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Proteolytic Cleavage of the E2 Glycoprotein of Murine Coronavirus: Host-Dependent Differences in Proteolytic Cleavage and Cell Fusion

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Cell fusion induced by infection with mouse hepatitis virus strain A59 (MHV-A59) varied markedly in extent and time course in four different murine cell lines. When inoculated at a multiplicity of 3 to 5 PFU per cell, the Sac⁻, L2, and DBT cell lines began to fuse by 7 h, were fused into confluent syncytia by 9 to 12 h, and peeled from the substrate by 10 to 14 h. These virulent virus-cell interactions were in striking contrast to the moderate interaction of MHV-A59 with the 17 Cl 1 cell line, in which only small syncytia were observed 18 h postinoculation, and >50% of the cells remained unfused by 24 h. The yield of infectious virus produced by 17 Cl 1 cells was 10-fold higher than the yields from the other three cell lines. The processing of the nucleocapsid protein, the membrane glycoprotein E1, and the peplomeric glycoprotein E2 were found to differ significantly in the four cell lines. Since the E2 glycoprotein is responsible for virus-induced cell fusion, we attempted to correlate differences in cellular processing of E2 with differences in fusion of infected cells. The predominant intracellular form of E2 in all cell lines was the 180K species. Pulse-chase experiments showed that a small portion of the 17 Cl 1 cell-associated 180K E2 was cleaved by 1 h after synthesis to yield 90K E2, shown in the preceding paper to consist of two different glycoproteins called 90A and 90B (L. S. Sturman, C. S. Ricard, and K. V. Holmes, J. Virol. 56:904–911, 1985). This cleavage occurred shortly before the release of virions from cells, as shown by pulse-chase experiments. After budding at intracellular membranes, virions released into the medium by the four cell lines contained different ratios of 180K to 90K E2. Virions from Sac⁻ cells, which contained 100% 90K E2, fused L2 cells rapidly without requiring virus replication, whereas virions from 17 Cl 1 cells, which had 50% 90K E2, required trypsin activation to induce rapid fusion (Sturman et al., J. Virol. 56:904–911, 1985). The addition of protease inhibitors to the medium markedly delayed L2 cell fusion induced by MHV infection. The extent of coronavirus-induced cell fusion does not depend solely upon the percent cleavage of the E2 glycoprotein by cellular proteases, since extensive fusion was induced by infection of L2 and DBT cells but not 17 Cl 1 cells, although all three cell lines cleaved E2 to the same extent. Differences observed between the molecular weights of the E2 cleavage products in several cell lines could result from host cell-dependent differences in glycosylation or cleavage of E2. Such changes in E2 processing could affect the cell-fusing activity of the glycoprotein. Cell lines also differ in susceptibility to the immediate cell-fusing effects of concentrated MHV (Sturman et al., J. Virol. 56:904–911, 1985). Thus, host-dependent differences in the precise location of the cleavage site of E2, the rate of transport of cleaved E2 to the cell membrane, or the response of the cell membranes to the fusing effects of cleaved E2 may also determine the extent of MHV-induced fusion of various cell types.

Coronaviruses exhibit a high degree of host dependence in replication, cytopathology, tissue tropism, and virulence (26, 44, 52). Genetic differences in host susceptibility to mouse hepatitis virus (MHV) strain 2 were demonstrated by Bang and his colleagues more than 20 years ago (4) but are not yet understood at the molecular level. It is unclear why some coronaviruses show stringent requirements for differentiated cells during virus isolation. For example, some human respiratory coronaviruses can only be isolated by growth in primary human fetal tracheal organ cultures (27, 51). Many strains of MHV differ in virulence, tissue tropism, and ability to grow in various murine cell lines (5, 8, 10, 14). The A59 strain of MHV (MHV-A59), which can be propagated readily in vitro, has been used extensively for biochemical studies (44). In the course of these studies, it has become apparent that there are important host-dependent determinants of the growth and cytopathogenic effects of MHV-A59. For example, MHV-A59 grows to high titer in transformed murine cell lines but not in related, nontransformed cell lines (48). Differences in plating efficiency, yield, and cytopathic effects of MHV-A59 have been noted even among different sublines of murine L929 cells (20, 28, 33).

The accompanying paper showed that proteolytic cleavage of the E2 glycoprotein of MHV-A59 was required for virus-induced cell fusion (47). In this paper, we have explored the role of the host cell in the cleavage of E2 and analyzed the correlation of E2 cleavage with virus yield and cytopathology. We observed differences in virus yield and cytopathic effects in four permissive cell lines, 17 Cl 1, Sac⁻ DBT, and L2 cells. Intracellular processing of the nucleocapsid protein, N, and the envelope glycoproteins, E1 and E2, differed in these cell lines. Detailed analysis of the host dependence of E2 synthesis, proteolytic cleavage, and assembly in these four cell lines showed that the ratio of 180K to 90K E2 on virions depended upon the host cell in which the virus was grown. In addition, there were host-dependent differences in the molecular weights of the E2 cleavage products. Such host-dependent differences in the processing of E2 glycoprotein may be of critical importance in the cytopathic effects, virulence, and tissue tropism of coronaviruses.

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MATERIALS AND METHODS

Cell cultures. The sources of the 17 Cl 1 and L2 lines of murine fibroblasts were previously described (48). The DBT cell line, derived from a Schmidt-Ruppin Rous sarcoma virus-induced mouse tumor (15), was obtained from Steven Stohlman of the University of Southern California, Los Angeles, and the Sac$^-$ line of Moloney sarcoma virus-transformed murine fibroblasts (39) was obtained from Ben van der Zeijst, University of Utrecht, The Netherlands. All of the cell lines were propagated in monolayer cultures by passage twice weekly in Dulbecco modified Eagle minimal essential medium with high glucose (DMEM; GIBCO Laboratories, Grand Island, N.Y.), 10% fetal calf serum (FCS), and 1% antibiotic-antimycotic mixture (GIBCO Laboratories, Grand Island, N.Y.).

Virus inoculation and propagation. MHV-A59 was propagated in 17 Cl 1 cells, and released virions were purified by sucrose density gradient ultracentrifugation as previously described (46). Plaque assays were performed in L2 cells (48). Virus inoculation of monolayer cultures was done at multiplicities of 3 to 10 PFU per cell, with an adsorption period of 1 h at 37°C. Control cultures were sham inoculated with an equal volume of DMEM with 10% FCS. The inocula were removed, and the cultures were incubated at 37°C in DMEM with 10% FCS. Cytopathic effects were observed with a Leitz inverted phase-contrast photomicroscope. At 13 h after inoculation, released virus was harvested and purified, and cytoplasmic extracts were prepared for analysis of viral proteins. For analysis of the capacity of concentrated virions to induce immediate cell fusion, gradient-purified virions released from Sac$^-$ cells were adsorbed to L2 cell monolayers at a multiplicity of >100 PFU per cell at 4°C for 45 min. The inoculum was removed, and the cells were incubated with DMEM–10% FCS at 37°C. Cell fusion was monitored by phase-contrast microscopy.

Analysis of viral proteins. Sucrose density gradient-purified virions were pelleted by ultracentrifugation and suspended in sample treatment mixture consisting of 2% sodium dodecyl sulfate (SDS), 5% mercaptoethanol, and 3 M urea in 62.5 mM Tris (pH 6.5)–bromphenol blue, boiled for 3 min, and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 5 to 15% gradient slab gels (21). Cytoplasmic extracts were prepared by washing infected or control monolayers in 60-mm petri dishes once with phosphate-buffered saline at 4°C and lysing cells with RIPA buffer (10 mM Tris (pH 7.4), 1% deoxycholate, 1% Nonidet P-40, 0.1% SDS, 0.15 M NaCl, 1% aprotinin, 0.1% phenylmethylsulfonyl fluoride) on ice for 10 min. The lysates were transferred to centrifuge tubes, and nuclei and debris were removed by centrifugation at 2,000 rpm. The supernatants were quick-frozen and stored at −70°C. For analysis by SDS-PAGE, equal volumes of cytoplasmic extract and sample treatment mixture were mixed and incubated at 37°C for 30 min. For some experiments, viral proteins in cell extracts were immunoprecipitated with rabbit antibody to gradient-purified, Nonidet P-40-disrupted MHV virions (anti-MHV) as previously described (46). Control immunoprecipitates utilized normal rabbit serum. Unlabeled viral proteins on SDS-PAGE were detected by immunoblotting with anti-MHV, prepared from a modification of the procedure of Towbin et al. (50), and a Bio-Rad electroblotting apparatus and by detecting the bound rabbit serum with radioiodinated staphylococcal protein A by autoradiography with Kodak X-ray film.

Pulse-labeling experiments. At 2 or 4 h after virus inoculation, MHV-infected and sham-inoculated control cell monolayers were treated with actinomycin D (5 μg/ml) in leucine- or methionine-deficient Eagle MEM with 5% dialyzed FCS for 4 h and then labeled for 15 min with [^{3}H]leucine or [^{35}S]methionine (20 μCi/ml) at 37°C. The cells were washed once, medium was replaced with DMEM–10% FCS, and the cultures were incubated at 37°C. At intervals after pulse-labeling, cytoplasmic extracts were prepared for radioimmunoprecipitation of viral proteins, and virions released into the medium over the cultures were pelleted through a 20% sucrose cushion in Tris-maleate saline buffer (pH 6.0) and analyzed by SDS-PAGE in 12-cm cylindrical gels. The gels were fractionated with an ISCO gel fractionator, and the radioactive label in each fraction was determined by counting in a Beckman scintillation counter.

RESULTS

Growth and cytopathic effects of MHV in various cell lines. Early studies on coronaviruses were hampered by difficulty in virus isolation and purification and low virus yields. For several coronaviruses, these problems have been overcome, and permissive cell types have been identified which provide high virus yields (38). For studies of the molecular biology of MHV, the following four cell lines have been used: 17 Cl 1 and L2 (42, 43, 46, 48), DBT (15, 36), and Sac$^-$ (39). The cytopathic effects of MHV-A59 in these four cell lines are compared in Fig. 1, and the yields of infectious virus are shown in Fig. 2. Infection of Sac$^-$, L2, and DBT cells caused rapid and extensive cell fusion. Syncytia were observed first at 6 to 7 h postinoculation (p.i.), and cell fusion progressed rapidly until by 9 to 12 h p.i. >90% of the cells in the monolayers were fused into confluent syncytia. These detached from the substrate and died within 10 to 14 h. Thus, MHV-A59 caused a rapid, virulent cytocidal infection of these three cell lines. In contrast, infection of confluent monolayers of 17 Cl 1 cells with MHV-A59 under the same conditions resulted in much slower formation of syncytia. Little or no fusion was observed 9 h p.i. (Fig. 1). Small syncytia were seen by 18 to 20 h p.i., and by 24 h p.i. as many as 50% of the cells remained unfused. Thus, MHV-A59 infection of 17 Cl 1 cells was more moderate than infection of the other three cell lines.

Analysis of the growth curves of MHV-A59 in these four cell lines showed that the virulent infections in Sac$^-$, DBT, and L2 cells yielded less virus than did the more moderate infection of 17 Cl 1 cells (Fig. 2). The latent period was 5 to 7 h in all four cell lines. The release of virus from Sac$^-$ and DBT cells was slower than that from L2 and 17 Cl 1 cells. Virus release from Sac$^-$, L2, and DBT cells ceased by 12 h p.i. when extensive cell fusion and death had occurred, but 17 Cl 1 cells continued to produce virus for up to 24 h p.i. Consequently, the maximal yield of virus from 17 Cl 1 cells was 10- to 100-fold higher than that of the three other cell lines.

Intracellular synthesis of viral structural proteins. The synthesis and processing of viral structural proteins were compared in the four cell lines. At intervals after virus inoculation, cytoplasmic extracts were prepared, and the intracellular virus-specific proteins were analyzed by SDS-PAGE and immunoblotting (Fig. 3). Processing of the 50K nucleocapsid protein N, the 20K to 30K membrane glycoprotein E1, and the 180K spike glycoprotein E2 differed in

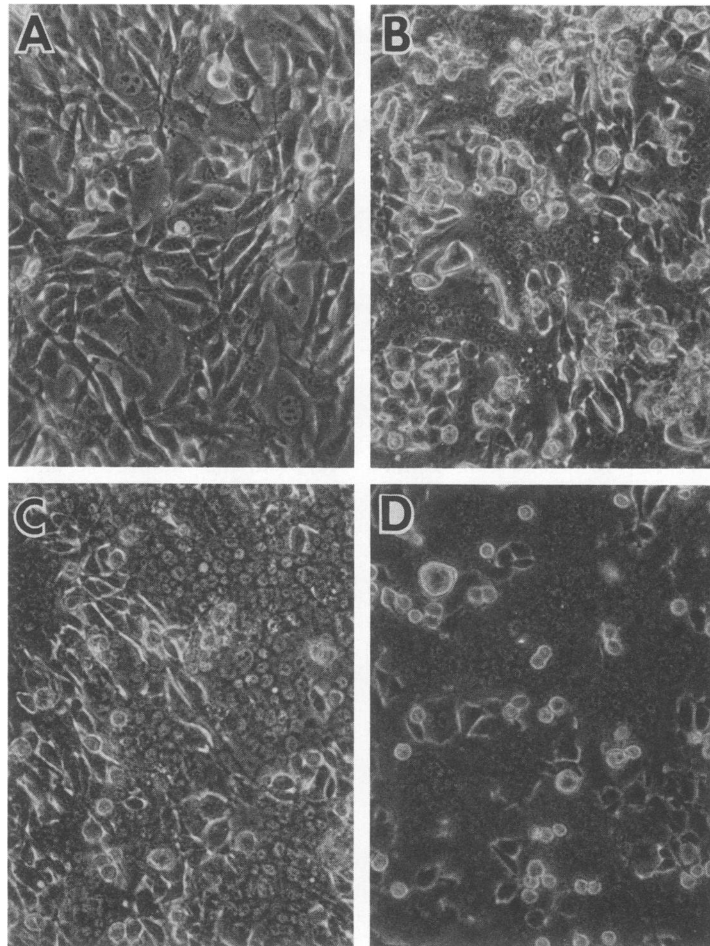

FIG. 1. Cytopathic effects of MHV-A59 in four murine cell lines. Nine hours after inoculation with MHV-A59 at a multiplicity of infection of 5 PFU per cell, the extent of cell fusion varied markedly in 17 Cl 1 (A), DBT (B), Sac^- (C), and L2 (D) cells.

the four cell lines. Intracellular N in 17 Cl 1, DBT, and Sac^- cells was predominantly a 50K protein, whereas in L2 cells, the majority of N protein was found in two faster migrating species which we call N′ and N″. At 18 to 24 h p.i., some N′ and N″ were detected in infected 17 Cl 1 cell extracts. Pulse-chase experiments in 17 Cl 1 cells showed that N′ and N″ were derived from N (data not shown). Immunoblotting and radioimmunoprecipitation with monospecific anti-N antibody confirmed that N′ and N″ are antigenically related to N (data not shown). In infected cell lines, the ratio of N′ and N″ to N increases with time after infection and depends upon the host cell line. N′ and N″ are not incorporated into virions, however. The ratio of glycosylated (23K) E1 to nonglycosylated (20K) E1 (31) was significantly higher in DBT and L2 cells than in 17 Cl 1 and Sac^- cells, suggesting faster transport of E1 to the Golgi apparatus where O-linked glycosylation occurs (31), more active O-glycosylation of E1, or slower release of glycosylated E1 into virions by DBT and L2 cells. The biological significance of these host-dependent differences in processing of N and E1 is unknown.

In virions from 17 Cl 1 cells, we previously showed that the E2 glycoprotein is found in equal amounts in 180K and 90K forms and that the 90K form could be generated from the 180K form by trypsin treatment of virions (43). In all four cell lines, we found that the majority of the intracellular E2 glycoprotein was in the 180K form (Fig. 3). We therefore examined the processing of 180K E2 in infected cells by using pulse-chase experiments. To determine the time course of processing of viral structural proteins, MHV-infected 17 Cl 1 cells were pulse-labeled for 15 min with $[^3H]$leucine at 6 h p.i., and cytoplasmic extracts were immunoprecipitated with antibody directed against viral structural proteins. At the end of the 15-min labeling period (Fig. 4, lane A) and a 30-min chase period (data not shown), no 90K E2 was detected. E2 was found only in the 180K form and in a slower migrating band which we believe is a dimer of E2 (Fig. 4, lane A). After a 45-minute chase period with excess unlabeled leucine, both the predominant 180K form and a small amount of 90K E2 were detected (Fig. 4, lane B). Similar results were obtained by pulse-labeling at 8 h p.i. (data not shown). Previous studies showed that all of the labeled E2 is chased out of 17 Cl 1 cells within 2 h after the pulse-labeling period (17). Efforts to determine the ratio of 180K to 90K E2 on the plasma membrane by surface labeling and radioimmunoprecipitation were unsuccessful because of coprecipitation of E2 from the numerous virions bound to the plasma membrane of the MHV-infected cells. Figure 4 also shows quantitative conversion of the membrane glycoprotein E1 from the 20K to the 23K form during the 45-min chase period, due to O-linked glycosylation in the Golgi apparatus (31).

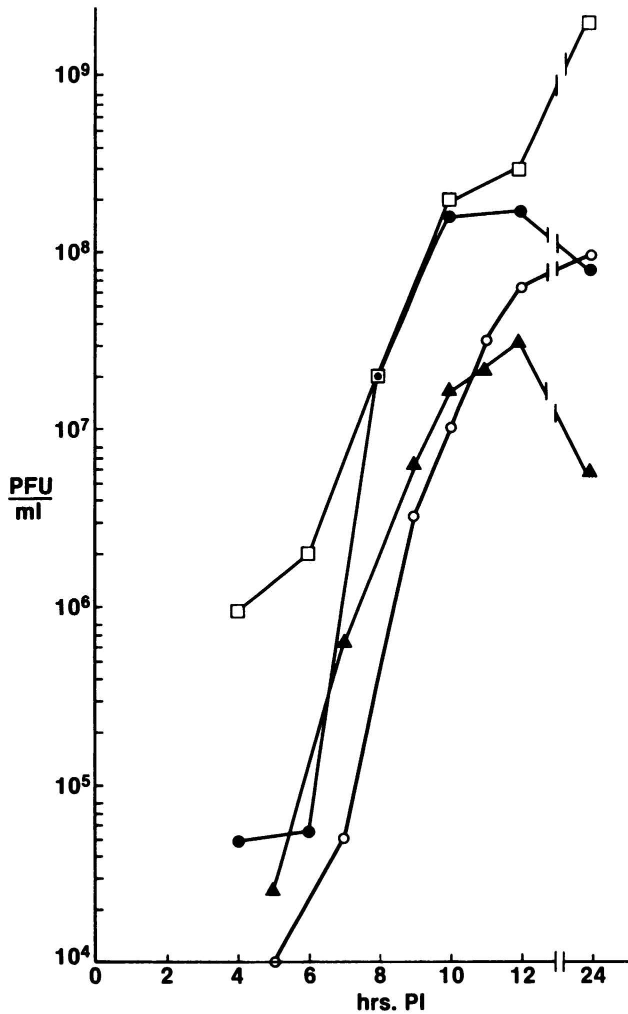

FIG. 2. Release of infectious MHV-A59 virions from four murine cell lines. The yield and rate of release of infectious virus were determined for 17 Cl 1 (□), Sac⁻ (▲), DBT (○), and L2 cells (●).

Host-dependent differences in cleavage of virion-associated E2. To study when cleavage of E2 occurs relative to virus maturation and release, MHV-infected 17 Cl1 and Sac⁻ cells were pulse-labeled with [^{35}S]methionine for 15 min at 8 h p.i., the label was removed, and medium containing unlabeled methionine was added. At intervals after the pulse, virions released into the supernatant medium were purified by ultracentrifugation and analyzed by SDS-PAGE. Labeled virus was first detectable in the supernatant fluids 30 min after the pulse, and release of radiolabeled virus increased up to 2.5 h, after which no additional labeled virus was released. Analysis of the virion-associated proteins by SDS-PAGE is shown in Fig. 5. Label released from 17 Cl 1 or Sac⁻ cells in extracellular virus was first detectable in the membrane glycoprotein E1 (seen here as the 23K monomer and a 38K dimer [43]), next in the nucleocapsid protein N, and last in the spike glycoprotein E2. From these data, it is not possible to determine the order in which proteins were incorporated into virions, since the order of appearance of label in virion proteins closely resembles the relative amounts of methionine incorporated into the viral proteins in an overnight labeling period (42). This experiment indicates that the incorporation of label into viral structural proteins

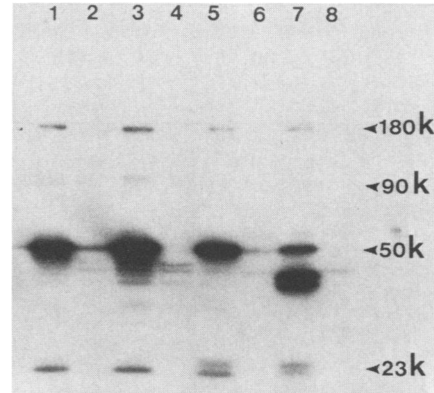

FIG. 3. Intracellular virus-specific proteins in four murine cell lines. Thirteen hours after inoculation with MHV-A59 at a multiplicity of infection of 5 PFU per cell, cytoplasmic extracts were prepared and analyzed by SDS-PAGE and immunoblotting with antibody directed against virion structural proteins, which are shown at right. Lanes 1, 3, 5, and 7, MHV-infected 17 Cl 1, Sac⁻, DBT, and L2 cells, respectively; lanes 2, 4, 6, and 8, corresponding sham-inoculated control cell lines.

and the assembly and release of virions follow similar kinetics in 17 Cl 1 cells and Sac⁻ cells. However, the E2 in virions released from Sac⁻ cells was nearly all in the 90K forms, whereas in virions from 17 Cl 1 cells about 50% of the E2 was in the 180K form (Fig. 5). Thus, the extent of cleavage of E2 on virions is host cell dependent.

This observation suggested that cleavage of E2 by host cell enzymes might determine the extent of MHV-induced fusion of various cell lines. The accompanying paper shows that complete cleavage of 180K E2 to 90K species by trypsin was required to activate the cell-fusing capacity of purified, concentrated MHV-A59 virions from 17 Cl 1 cells (47). To determine whether virions from Sac⁻ cells which contained only 90K E2 could fuse cells directly without protease

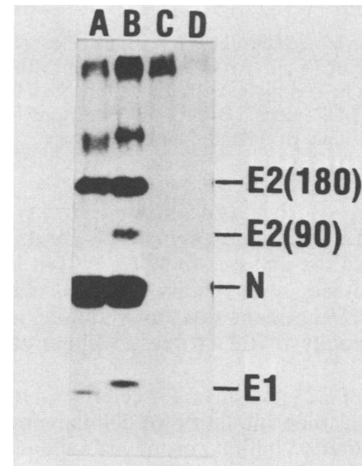

FIG. 4. Pulse-labeling of intracellular virus-specific proteins. MHV-specificproteins in cytoplasmic extracts of [^{3}H]leucine-labeled 17 Cl 1 cells were immunoprecipitated with rabbit antibody directed against the structural proteins E1, E2 and N. Lanes A and C, MHV-infected and control cells, respectively, at the end of a 15-min pulse-label 6 h p.i.; lanes B and D, MHV-infected and control cells, respectively, 45 min after this pulse-labeling period. Viral proteins are labeled on the right.

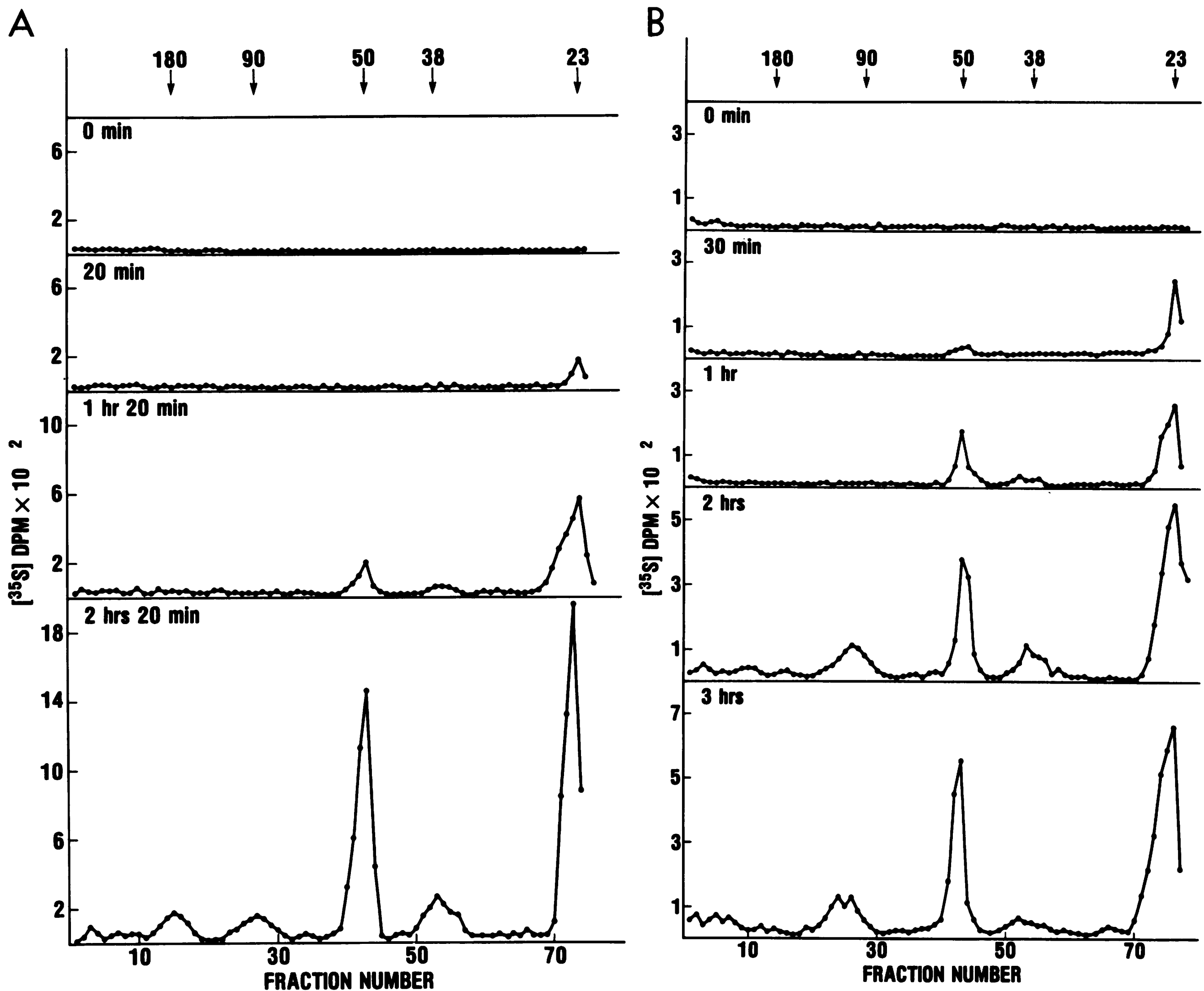

FIG. 5. Pulse-labeled proteins in virions released from two murine cell lines. The viral proteins in 17 Cl 1 cells were pulse-labeled with [^{35}S]methionine for 15 min 8 h after inoculation with MHV-A59, and virions released from the cells were purified by pelleting through a 20% sucrose cushion in Tris-maleate saline buffer (pH 6.0). Viral proteins were analyzed by SDS-PAGE in cylindrical gels. (A) Proteins of virions released from 17 Cl 1 cells; (B) proteins of virions released from Sac$^-$ cells. Times of harvest are indicated at the left, and the migration of marker viral proteins is indicated across the top.

activation, we adsorbed concentrated virions purified from Sac$^-$ cells to L2 cell monolayers at 4°C for 45 min and then rapidly warmed the cultures to 37°C. Within 1 h and 45 min at 37°C, cell fusion was readily detected (Fig. 6a and b). Thus, protease treatment was not required to activate the cell-fusing capacity of E2 on the envelope of virions from Sac$^-$ cells.

If cleavage of E2 is required for cell fusion to occur during MHV infection, then inhibition of cellular protease activity might be expected to inhibit coronavirus-induced cell fusion. When leupeptin, a potent inhibitor of serine and thiol proteases (1), was added to the growth medium of L2 cells 2 h after virus inoculation, the onset of cell fusion was delayed by 4 to 6 h (Fig. 6c and d), and the size of giant cells was markedly reduced. Similar delays in fusion of MHV-infected L2 cells resulted from the addition of 10- to 100-μg/ml concentrations of tolylsulfonyl phenylalanyl chloromethyl ketone to the medium 4 to 5 h p.i. It is not known whether these protease inhibitors act only at the plasma membrane or also within the cells under these conditions.

The marked difference between the percentage of E2 cleaved in virions from Sac$^-$ cells and 17 Cl 1 cells was confirmed by immunoblot analysis (Fig. 7). Virions released from DBT and L2 cells resembled those from 17 Cl 1 cells in that 50% of the E2 was cleaved. Since DBT and L2 cells, unlike 17 Cl 1 cells, are rapidly fused during infection with MHV-A59, it is clear that the extent of cell fusion resulting from virus infection does not correlate with the extent to which a particular cell line cleaves the E2 glycoprotein.

The cleavage products of E2 on virions from various cell lines showed slight but significant differences in electrophoretic mobility (Fig. 7). Cleavage of E2 in Sac$^-$ cells (Fig. 7, lane 2) yielded two species with very similar mobilities which migrated more slowly than did the E2 cleavage products from other cell lines. Faster migrating species of cleaved E2 were detected in virions from 17 Cl 1 and DBT

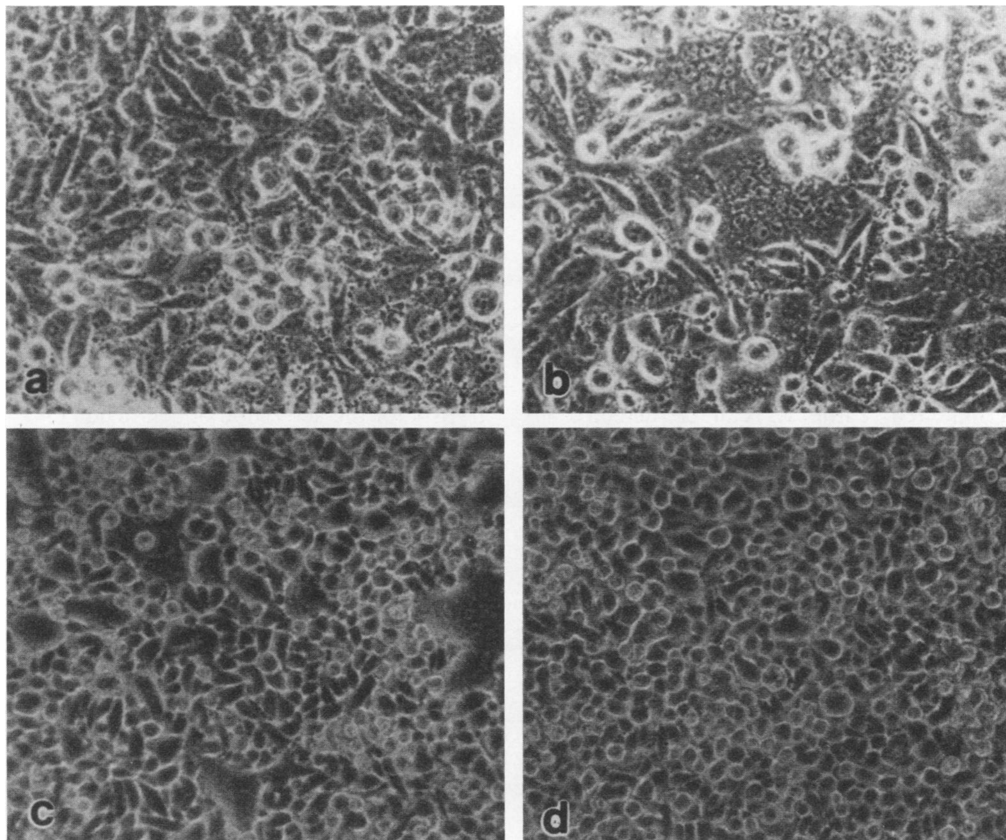

FIG. 6. Direct fusion of cells by concentrated virions from Sac⁻ cells and effect of the protease inhibitor leupeptin on fusion of infected cells. Concentrated, gradient-purified virions released from Sac⁻ cells fused monolayers of L2 cells after 1 h 45 min at 37°C (b). Control sham-inoculated cells are unfused (a). L2 cells 6 h after inoculation with 3 PFU per cell show the formation of small multinucleate syncytia (c), but this fusion is inhibited by the addition to the medium of leupeptin, an inhibitor of serine and thiol proteases (d).

cells (Fig. 7, lanes 1 and 3). These host-dependent differences in the electrophoretic mobilities of the E2 cleavage products could be due to differences in glycosylation of E2 or, alternatively, to differences in the site of proteolytic cleavage of E2 in the four cell lines. We have found that treatment of purified virions from 17 Cl 1 cells with thermolysin also yields 90K cleavage products (data not shown), indicating that there is a thermolysin cleavage site near the trypsin cleavage site of the E2 molecule. It will be of considerable interest to determine the effects of various proteases on the activation of MHV infectivity and cell-fusing capacity.

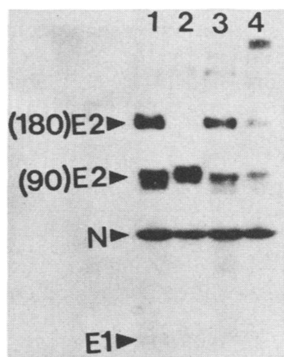

FIG. 7. Proteins in virions released from four murine cell lines. Virions released from cells 13 h after inoculation were purified by sucrose density gradient ultracentrifugation and analyzed by SDS-PAGE and immunoblotting with antibody to the structural proteins. Virions were released from 17 Cl 1 (lane 1), Sac⁻ (lane 2), DBT (lane 3), and L2 (lane 4) cells.

DISCUSSION

The outcome of MHV infection of cultured cells is strikingly dependent upon the host cell (10, 20, 24, 28, 33, 48). The genetic susceptibility to MHV of the mouse from which cells are derived can determine whether infection is productive or abortive (3). MHV-A59 grows to high titer in transformed murine cell lines but not in related, nontransformed cell lines (48). Differences in plaquing efficiency, yield, and cytopathic effects of MHV-A59 have been noted even among various sublines of permissive 17 Cl 1 and L929 cells (20, 28). Studies on the molecular biology of MHV are needed to identify host-dependent differences in virus-cell interactions which lead to productive, abortive, or persistent infection.

We have studied productive infection of MHV-A59 in four permissive murine cell lines to identify host-dependent determinants of coronavirus-induced cell fusion, which is mediated by the viral spike glycoprotein E2 (9, 17, 18). Analysis of the synthesis, proteolytic cleavage, and assembly of the E2 glycoprotein of MHV-A59 in 17 Cl 1, Sac⁻, DBT, and L2 cells revealed the following: cleavage of E2 was host dependent; the ratio of uncleaved (180K) to cleaved

(90K) E2 on virions depended on the host cell; and there were host-dependent differences in the molecular weights ofthe E2 cleavage products. The susceptibility of a cell to MHV-induced fusion is not solely determined by the ability of the cell to cleave the E2 glycoprotein, however. Some cells are more susceptible to immediate fusion by concentrated, trypsin-activated MHV than others (47), a result which is apparently due to differences in the response of the membrane to the fusion determinant on the virus envelope.

The major features of the synthesis, transport, processing, and assembly of the E2 glycoprotein of MHV are becoming clear (31, 32, 45). Translation of mRNA 3 on membrane-bound ribosomes results in cotranslational insertion of E2 into the RER membrane (34, 37) and cotranslational glycosylation in the RER. In the presence of tunicamycin, glycosylation is inhibited. Little E2 can be detected in infected cells, and E2 is not incorporated into the virions released from the cell (17, 18, 32). The nonglycosylated E2 may be degraded in an accelerated manner, or translation of E2 may be arrested by abnormal processing of the glycoprotein. E2 is transported to the Golgi apparatus where the oligosaccharides are trimmed, additional terminal sugars are added (32), and the 90A domain of E2 is acylated (11, 45). Assembly of E2 into the envelopes of budding viruses occurs at Golgi-associated and RER membranes. Pulse-labeling studies showed that during exponential release of virions from 17 Cl 1 cells, all of the newly synthesized E2 is incorporated into virions and released from cells within 2 h (17). Thus, unlike paramyxo- and orthomyxoviruses, MHV in 17 Cl 1 cells synthesizes little excess spike glycoprotein. In contrast, in Sac$^-$ cells, E2 is not quantitatively released from the cells, and most of it remains cell associated (37). This host-dependent difference in the release of E2 could be due to altered intracellular transport of E2, inefficient virus assembly, or slower secretion of virions in Sac$^-$ cells than in 17 Cl 1 cells. In the four cell lines which we studied, the bulk of E2 in cytoplasmic extracts is in the uncleaved 180K form, although E2 in released virions is 50 to 100% cleaved. These observations suggest that cleavage occurs at or shortly before the time of virus release from the infected cells. Further support for the idea that E2 cleavage is a late step in the transit of E2 through the cell comes from the observation that monensin, which arrests intracellular transport of proteins at the level of the Golgi apparatus, prevents the cleavage of E2 (31). Excess E2 not incorporated into virions is transported to the plasma membrane (17), where it could render the cell susceptible to immunological attack or participate in cell fusion or lysis. Thus, in Sac$^-$ cells which fuse rapidly upon MHV infection, there is more E2 on the plasma membrane, and the E2 is more likely to be cleaved than in 17 Cl 1 cells in which less fusion is observed.

The demonstration that cleavage of the fusion glycoprotein of a coronavirus is host cell dependent and required for the activation of cell fusion bears a striking similarity to the characteristics of fusion glycoproteins of orthomyxo- and paramyxoviruses. These negative-strand RNA viruses have fusion glycoproteins in the viral envelope which must be cleaved by proteolytic enzymes of the host cell to activate both viral infectivity (19, 22, 23, 29, 30) and cell-fusing activity (22, 25, 53). Host cell proteolytic cleavage of fusion glycoproteins can determine the tissue tropism, pathogenesis, and virulence of these viruses (6, 12, 30, 35). The functional similarity and requirement for proteolytic activation of the glycoproteins from positive-strand coronaviruses and negative-strand orthomyxo- and paramyxoviruses suggests a possible evolutionary relationship between these structurally similar virus groups, although their replication strategies differ significantly. In addition, important cellular constraints on glycoprotein processing may be imposed upon all of these virus groups (13).

Cleavage of the E2 glycoprotein of coronaviruses is not limited to MHV-A59. In avian infectious bronchitis virions released from primary chicken embryo cells, there are two E2 glycoproteins, namely, the 84K and 90K glycoproteins (7). These are probably cleavage products of the large 155K glycoprotein precursor found in infected cells (40).

The addition of exogenous protease to the growth medium can enhance cell fusion by other coronaviruses. The bovine enteric coronavirus did not produce plaques in bovine brain or thyroid cells unless trypsin was added to the medium (41). Similarly, a cold-sensitive variant of MHV strain S required trypsin for the activation of cell fusion (54). The addition of the protease inhibitor leupeptin reduced the yield of infectious HCV strain 229E virus (2).

In the paramyxo- and orthomyxovirus systems, proteolytic cleavage of the viral glycoprotein is required for cell fusion but is not a sufficient condition to guarantee fusion. One factor which affects fusion is the susceptibility of the plasma membrane to the direct fusing effects of the cleaved fusion glycoprotein. The paramyxovirus SV5 causes rapid fusion and death of BHK-21-F cells but induces persistent, productive, noncytocidal infection of primary monkey cells due to differences in the response of the cell membranes to the viral fusion glycoprotein (16). The difference in susceptibility of L2 and 17 Cl 1 cells to direct fusion by trypsin-activated 17 Cl 1 virus is similar (47). Thus, whether a virus causes moderate or cytocidal infection depends upon intrinsic characteristics of the plasma membrane. The nature of these membrane differences has not yet been determined.

Different susceptibilities of cell lines to virus-induced fusion may also be related to the site of cleavage on the fusion glycoprotein. In influenza, several different proteases can cleave the hemagglutinin glycoprotein generating similar sized glycopeptides, but cleavage at only one specific site activates infectivity (13, 22). The observation reported here that the cleavage products of MHV E2 generated in different cell lines differ in electrophoretic mobility raises the possibility that different sites on E2 may be cleaved by these cells, resulting in different levels of activation of cell fusion.

An understanding of host-dependent differences in E2 maturation and processing provides new perspective on several perplexing problems of coronavirus cultivation, cell tropism, and pathogenesis. It is now clear that for some virus-host cell combinations, the outcome of coronavirus infection is determined by the ability of the host cell to activate the viral fusion factor by proteolytic cleavage of E2. However, other factors also affect the outcome of coronavirus infection. These include differences in intracellular transport of E2, the degree of cell membrane susceptibility to the fusing effects of E2, the extent of cleavage of E2, and possibly the site of cleavage on the E2 molecule. These studies have focused upon the role of the E2 glycoprotein in cellular permissivity for coronavirus replication. To fully understand the host cell restrictions on coronavirus replication which are affected by the age and strain of the murine host, the virus strain, and the cell type (4, 5, 49), it will also be necessary to analyze host cell factors which may affect coronavirus transcription and replication.

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LITERATURE CITED

1. **Aoyagi, T., and H. Umezawa.** 1975. Structures and activities of protease inhibitors of microbial origin, 429–453. *In* E. Reich, D. B. Rifkin, and E. Shaw (ed.), Cold Spring Harbor Conferences on Cell Proliferation, vol. 2. Proteases and biological control. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
2. **Appleyard, G., and M. Tisdale.** 1985. Inhibition of the growth of human coronavirus 229E by leupeptin. J. Gen. Virol. **66:**363–366.
3. **Bang, F. B.** 1981. The use of a genetically incompatable combination of host and virus (MHV) for the study of mechanisms of host resistance. Adv. Exp. Med. Biol. **142:**359–373.
4. **Bang, F. B., and A. Warwick.** 1960. Mouse macrophages as host cells for the mouse hepatitis virus and the genetic basis of their susceptibility. Proc. Natl. Acad. Sci. USA **46:**1065–1075.
5. **Barthold, S. W., and A. L. Smith.** 1984. Mouse hepatitis virus strain-related patterns of tissue tropism in suckling mice. Arch. Virol. **81:**103–112.
6. **Bosch, F., M. Orlich, H.-D. Klenk, and R. Rott.** 1979. The structure of the hemagglutinin, a determinant for the pathogenicity of influenza viruses. Virology **95:**197–207.
7. **Cavanagh, D.** 1981. Structural polypeptides of coronavirus IBV. J. Gen. Virol. **53:**93–103.
8. **Cheley, S., V. L. Morris, M. Cupples, and R. Anderson.** 1981. RNA and polypeptide homology among murine coronaviruses. Virology **115:**310–321.
9. **Collins, A. R., R. L. Knobler, H. Powell, and M. J. Buchmeier.** 1982. Monoclonal antibodies to murine hepatitis virus 4 (strain JHM) define the viral glycoprotein responsible for attachment and cell-cell fusion. Virology **119:**358–371.
10. **Dubois-Dalcq, M. E., E. W. Doller, M. V. Haspel, and K. V. Holmes.** 1982. Cell tropism and expression of mouse hepatitis viruses (MHV) in mouse spinal cord cultures. Virology **119:**317–331.
11. **Dunphy, W. G., E. Fries, L. J. Urbani, and J. E. Rothman.** 1981. Early and late functions associated with the Golgi apparatus reside in distinct compartments. Proc. Natl. Acad. Sci. USA **78:**7453–7457.
12. **Garten, W., W. Berk, Y. Nagai, R. Rott, and H.-D. Klenk.** 1980. Mutational changes of the protease susceptibility of glycoprotein F of Newcastle disease virus: effects on pathogenicity. J. Gen. Virol. **50:**135–147.
13. **Garten, W., F. X. Bosch, D. Linder, R. Rott, and H.-D. Klenk.** 1981. Proteolytic activation of the influenza virus hemagglutinin: the structure of the cleavage site and the enzymes involved in cleavage. Virology **115:**361–374.
14. **Hartley, J. W., and W. P. Rowe.** 1963. Tissue culture cytopathic and plaque assays for mouse hepatitis viruses. Proc. Soc. Exp. Biol. Med. **113:**403–406.
15. **Hirano, N., K. Fujiwara, S. Hino, and M. Matumoto.** 1974. Replication and plaque formation of mouse hepatitis virus (MHV-2) in mouse cell line DBT culture. Arch. Gesamte Virusforsch. **44:**298–302.
16. **Holmes, K. V., and P. W. Choppin.** 1966. On the role of the response of the cell membrane in determining virus virulence. Contrasting effects of the parainfluenza virus SV5 in two cell types. J. Exp. Med. **124:**501–520.
17. **Holmes, K. V., E. W. Doller, and J. N. Behnke.** 1981. Analysis of the functions of coronavirus glycoproteins by differential inhibition of synthesis with tunicamycin. Adv. Exp. Med. Biol. **142:**133–142.
18. **Holmes, K. V., E. W. Doller, and L. S. Sturman.** 1981. Tunicamycin resistant glycosylation of a coronavirus glycoprotein: demonstration of a novel type of viral glycoprotein. Virology **115:**334–344.
19. **Klenk, H.-D., R. Rott, M. Orlich, and J. Blodorn.** 1975. Activation of influenza A viruses by trypsin treatment. Virology **68:**426–439.
20. **Koolen, M. J. M., D. M. E. Osterhaus, G. van Steenis, M. C. Horzinek, and B. A. M. van der Zeijst.** 1983. Temperature sensitive mutants of mouse hepatitis virus strain A59: isolation, characterization and neuropathogenic properties. Virology **125:**393–402.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
22. **Lazarowitz, S. G., and P. W. Choppin.** 1975. Enhancement of the infectivity of influenza A and B viruses by proteolytic cleavage of the hemagglutinin polypeptide. Virology **68:**440–454.
23. **Lazarowitz, S. G., R. W. Compans, and P. W. Choppin.** 1973. Proteolytic cleavage of the hemagglutinin polypeptide of influenza virus. Function of the uncleaved polypeptide HA. Virology **52:**119–212.
24. **Lucas, A., W. Flintoff, R. Anderson, D. Percy, M. Coulter, and S. Dales.** 1977. In vivo and in vitro models of demyelinating diseases: tropism of the JHM strain of murine hepatitis virus for cells of glial origin. Cell **12:**533–560.
25. **Maeda, T., K. Kawasaki, and S. Ohnishi.** 1981. Interactions of influenza virus hemagglutinin with target membrane lipids is a key step in virus-induced hemolysis and fusion at pH 5.2. Proc. Natl. Acad. Sci. USA **78:**4133–4137.
26. **McIntosh, K.** 1974. Coronaviruses. A comparative review. Curr. Top. Microbiol. Immunol. **63:**85–129.
27. **McIntosh, K., H. J. Dees, W. B. Becker, A. Z. Kapikian, and R. M. Chanock.** 1967. Recovery in tracheal organ cultures of novel viruses from patients with respiratory disease. Proc. Natl. Acad. Sci. USA **57:**933–940.
28. **Mizzen, L., S. Cheley, M. Rao, R. Wolf, and R. Anderson.** 1983. Fusion resistance and decreased infectability as major host cell determinants of coronavirus persistence. Virology **128:**407–417.
29. **Nagai, Y., and H.-D. Klenk.** 1977. Activation of precursors to both glycoproteins of NDV by proteolytic cleavage. Virology **77:**125–134.
30. **Nagai, Y., H.-D. Klenk, and R. Rott.** 1976. Proteolytic cleavage of the viral glycoproteins and its significance for the virulence of Newcastle disease virus. Virology **72:**494–508.
31. **Niemann, H., B. Boschek, D. Evans, M. Rosing, T. Tamura, and H.-D. Klenk.** 1982. Post-translational glycosylation of coronavirus glycoprotein E1: inhibition by monensin. EMBO J. **1:**1499–1504.
32. **Niemann, H., and H.-D. Klenk.** 1981. Coronavirus glycoprotein E1: a new type of viral glycoprotein. J. Mol. Biol. **153:**993–1010.
33. **Robb, J. A., and C. W. Bond.** 1979. Pathogenic murine coronaviruses. I. Characterization of biological behaviour in vitro and virus-specific intracellular RNA of strongly neurotropic JHMV and weakly neurotropic A59V viruses. Virology **94:**352–370.
34. **Rottier, P. J. M., W. J. M. Spaan, M. C. Horzinek, and B. A. M. van der Zeijst.** 1981. Translation of three mouse hepatitis virus strain A59 subgenomic RNAs in *Xenopus laevis* oocytes. J. Virol. **38:**20–26.
35. **Scheid, A., and P. W. Choppin.** 1976. Protease activation mutants of Sendai virus. Activation of biological properties by specific proteases. Virology **69:**265–277.
36. **Siddell, S. G., H. Wege, A. Barthel, and V. ter Meulen.** 1980. Coronavirus JHM: cell-free synthesis of structural protein p60. J. Virol. **33:**10–17.
37. **Siddell, S., H. Wege, A Barthel, and V. ter Meulen.** 1981. Coronavirus JHM: intracellular protein synthesis. J. Gen. Virol. **53:**145–155.
38. **Siddell, S., H. Wege, and V. ter Meulen.** 1982. The structure and replication of coronaviruses. Curr. Top. Microbiol. Immunol. **99:**131–163.
39. **Spaan, W. J. M., P. J. M. Rottier, M. C. Horzinek, and B. A. M. van der Zeijst.** 1981. Isolation and identification of virus-specific

mRNAs in cells infected with mouse hepatitis virus (MHV-A59). Virology **108**:424–434.

40. **Stern, D. F., and B. M. Sefton.** 1982. Coronavirus proteins: biogenesis of avian infectious bronchitis virus virion proteins. J. Virol. **44**:794–803.
41. **Storz, J., R. Rott, and G. Kaluza.** 1981. Enhancement of plaque formation and cell fusion of an enteropathogenic coronavirus by trypsin treatment. Infect. Immun. **31**:1214–1222.
42. **Sturman, L. S.** 1977. Characterization of a coronavirus. I. Structural proteins: effects of preparative conditions on the migration of protein in polyacrylamide gels. Virology **77**:637–649.
43. **Sturman, L. S., and K. V. Holmes.** 1977. Characterization of a coronavirus. II. Glycoproteins of the viral envelope: tryptic peptide analysis. Virology **77**:650–660.
44. **Sturman, L. S., and K. V. Holmes.** 1983. The molecular biology of coronaviruses. Adv. Virus Res. **28**:35–112.
45. **Sturman, L. S., and K. V. Holmes.** 1985. The novel glycoproteins of coronaviruses. Trends Biochem. Res. **10**:17–20.
46. **Sturman, L. S., K. V. Holmes, and J. N. Behnke.** 1980. Isolation of coronavirus envelope glycoproteins and interaction with the viral nucleocapsid. J. Virol. **33**:449–462.
47. **Sturman, L. S., C. S. Ricard, and K. V. Holmes.** 1985. Proteolytic cleavage of the E2 glycoprotein of murine coronavirus: activation of cell-fusing activity of virions by trypsin and separation of two different 90K cleavage fragments. J. Virol. **56**:904–911.
48. **Sturman, L. S., and K. K. Takemoto.** 1972. Enhanced growth of a murine coronavirus in transformed mouse cells. Infect. Immun. **6**:501–507.
49. **Taguchi, R., A. Yamada, and K. Fujiwara.** 1979. Factors involved in the age-dependent resistance of mice infected with low-virulence mouse hepatitis virus. Arch. Virol. **62**:333–340.
50. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
51. **Tyrrell, D. A. J., and J. D. Almeida.** 1967. Direct electron-microscopy of organ cultures for the detection and characterization of viruses. Arch. Gesamte Virusforsch. **22**:417–425.
52. **Wege, H., S. Siddell, and V. ter Meulen.** 1982. The biology and pathogenesis of coronaviruses. Curr. Top. Microbiol. Immunol. **99**:165–200.
53. **White, J., K. Matlin, and A. Helenius.** 1981. Cell fusion by Semliki Forest virus, influenza virus, and vesicular stomatitis virus. J. Cell Biol. **89**:674–679.
54. **Yoshikura, H., and S. Tejima.** 1981. Role of protease in mouse hepatitis virus-induced cell fusion. Virology **113**:503–511.