MOLECULAR CHARACTERIZATION OF THE 229E STRAIN OF HUMAN CORONAVIRUS

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ABSTRACT

Human coronaviruses (HCV) cause various respiratory, gastrointestinal and possibly neurological disorders. Very little is known of the molecular biology of these ubiquitous pathogens. We have undertaken the molecular characterization of the prototype 229E strain of HCV. The virus grew to the highest titers on a human embryonic lung cell line (L132) at 33°C and purification was optimal on Renografin-60® gradients. Metabolic labeling with [³⁵S]methionine or [³H]glucosamine or galactose and analysis by SDS-PAGE revealed at least five structural proteins, which could be identified by analogy with murine coronaviruses as follows: the spike glycoprotein (E2/S), in both monomeric (88-97 kDa) and dimeric (190-200) forms, the nucleoprotein (N) at 52-53 kDa and the matrix protein (E1/M), in both glycosylated (25-26 kDa) and non-glycosylated (20-22 kDa) forms. Monomeric, dimeric and multimeric (>200 kDa) forms of E2/S incorporated glucosamine and galactose, whereas only galactose was incorporated into E1/M. Multimers of E1/M, with apparent molecular masses of 44, 74 and 140 kDa, were formed in the absence of a reducing agent.

INTRODUCTION

Human coronaviruses belong to either one of two antigenic groups, represented by the prototype strains 229E and OC43¹. They are responsible for as much as 25% of common colds², and a possible involvement in neurological disorders was suggested by the observation of corona-like virus particles in the brain of one multiple sclerosis (MS) patient³, the isolation of two coronaviruses from MS brain tissue passaged in mice⁴ and the detection of intrathecal antibodies against coronaviruses in MS patients⁵. Nevertheless, our limited knowledge of the molecular structure and biology of human coronaviruses and the lack of molecular probes have not yet allowed a verification of their medical importance. We report here our initial studies on the molecular structure of the 229E strain of human coronavirus.

METHODS

Cells and virus

The L132 human embryonic lung cell line^{6,7}, the Vero african green monkey kidney cell line and the 229E strain of HCV (HCV-229E) were obtained from the American

Type Culture Collection (ATCC; Rockville, MD, U.S.A.). The RD151 human rhabdomyosarcoma^{8,9} and the HRT18 human adenocarcinoma¹⁰ cell lines were kindly provided by Dr. Arlene R. Collins (State University of New York, Buffalo, NY, U.S.A.) and Dr. David A. Brian (University of Tennessee, Knoxville, TN, U.S.A.), respectively. The IMHP human embryonic lung cell line was obtained from the Tissue Culture Service of this Institute. Cells were grown as monolayers at 37 °C in Earle's minimum essential medium : Hank's M199 (1:1, v/v) containing 0.13 % (w/v) sodium bicarbonate, 50 µg/ml of gentamicin and 5% (v/v) fetal bovine serum (FBS). The virus was plaque-purified twice and a stock obtained by three serial passages at an MOI of 0.001. Infectious titers were determined by plaque assay as described¹¹, except that plaques were revealed after a 7-day incubation period of infected L132 cells at 33°C. Viral infections were performed on half-confluent cell monolayers in medium which had a FBS content reduced to 1% (v/v). For viral growth curves in L132 cells, infectious titers were obtained by plaque assay with both culture medium clarified at 1500 x g for 7 min (extracellular virus) and cell monolayers lysed by three cycles of freezing at -70°C and thawing at 37°C (intracellular virus).

Radiolabeling of viral polypeptides

The infectious titer of the HCV-229E stock was concentrated after a fourth passage at low MOI in L132 cells. The medium, harvested at 43 hrs p.i., was clarified by centrifugation at 10,000 x g for 20 min and virus pelleted at 100,000 x g for 90 min. Resuspension into a small volume of culture medium boosted the viral titer from 1.2×10^6 to 2.6×10^8 PFU/ml. This allowed infection of cells at a higher MOI, in order to optimize the incorporation of radiolabeled precursors into viral polypeptides. At 4 hrs after infection of cells with HCV-229E at an MOI of 1, either 0.33 mCi/ml of [³⁵S]methionine (³⁵S-*E. coli* hydrolysate containing 70 % methionine) or 70 µCi/ml of [³H]glucosamine or [³H]galactose (ICN, Ville St-Laurent, PQ, Canada) was added to the culture medium.

Virus purification

Infected fluids were harvested at 43 hrs p.i., clarified by centrifugation at 10,000 x g for 20 min, and virus precipitated with 10 % (w/v) polyethylene glycol in the presence of 0.5 M NaCl. After centrifugation at 10,000 x g for 30 min, the pellet was resuspended in TMEN buffer (50 mM Tris-acid-maleate, pH 6.2, 0.1 M NaCl, 1 mM EDTA) and applied to a discontinuous 10 and 50% (v/v) Renografin-60[®] (Squibb Canada, Montréal, PQ, Canada) gradient, which was centrifuged at 148,000 x g for 2 hrs. The viral band was further purified on a continuous gradient of the same material, centrifuged at the same speed for 18 hrs. In one experiment, the following gradient materials were compared to Renografin-60[®] for the optimization of viral purification: 10-50 % (w/w) sucrose or potassium tartrate or 10-50% (w/v) Nycodenz[®] (Accurate Chemicals, Westbury, NY, U.S.A.).

Polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were resuspended in sample buffer [50 mM Tris-HCl, pH 6.8, 1% (w/v) sodium dodecyl sulfate (SDS), 1% (v/v) 2-mercaptoethanol (omitted in one experiment), 10% (v/v) glycerol, 0.003% (w/v) bromophenol blue] and treated at 100 °C for 2 min. Polypeptides were separated on 7 to 15% (w/v) polyacrylamide gels¹² and revealed by fluorography with Enlightning[®] (Dupont, Montréal, PQ, Canada). Radiolabeled molecular weight standards were used to estimate the size of viral polypeptides.

RESULTS

The initial step in our molecular characterization of HCV-229E consisted in optimizing virus growth and purification conditions. A viral growth curve was established in L132 cells at 37°C, as shown in Fig. 1. After infection at low MOI, infectious virus was detectable by 10 hrs p.i. and started to appear in the culture medium at 14 hrs p.i., before reaching a peak at 42-44 hrs p.i.. However, much higher infectious titers could be obtained

Fig. 1. Growth curve of HCV-229E on L132 cells at 37 °C (MOI = 0.001)

after infection at 33°C. Growth curves were obtained at this temperature for both intracellular (Fig. 2) and extracellular (Fig. 3) virus. Even though infectious titers were as much as 1000-fold higher at 33°C, the growth curves showed a similar time-dependence on the production of infectious virus particles. Moreover, there was no difference in the infectious virus titers obtained at 42-44 hrs after infection at MOIs of 0.01, 0.1 or 1, although extracellular virus titers were slightly reduced after infection at an MOI of 0.001. Thus, an MOI of 0.01 was chosen for virus passages, and a time of 43 hrs p.i. determined to be optimal for the obtention of extracellular virions. We then compared the infectious virus titers which could be obtained after infection of various cell lines. As shown in Table 1, given the fact that IMHP cells were more difficult to grow, L132 cells represented the best of the cell lines tested. Finally, of four gradient materials tested for virus purification, Renografin-60® allowed the best recovery of infectious virus and integrity of virions as seen in the electron microscope (data not shown)

Table 1. Growth of HCV-229E on various cell lines

Cell line	Temperature (°C)	Infectious virus titer (PFU/ml)	n ¹
L132	37	$1.3 \pm 0.7 \times 10^5$	7
IMHP	37	$2.8 \pm 0.2 \times 10^5$	2
RD151	37	1.2×10^4	2
Vero	37	<20	2
HRT18	37	<20	2
L132	33	$1.2 \pm 0.9 \times 10^6$	9

¹ number of experiments

Fig. 2. Growth curves of HCV-229E on L132 cells at 33°C and various MOIs (intracellular virus)

Fig. 3. Growth curves of HCV-229E on L132 cells at 33°C and various MOIs (extracellular virus)

Fig. 4. Effect of reducing agent on [^{35}S]methionine-labeled HCV-229E polypeptides. Sample buffer for SDS-PAGE contained (lane 1) or was free of (lane 2) 2-mercaptoethanol. The migration of radiolabeled molecular weight standards is shown on the left and the calculated molecular weights of observed viral polypeptides is shown on the right.

Having optimized virus growth and purification conditions, we undertook the molecular characterization of the viral proteins. Labeling of HCV-229E with [^{35}S]methionine and analysis of purified virions by SDS-PAGE showed four major viral polypeptides which migrated with apparent molecular masses of 200, 53, 26 and 21 kDa. These masses varied slightly between experiments and the following ranges were observed: 190-200, 52-53, 25-26 and 20-22 kDa. An additional polypeptide of 88-97 kDa was occasionally observed. Omission of the reducing agent caused a disappearance of the 26 and 21 kDa molecules and the appearance of 44, 74 and 140 kDa polypeptides.

Fig.5. Labeling of HCV-229E glycoproteins with [^3H]glucosamine. The migration of radiolabeled molecular weight standards is shown on the left and the calculated molecular weights of observed viral polypeptides is shown on the right.

Finally, the incorporation of radiolabeled sugars was used to identify viral glycoproteins. As shown in Fig. 5, polypeptides of 97 and 190, as well as two large species which migrated above the 200 kDa marker, incorporated [^3H]glucosamine. On the other hand, [^3H]galactose was also incorporated into a 26 kDa viral polypeptide (data not shown).

DISCUSSION

We have shown that the growth of HCV-229E in the human embryonic lung cell line L132 at 33°C after infection at an MOI of at least 0.01, followed by purification on Renografin-60[®] gradients, represented the optimal experimental conditions tested for the most efficient recovery of infectious viral particles needed for molecular studies. Growth curves allowed the determination of optimal harvesting times for the analysis of viral structural proteins and will prove invaluable for the study of non-structural proteins. After metabolic radiolabeling with [^{35}S]methionine, four to five structural viral proteins could be identified. By analogy with murine coronaviruses, they could be identified as the spike glycoprotein (E2/S), in both monomeric (88-97 kDa) and dimeric (190-200) forms, the nucleoprotein (N) at 52-53 kDa and the matrix protein (E1/M), in both glycosylated (25-26 kDa) and non-glycosylated (20-22 kDa) forms.

Other authors have identified putative E2/S glycoprotein dimers at 160-196 kDa^{13,14,15} and monomers at 105 kDa^{13,14}, which is consistent with our observation of

190-200 and 88-97 kDa. However, the putative E2/S multimers which migrated at >200 kDa after labeling with [^3H]glucosamine have not been previously observed, although their significance is unclear since they could not be detected after labeling with [^{35}S]methionine. The inconsistent appearance of the putative E2/S monomer of 88-97 kDa may relate to variations in the presumed proteolytic cleavage of the dimeric form of the molecule.

On the other hand, we have observed an apparently larger nucleoprotein than the previously reported 47-50 kDa molecule^{13,14,15}, as well as larger E1/M polypeptides when compared to the previously reported 17-24 kDa molecules^{13,14,15}. The only study which reported on the glycosylation of the E1/M protein showed incorporation of [^{14}C]glucosamine into a 17 kDa molecule¹³. We have been unable to detect incorporation of glucosamine into the 25-26 kDa putatively glycosylated form of E1/M, although we could confirm glycosylation by the incorporation of [^3H]galactose. Moreover, we could not observe the 165 and 31 kDa glycoproteins that were previously reported¹³, which is consistent with other studies^{14,15}.

The apparent multimerization of the E1/M matrix protein in the absence of a reducing agent has not to our knowledge been observed to date with any coronaviruses. The apparent molecular masses of the observed putative multimers suggest dimers (44 kDa), trimers (74 kDa) and hexamers (140 kDa). Putative dimers were most prominent and their absence in the presence of 2-mercaptoethanol is contrary to the reducing agent - enhanced appearance of a 38 kDa polypeptide of MHV-A59¹⁶. Moreover, the predicted primary sequence of the E1/M protein of HCV-229E, deduced from the nucleotide sequence of the cloned gene reveals, unlike other coronaviruses, the presence of only one cysteine residue (P. Jouvienne *et al.*, submitted for publication), which may be available to the formation of intermolecular disulfide bridges. Previous studies with MHV-A59 and HCV-OC43 have estimated the molar ratios of E1/M to E2/S to be 16:1¹⁷ or 33:4¹⁸, respectively. Presumably, several E1/M molecules could be joined by intermolecular disulfide bridges and other molecular links and interact with E2/S glycoproteins to stabilize viral peplomers. Thus, it would be conceivable that mutations in the E1/M gene could alter the stability of peplomers, which could alter virus pathogenesis. This would further emphasize the important role of this protein in virus biology, as was recently exemplified by the observation of passive anti- E1/M monoclonal antibody protection from MHV-JHM - induced encephalitis¹⁹, and which would be consistent with the extensive antigenic conservation of E1/M already observed among various strains of murine coronaviruses²⁰.

Further molecular studies of human coronaviruses are important to gain a better understanding of their biology and involvement in human disease. Such studies are now in progress.

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