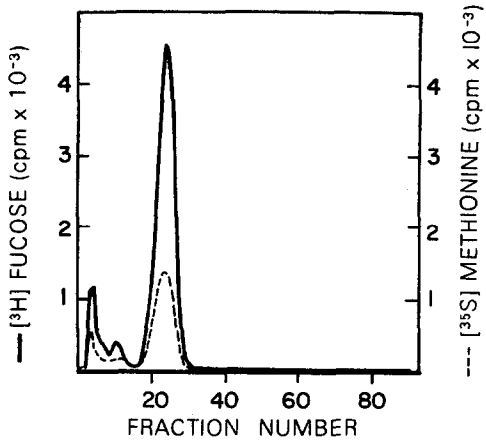

Fig. 2. SDS-PAGE analysis of the aqueous phase, derived from Triton X-114 solubilization of coronavirus, radiolabeled with [^{3}H]fucose and [^{35}S]methionine. The sample containing 2% SDS was heated at 100°C for 2 min before analysis.

as a result of heating the sample at 100°C in SDS. Even larger aggregates were obtained by heating in the presence of reducing agents[6]. Although E1 aggregation could be prevented by avoiding these conditions, complete solubilization of E2 in SDS was not obtained without heating. The most serious limitation, however, was the failure to resolve the trypsin cleavage products of E2. Only a single peak was detected at approximately 90 kD (Fig. 1). Therefore, another approach had to be taken to separate E2 from the other coronavirus structural proteins and to isolate E2 subunits which were not resolved by SDS-PAGE.

Purified virions were solubilized with Triton X-114 and the constituents were phase-fractionated. An SDS-PAGE profile of the protein which partitioned to the aqueous phase is shown in Fig. 2. The only species detected was E2, which greatly simplified the task of purification. Approximately 85% of E2 was recovered in the aqueous phase. The same result was obtained with either trypsin-treated or native virions. Both 90 kD and 180 kD species, as well as undissociated aggregates of E2, partitioned to the aqueous phase. The other two structural proteins, E1 and N, as well as viral RNA and lipid partitioned to the Triton X-114 phase. Less than 0.1% of E1 and N, and less than 3% of the viral RNA and lipid were found in the aqueous phase. Although E2 is a membrane-associated glycoprotein, the hydrophobic domain may be small or inaccessible to the detergent micelles under the conditions employed. The behavior of N, which is a hydrophilic protein, was somewhat surprising, as newly synthesized N had been shown previously to partition to the aqueous phase[10]. However, when E1 was solubilized by NP40 (another alkylphenol–polyethylene oxide detergent of the Triton series), this glycoprotein formed a complex with the viral nuclecapsid at temperatures above 20°C[6]. The interaction between E1 and viral RNA presumably caused the N protein bound to the viral genome to enter the detergent phase.

Size-exclusion HPLC of E2

In order to purify both species of E2, 180 kD and 90 kD forms were separated

by size-exclusion HPLC. Elution profiles of E2 from trypsin-treated and non-trypsin-treated (native) virions on TSK 4000 columns in the presence of SDS are shown in Fig. 3. Native virions contained a mixture of 180 kD and 90 kD species.

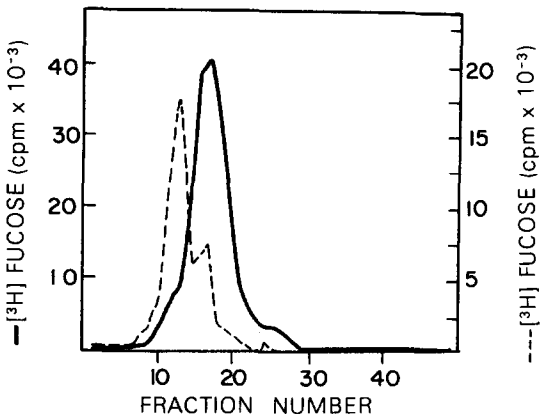

Fig. 3. Size-exclusion chromatographic profiles of 180 kD and 90 kD E2. Composite of chromatographic separations on TSK 4000 (600 × 7.5 mm) of [^{3}H]fucose-labeled E2 from trypsin-treated (———) and non-trypsin-treated (– – –) coronavirus.

Hydroxyapatite HPLC of 90A and 90B subunits of E2

Although the adsorption of native proteins is based on the number of available carboxylic groups[9], the mechanism underlying resolution of proteins by hydroxyapatite chromatography in the presence of SDS is not clear. However, this method has been employed successfully for the separation of structural proteins of diverse viruses[8,11–13], and in several cases proteins have been resolved which have identical electrophoretic mobilities when analyzed by SDS-PAGE[8,14,15]. The 90 kD species of E2 obtained from size-exclusion HPLC were separated by hydroxyapatite HPLC in the presence of SDS. The elution profile revealed two well-separated peaks (Fig. 4). The ratio of fucose to methionine was 2.0 and 1.2 in the first and second peaks respectively.

Analysis of the same subunits on two types of conventional hydroxyapatite columns is shown in Fig. 5. With BioGel HT, flow-rates varied between 0.025 and 0.15 ml/min in different experiments. The analyses shown in Fig. 5A and B required 16 to 60 h, compared to 90 min by HPLC.

Palmitic acid is covalently bound to E2 and, by analogy and other acylated viral glycoproteins, the fatty acid is probably associated with the region of the protein which is inserted in the viral envelope[16,17]. Separation of the two 90 kD subunits of E2 by hydroxyapatite HPLC allowed identification of the acylated portion. When viral proteins were labeled with palmitic acid and methionine, the palmitic label was found associated only with the second peak (Fig. 6). This subunit was designated 90A, and the other subunit, which was not acylated and eluted first, was designated 90B.

Proteins which differ significantly in size and structure may be eluted unre-

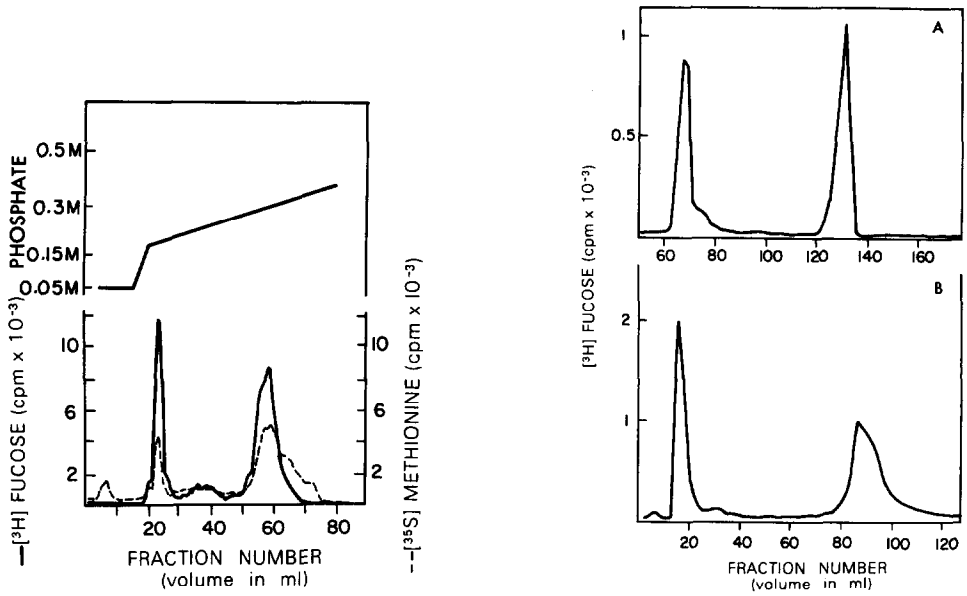

Fig. 4. Separation of [³H]fucose- and [³⁵S]methionine-labeled 90A and 90B subunits of E2 by hydroxyapatite HPLC on HPHT (BioRad). The samples were eluted in a gradient of 0.15–0.5 *M* sodium phosphate buffer, pH 6.8, containing 0.1% SDS at a flow-rate of 1.0 ml/min., Prior to analysis on HPHT, E2 was solubilized from virions with Triton X-114, and the 90 kD subunits were purified by size-exclusion chromatography on TSK 4000.

Fig. 5. Separation of [³H]fucose-labeled 90A and 90B subunits of E2 by conventional hydroxyapatite chromatography. The proteins were eluted with a gradient of 0.15–0.5 *M* sodium phosphate buffer, pH 6.8, containing 0.1% SDS. (A) BioGel HT (BioRad) 20 × 0.9 cm, flow-rate: 0.10 ml/min. (B) Ultrogel HA (LKB) 10.5 × 0.9 cm, flow-rate: 0.20 ml/min.

solved from hydroxyapatite in SDS. In the present application, the native 180 kD and 90A subunit of E2 exhibited similar elution profiles with hydroxyapatite (data not shown). This suggests that adsorption of intact E2 to hydroxyapatite is determined by reactive groups on the 90A subunit. For this reason, size-exclusion HPLC was performed prior to hydroxyapatite HPLC separation of the subunits.

To avoid dissolution of the column matrix, it is recommended that buffers contain calcium and phosphate at the level of the solubility product of calcium phosphate. However, a precipitate is formed when SDS is added to such buffers. In order to utilize SDS with hydroxyapatite, calcium was omitted from the phosphate buffer gradient. This reduced the useful life span of the hydroxyapatite column. Conventional columns were discarded after a single use. HPLC columns exhibited a gradual increase in head space and back pressure. Each column was useful for a only a limited number of separations, which varied from 12 to 15.

Limited protease digestion of E2 subunits which have been isolated by the method described in this report has revealed that 90A and 90B produce different peptide patterns and have different different amino acid compositions[18,19]. Further analysis of the primary and secondary structure of these subunits and their role in coronavirus-induced cell fusion is currently under investigation.

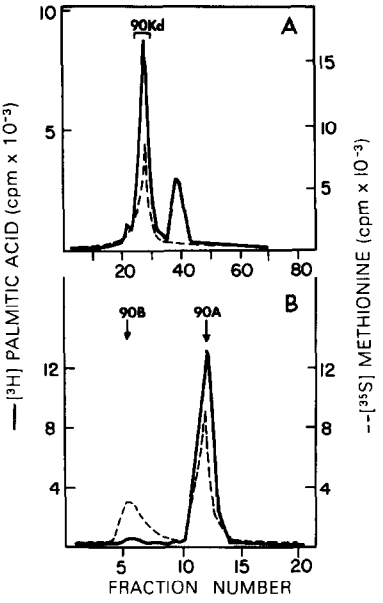

Fig. 6. Characterization of [^{3}H]palmitic acid- and [^{35}S]methionine-labeled 90A and 90B subunits of E2. (A) Size-exclusion chromatography of a mixture of 90A and 90B E2 species. Peak fractions designed by the bracket were pooled and introduced into an HPHT column. (B) Separation of the 90 kD, 90A and 90B subunits of E2 on HPHT in the presence of 0.1% SDS.

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