Coronavirus JHM: Tryptic Peptide Fingerprinting of Virion Proteins and Intracellular Polypeptides

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SUMMARY
The virion proteins and intracellular polypeptides of the murine coronavirus MHV-JHM have been analysed by two-dimensional fingerprinting of their L35Smethionine-containing tryptic peptides. The analysis shows that the virion proteins gp98, gp65, pp60 and p23 are distinct. Virion protein gp25 has the same polypeptide component as p23, and virion protein gp170 has a polypeptide component related to gp98. The six virus polypeptides synthesized in infected cells, 150K, 65K, 60K, 30K, 23K and 14K are also distinct. The 170K and 98K species, which are produced by processing, are related to 150K. The 25K species is a processed form of 23K. The identity of corresponding species in the cell and in the virion has been shown and a model describing the genesis of coronavirus JHM proteins can now be proposed.

INTRODUCTION
Coronaviruses are associated with a wide variety of diseases in animals and humans (for review, see Wege et al., 1982). They are enveloped viruses with a large RNA genome which is single-stranded, unsegmented and infectious. The virions contain relatively few proteins: characteristically, a phosphorylated nucleocapsid protein [50 × 10^3 to 60 × 10^3 (50K to 60K) mol. wt.], a matrix protein(s) (20K to 35K) and a high mol. wt. glycoprotein(s) (90K to 180K) which constitutes the virus peplomer (for review, see Siddell et al., 1982). It has recently been shown that the coronavirus replication strategy involves the production of a 3' co-terminal nested set of subgenomic mRNAs, each of which (excluding the smallest) is structurally polycistronic, but functionally appears to initiate translation independently (Cheley & Anderson, 1981) and directs the synthesis of only one protein (Stern & Kennedy, 1980a, b; Lai et al., 1981; Spaan et al., 1981; Cheley et al., 1981; Siddell et al., 1980, 1981b; for review, see Siddell et al., 1982).

The murine coronavirus mouse hepatitis virus JHM (MHV-JHM) is of particular interest in that it has the ability to induce central nervous system disorders in mice and rats and can be used as a model for virus-induced demyelination (Nagashima et al., 1978, 1979). This virus has a genomic RNA of 6.1 × 10^6 to 6.7 × 10^6 mol. wt. (Leibowitz et al., 1981; Wege et al., 1981) and six major virion proteins, four of which are glycoproteins, with mol. wt. ranging from 23K to 170K (Siddell et al., 1981a; Lai & Stohlman, 1981). These proteins conform to the characteristic coronavirus pattern.

In infected cells seven virus-specific RNA species, with mol. wt. ranging from 0.6 × 10^6 to 6.0 × 10^6 have been found. These RNAs are structurally related in the predicted nested set fashion, are polyadenylated and are associated with polysomes (Leibowitz et al., 1981; Wege et al., 1981; Cheley et al., 1981). Also, the synthesis of six polypeptides has been detected in JHM-infected cells and, during post-translational modification, two of these polypeptides are processed and a further three polypeptides are found (Siddell et al., 1981a). The
electrophoretic, immunological and physical characterization of these polypeptides suggests that they include the cellular counterparts of the virion proteins as well as some polypeptides not found in the virion (Siddell et al., 1981a).

Finally, with the exception of the intracellular protein 65K, studies on the in vitro translation of size-fractionated poly(A)-containing RNA from JHM-infected cells have correlated the synthesis of each of the known virus-specific primary translation products with one of the virus-specific subgenomic sized mRNAs. The size of the non-overlapping sequences in each mRNA and the size of its translation product suggest that each polypeptide is encoded at the 5' terminus of its mRNA (Siddell et al., 1981b).

These findings, together with the results of similar experiments with infectious bronchitis virus (IBV) and murine hepatitis virus A59 (MHV-A59) (Stern et al., 1980a, b; Lai et al., 1981; Spaan et al., 1981, 1982; Rottier et al., 1981a, b; Anderson et al., 1979; Bond et al., 1981; Lai et al., 1982; Cheley & Anderson, 1981; Cheley et al., 1981), have led to a model for coronavirus replication (see Siddell et al., 1982), the details of which can now be rigorously tested. The aim of the experiments reported here was to establish by tryptic peptide fingerprinting the relationships between the intracellular polypeptides and virion proteins of coronavirus JHM and thereby to consolidate one aspect of this model.

METHODS

**Virus and cells.** Sac(-) cells were grown in suspension or as monolayers and MHV-JHM virus stocks were obtained and propagated as described previously (Siddell et al., 1980). Virus was labelled with [35S]methionine (SJ204; Amersham/Buchler, Braunschweig, F.R.G.), purified by polyethylene glycol precipitation and a combination of velocity and equilibrium centrifugation and stored also as described previously (Siddell et al., 1980).

**Pulse labelling of intracellular polypeptides.** Sac(-) cells were infected at an m.o.i. of 6, and 10 h later pulse-labelled for 45 min with minus-methionine medium containing 100 μCi/ml [35S]methionine. These procedures and the subsequent preparation of total cell lysates for one-dimensional polyacrylamide gel electrophoresis (PAGE) have been described previously (Siddell et al., 1980). For the immunoprecipitation of polypeptides, cells were infected and labelled as described above and cytoplasmic lysates prepared at 4 °C (Siddell et al., 1981a).

**Pulse–chase labelling of intracellular polypeptides.** Cells were infected and pulse-labelled as described above. At the end of the labelling period the radioactivity was chased through the cells by removing the medium, washing the cells twice in complete medium containing an additional 1 mM-methionine and incubating the cells in this medium at 37 °C for 2 h. Cytoplasmic lysates were then prepared for immunoprecipitation as described below.

**Immunoprecipitation.** Immunoprecipitation of cytoplasmic lysates with hyperimmune serum raised against purified virus (Siddell et al., 1980) was as described previously (Siddell et al., 1981a) with the exception that the immunoprecipitates were not boiled before electrophoresis.

**Gel electrophoresis.** Samples were mixed with an equal volume of electrophoresis sample buffer (Siddell et al., 1980). Those samples containing total cell lysates were boiled, whilst virus- or immunoprecipitate-containing samples were heated to 37 °C, in both cases for 2 min, before electrophoresis on 15% discontinuous SDS–polyacrylamide gels as described by Laemmli (1970). Gels were dried under vacuum without fixation and then exposed to Fuji RX film at room temperature.

**Tryptic peptide mapping.** The portions of the gel containing the relevant protein were excised, the protein recovered, performic acid-oxidized and digested with trypsin as described by Hutchinson et al. (1978) except that the gel homogenization buffer contained additionally 20 μg/ml human IgG (Miles Laboratories). Two-dimensional mapping was performed as
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Fig. 1. Autoradiograph of \(^{35}\)S\textsuperscript{m}ethionine-labelled MHV-JHM structural proteins separated by SDS–PAGE. O, Origin; BB, bromophenol blue dye marker.

described by Siddell (1978) with the separation of peptides on thin-layer cellulose plates (Eastman Kodak Sheet 13255) by electrophoresis in pH 2.1 buffer and ascending chromatography in butanol–acetic acid–water–pyridine. The plates were dried, in some cases after dipping in 7\% (w/v) PPO (2,5-diphenyloxazole) dissolved in ether, and exposed to Fuji RX film at room temperature or at \(-70\) °C as appropriate.

RESULTS

Virion proteins

MHV-JHM virions contain six \(^{35}\)S\textsuperscript{m}ethionine-containing proteins, four of which are glycoproteins. These are gp170, gp98, gp65, pp60, gp25 and p23 (Siddell et al., 1981a). Quite often the gp25 species is detected as multiple bands. Rottier et al. (1981b), studying the closely related MHV-A59, found a similar pattern of virion proteins except gp65, which they did not describe. These authors, however, did describe as \(^{35}\)S\textsuperscript{m}ethionine-containing virion proteins two additional polypeptides, p22/X and p14.5/Y. Fig. 1 shows an overexposed autoradiograph of MHV-JHM labelled with \(^{35}\)S\textsuperscript{m}ethionine and purified as described in Methods. No proteins additional to those already described were detected and, if present, they must either have been minor components of MHV-JHM virions or selectively lost during the purification procedure used.
Fig. 2. [$^{35}$S]methionine-containing tryptic peptide fingerprints of MHV-JHM proteins. (a) gp170; (b) gp98; (c) gp65; (d) pp60; (e) gp25; (f) p23.

Tryptic peptide analysis of virion proteins

Fig. 2 (a to f) shows the [$^{35}$S]methionine-containing tryptic peptide fingerprints of all six MHV-JHM virion proteins. This analysis showed that the proteins gp98, gp65, pp60 and p23...
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Fig. 2 (continued). \[^{35}S\]methionine-containing tryptic peptide fingerprints of intracellular MHV-JHM polypeptides prepared from immunoprecipitated samples. (g) 150K; (h) 98K; (i) 65K; (j) 60K; (k) 25K; (l) 23K.

were structurally distinct. In contrast, gp25 appeared to have essentially the same polypeptide component as p23, and most although not all of the major tryptic peptides found in gp98 were also found in gp170. Mixing experiments were performed and confirmed that with respect to
the major peptides the fingerprints of gp25 and p23 were identical and that the peptides designated 1, 2, 3, C and E in the fingerprints of gp170 and gp98 were identical. None of the tryptic peptides found in the fingerprints of the four structurally distinct polypeptides had the same mobility (data not shown).

**Tryptic peptide mapping of intracellular proteins**

We have described the synthesis of six primary translation products (150K, 65K, 60K, 30K, 23K and 14K) and three post-translationally modified polypeptides (170K, 98K and 25K) in MHV-JHM-infected cells (Siddell et al., 1981a). Fig. 2 (g to m, p) shows the \(^{35}\)S methionine-containing tryptic peptide fingerprints of all these species, with the exception of 170K. Comparison of these fingerprints with each other and with the fingerprints of virion proteins shows, first, that the fingerprints of the primary translation products 150K, 65K, 60K, 30K, 23K and 14K are distinct (Fig. 2g, i, j, m, l, p respectively). Second, the intracellular proteins 150K and 98K have identical fingerprints (Fig. 2g, h). These fingerprints shared the peptides designated A, B, C, D and E with the fingerprint of gp98 (Fig. 2b). Peptides 1, 2 and 3 were also present, although they were not well resolved in the fingerprints of the intracellular proteins. Third, the fingerprint of 65K (Fig. 2i) contained two of the major peptides of the gp65 fingerprint (designated \(\alpha\) and \(\beta\)) (Fig. 2c). The third major peptide of gp65 (\(\delta\)) was present, but for an unknown reason under-represented in the fingerprint of 65K. Fourth, with the exception of one peptide (I) which appeared to vary in mobility (see below), the fingerprints of 60K (Fig. 2j) and pp60 (Fig. 2d) were identical. Finally, the fingerprints of 25K and 23K (Fig. 2k, l respectively) were found to be identical and, in spite of the poor resolution of these fingerprints, it was also possible to clearly recognize identity with the fingerprints of the virion gp25 and p23 (Fig. 2e, f).

These findings have also been confirmed by many mixing experiments (data not shown). Two such experiments are, however, illustrated in Fig. 2 (o, q) which shows fingerprints of mixtures of 150K and 98K (Fig. 2o) and of 60K and 14K (Fig. 2q). In one case (150K/98K) complete identity of the fingerprints can be seen, whilst in the other (60K/14K) complete non-identity of the fingerprints can be demonstrated. As mentioned above, the 60K peptide, I, appeared to show a variable mobility and in the mixed fingerprint it migrated to a position corresponding to the virion peptide rather than the intracellular peptide. The explanation for this variability is not known.

Fig. 2(n) shows the \(^{35}\)S methionine-containing tryptic peptide fingerprint of one further virus protein which has been found in infected cells. This polypeptide, designated Pd, was recognized when the fingerprint of the pulse–chase polypeptide(s) from the 65K region of the gel was prepared and it was found to contain the peptides characteristic of gp65 (\(\alpha, \beta\) and \(\delta\)), three peptides characteristic of the 150K/98K species (A, C and E) and four novel peptides (a, b, c and d). It is possible to conclude, therefore, that Pd is a product of the intracellular precursor(s) of the virion peplomer protein, although its precise relationship is uncertain.

**DISCUSSION**

The conclusions of this study can be summarized diagrammatically in a model describing the genesis of MHV-JHM proteins (Fig. 3). In addition to the tryptic fingerprinting data presented here, there are many data which lend support to these conclusions. These data can be considered in relation to each of the classes of coronavirion proteins.

In Fig. 3 it is concluded that both gp170 and gp98 are derived from a primary translation
product 150K, which is processed intracellularly to yield 170K and 98K, the equivalents of the virion proteins gp170 and gp98. Sturman and co-workers (Sturman, 1977, 1981; Sturman & Holmes, 1977) have also concluded that the two high molecular weight glycoproteins of MHV-A59, gp180 and gp90 are related. Their conclusion is based on the incorporation of a variety of amino acids and sugars, the susceptibility of these proteins to proteolytic degradation in the virion, fingerprinting of \[^{35}S\]methionine-containing tryptic peptides and the ability to cleave gp180 \textit{in vitro} with trypsin to yield gp90. Both Siddell \textit{et al.} (1981a) and Rottier \textit{et al.} (1981b) have demonstrated by pulse-chase experiments a temporal relationship in the infected cell between the synthesis and processing of these MHV polypeptides. Two-dimensional PAGE of the JHM species (Siddell \textit{et al.}, 1981a) also supports this model.

Although the relatedness of these species is evident the precise pathway of processing events involved in their generation has not yet been demonstrated. Sturman (1981) has proposed that the pathway proceeds via the cleavage of the larger glycoprotein to yield two dissimilar polypeptides, which have the same electrophoretic mobility. This model suggests that the primary translation product undergoes modification at a late stage of maturation to yield the larger virion protein, which is subsequently cleaved. In Fig. 3 it is indicated that the primary translation product (150K), which is a glycoprotein (Rottier \textit{et al.}, 1981b; S. G Siddell, unpublished results), contains a polypeptide core with a mol. wt. of 120K. Such a species has been found in MHV-infected cells treated with tunicamycin (Siddell \textit{et al.}, 1981b; Rottier \textit{et al.}, 1981b; Niemann & Klenk, 1981) and in translates of JHM-infected cell mRNA in cell-free systems (Siddell \textit{et al.}, 1980). The conversion of the primary translation product to the larger virion peplomer protein, therefore, possibly involves further modifications of the carbohydrate side chains which were added co-translationally to the 150K polypeptide (Niemann & Klenk, 1981). This extensive modification, which may interfere with the tryptic digestion, could also explain the absence of three peptides in the fingerprint of gp170 which are present in its postulated precursor, 150K. It is noteworthy that a large amount of radioactive material still remains at the origin of these fingerprints. If the cleavage of the 170K polypeptide is within, or adjacent to, a \[^{35}S\]methionine-lacking tryptic peptide then the data presented here are also compatible with this pathway.

JHM virion gp65 has tentatively been identified as a surface protein (Wege \textit{et al.}, 1979; Siddell \textit{et al.}, 1981a) and (glyco)proteins of a similar size have been reported for other coronaviruses, namely MHV-S (Lai & Stohlman, 1981), BCV-Mebus (Storz \textit{et al.}, 1981), and HEV-VW (Callebaut & Pensaert, 1980). Here, the identity of the virion and intracellular species described previously (Siddell \textit{et al.}, 1981a) has been confirmed and it has been
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demonstrated that they are unrelated to other virus proteins. However, because of the absence of such a protein from the majority of coronaviruses, including the closely related MHV-A59 (Sturman, 1977; Rottier et al., 1981 b), and because of the inability to characterize a coronavirus mRNA which directs the synthesis of this protein in vitro (Siddell et al., 1981 b; S. G. Siddell, unpublished observations) it is not yet possible to decide whether this MHV-JHM protein is host cell-coded and incorporated into virions, or the product of an as yet uncharacterized coronavirus gene.

The results presented here confirm earlier studies (Siddell et al., 1980, 1981 b; Cheley & Anderson, 1981; Anderson et al., 1979; Rottier et al., 1981 b; Bond et al., 1979, 1981; Gerdes et al., 1981; Holmes et al., 1981) which show that the major polypeptide synthesized in MHV-infected cells is the intracellular form of the phosphorylated nucleocapsid protein.

Fig. 3 suggests that the polypeptide component of the matrix protein is synthesized as a 23K species, which is subsequently modified to a 25K species before both proteins are incorporated into the virion. Pulse–chase labelling experiments, in vitro translation studies and two-dimensional PAGE of these species are all consistent with this conclusion (Siddell et al., 1980, 1981 a). Because it has been shown for MHV-A59 or MHV-JHM that the higher molecular weight form of both the intracellular and virion matrix protein species are glycosylated (Siddell et al., 1981 b; Sturman & Holmes, 1977; Neimann & Klenk, 1981; Macnaughton, 1980; Rottier et al., 1981 b), it is logical to conclude that this modification is due to glycosylation. A number of groups have now demonstrated that this glycosylation event is not, however, inhibited by the drug tunicamycin (Niemann & Klenk, 1981; Rottier et al., 1981 b; Siddell et al., 1981 b; Holmes et al., 1981; Cheley & Anderson, 1981) and chemical analysis conclusively shows that the glycosidic linkage is of the O type (Niemann & Klenk, 1981). Recently, by a combination of tryptic peptide mapping and partial proteolytic analysis, Stern et al. (1982) have concluded that the analogous proteins in the infectious bronchitis virion, i.e. the ‘p23 family’, also have a common polypeptide core, but differ in their degrees of glycosylation. Furthermore, the same authors have localized the variability in glycosylation to sites at the amino-terminal domain of the polypeptide.

This study has characterized as unique two further JHM-specific polypeptides, i.e. 30K and 14K. As shown, neither of these polypeptides has been detected in purified MHV-JHM virions. However, as both Rottier et al. (1981 b) and Stern et al. (1982) have found minor proteins in MHV-A59 and IBV virions with mol. wt. of 14 000, further studies to clarify this apparent discrepancy have been undertaken.

The remaining virus-specific polypeptide detected in the infected cell had a mol. wt. of 65 000 and has been designated as Pd. This polypeptide is not found in virions, but contains a subset of the [35S]methionine-containing tryptic peptides characteristic of the virion peplomer protein(s). The significance, if any, of this polypeptide is unknown.

This paper discusses translational aspects of the replication of MHV-JHM. In infected cells, six primary translation products have now been rigorously identified. Of these, four and possibly a fifth are incorporated into virions either directly or after processing. To establish the identity of the mRNAs which encode these proteins and correlate them with the virus-specific RNAs found in infected cells will ultimately require sequencing of both RNA and protein molecules. However, experiments with size-fractionated poly(A)-containing RNA in cell-free translation systems (Siddell et al., 1980, 1981 b; Leibowitz & Weiss, 1981) or in ovo (Rottier et al., 1981 a) have already provided tentative coding assignments for the major structural proteins of both MHV-JHM and MHV-A59. In the case of MHV-JHM further confirmation of these assignments can now be provided by comparison of the tryptic peptide fingerprints of these in vitro translation products with those reported here (S. G. Siddell et al., unpublished observations). As the subgenomic mRNAs of MHV have already been mapped on to the genome (Lai et al., 1981) it will soon be possible to produce a genetic map of MHV.
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