Physiological Characterization of Influenza Virus Temperature-sensitive Mutants Defective in the Haemagglutinin Gene

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SUMMARY

We have characterized the physiological defect in two temperature-sensitive mutants of the WSN strain of influenza virus which possessed a lesion in the haemagglutinin (HA) gene. In mutant virus-infected cells at the non-permissive temperature, the precursor HA polypeptide containing predominantly mannose-rich carbohydrate chains was not converted to the mature, functional HA polypeptide. Immunofluorescence showed that the HA polypeptide did not appear on the cell surface but was confined largely to the Golgi apparatus. It was concluded that the major physiological defect of these mutants was a block in the transport of the HA polypeptide beyond the Golgi apparatus. The block could be reversed, however, by lowering the temperature to 34 °C, resulting in normal processing of the precursor polypeptide and emergence of infectious progeny virus within 30 min. The HA activity of the two mutants, but not wild-type virus, was rapidly inactivated at 51 °C. Most, but not all, revertants derived from these mutants had HA with the heat stability of wild-type virus, suggesting that the temperature sensitivity and the heat lability of HA were two pleiotropic manifestations of a single lesion in the HA gene.

INTRODUCTION

Temperature-sensitive (ts) mutants of the WSN strain of influenza A virus have been classified into seven groups (I to VII) by recombination tests (Sugiura et al., 1972, 1975). Each group represents the defect in each of RNA gene segments 1 to 7 (for review, see Palese, 1977). The mutant (ts-61S) belonging to group VI was defective in the haemagglutinin (HA) gene. Virions of ts-61S formed at the permissive temperature (34 °C) had unusually heat-labile HA. No HA activity was found in cells infected with ts-61S and incubated at the non-permissive temperature (39.5 °C) and the band corresponding to the HA polypeptide was missing when infected cells were analysed by SDS-PAGE (Ueda & Kilbourne, 1976). Subsequently, however, Ritchey & Palese obtained evidence which suggested that the mutant did synthesize an incompletely glycosylated precursor of HA polypeptide (cited by Palese, 1978). With the availability of antiserum specific to the HA of the WSN strain, we have in this report re-investigated the mutant using in addition another group VI mutant (ts-134), phenotypically very similar to ts-61S, which had been obtained independently from ts-61S. The study confirms that both ts-61S and ts-134 synthesize an incompletely glycosylated precursor of HA polypeptide which is not transported beyond the Golgi apparatus at the non-permissive temperature.

METHODS

Cells and viruses. Madin–Darby bovine kidney (MDBK) cells were used throughout this study. Cells were grown and maintained as described by Sugiura et al. (1972, 1975). Wild-type virus (ts*) and ts-61S of the WSN strain of influenza virus have been described by Ueda & Kilbourne (1976). Mutant ts-134 was isolated from the ts* virus stock mutagenized with 5-fluorouracil by K. Nakajima in our laboratory. Standard recombination tests placed the lesion of ts-134 in group VI. The permissive and non-permissive temperatures for these mutants were 34 °C and 39.5 °C, respectively.
Antiserum. Rabbits were immunized with the fusion protein consisting of 190 amino acids from the trp-LE protein of Escherichia coli and 308 amino acids from the WSN HA (residues 1 to 308 of polypeptide HA) produced by E. coli through expression of cloned DNA of the HA gene. The antibody, though devoid of haemagglutination-inhibiting and virus-neutralizing activities, did bind to detergent-treated HA as well as to HA on intact virions or the surface of infected cells (Davis et al., 1983). The serum was treated with receptor-destroying enzyme and inactivated at 56 °C for 30 min.

Radiolabelling of polypeptides in infected cells. MDBK cells (2 × 10⁶ cells) were infected with ts+, ts-61S or ts-134 at an m.o.i. of about 2 p.f.u./cell. Infected or mock-infected cells were labelled with 10 μCi/ml [³⁵S]cysteine (sp. act. 1170 Ci/mmol, Amersham) in 3 ml cysteine-free maintenance medium for 1 h at 5 h post-infection. For chase experiments, after 30 min labelling, the labelling medium was replaced with fresh maintenance medium containing twice the normal concentration of cysteine and the cells were incubated for an additional 1.5 h. Glycoproteins were labelled by incubating infected cells in 15 μCi/ml [¹⁴C]mannose (sp. act. 279 mCi/mmol, Amersham) from 3 to 6 h post-infection. The cells were scraped off and washed three times with phosphate-buffered saline (PBS) by centrifugation. The final pellet was resuspended in 0.1 ml of sample buffer which consisted of 0.0625 M-Tris-HCl pH 6.8, 1% SDS, 2% 2-mercaptoethanol and 5% glycerol, boiled for 3 min, and subjected to PAGE.

Endoglycosidase H (endo H) digestion. The procedure of Person et al. (1982) was followed. [¹⁴C]Mannose-labelled cells were lysed in buffer containing 0.15 M-Tris-HCl pH 8.8, 2% 2-mercaptoethanol, and 1% SDS. Deoxyribonuclease I (Sigma), 50 μg in 50 μl of PBS, was added to 0-2 ml of lysed cells, and the mixture was incubated at 37 °C for 30 min, and boiled for 3 min. A 0.4 ml amount of sodium citrate (0.3 M, pH 5.5), and 0.01 unit of endo H (Seikagaku Kogyo Co., Tokyo, Japan) were added and the mixture was incubated overnight at 37 °C. Protein was precipitated by cold acetone and the precipitate was subjected to electrophoresis.

SDS–polyacrylamide slab gel electrophoresis. Electrophoresis was done in 10% polyacrylamide gel by the method of Maizel (1971). This method was adopted because it reproducibly enabled clear separation of the HA and HA' polypeptides to be described below. Gels were treated with 1 M-sodium salicylate as described by Chamberlain (1979), dried and fluorographed on Fuji X-ray film (RXO) at -70 °C.

Immunoprecipitation with anti-HA serum. Infected MDBK cells labelled with [³⁵S]cysteine as described above were lysed in 10 mM-Tris–HCl pH 7.4, 0.15 M-NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM-phenylmethylsulphonyl fluoride (lysis buffer), and clarified by centrifugation at 7000 g for 1 min. For immunoprecipitation, 200 μl of the lysate was mixed with 2 μl of 35% bovine serum albumin and 5 μl of rabbit anti-HA serum. The mixture was held at 37 °C for 1 h and then at 4 °C overnight. Twenty μl of a slurry of Protein A–Sepharose (Pharmacia) was added and the mixture was incubated for 45 min at room temperature with occasional shaking. The Protein A–Sepharose immune complex was washed three times with the lysis buffer, resuspended in the sample buffer for electrophoresis, boiled for 3 min, and centrifuged at about 200 g for 10 s. The supernatant was analysed by SDS–PAGE.

Immunofluorescence. MDBK cells grown on round 18 mm coverslips were infected at an m.o.i. of 2 p.f.u./cell and incubated in Petri dishes in a water-bath set to either 34 °C or 39.5 °C. After 6 h incubation, the cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. The cells were then treated successively with rabbit anti-HA serum, biotinylated goat anti-rabbit IgG serum, and fluorescein-conjugated avidin (the latter two purchased from Vector Laboratories, Burlingame, Ca., U.S.A.), for 20 min at room temperature for each, followed by thorough washing with PBS each time. The reagents were diluted in 0.14 M-NaCl, 0.01 M-Tris–HCl pH 7.4, and 3% bovine serum albumin. For surface staining, cells were processed directly after paraformaldehyde fixation. For staining of the interior of cells, fixed cells were permeabilized by treatment with 0.05% Triton X-100 for 30 min. To locate the subcellular organelles, mock-infected cells fixed and treated as above were stained with fluorescein-conjugated wheat germ agglutinin (Miles Laboratories) (Virtanen et al., 1980). Coverslips were mounted in 50% glycerol in PBS and examined and photographed with an Olympus transmission fluorescence microscope.

Isolation of revertants. Revertants of ts-61S and ts-134 were isolated from plaques formed at 39.5 °C, recloned, and grown in MDBK cells at 34 °C under fluid medium. Plaquing efficiency of the virus preparation was determined as described (Sugiura et al., 1972). Heat stability of the haemagglutinating activity was examined as described in Table 1.

RESULTS

Polypeptide synthesis in mutant-infected cells

Polypeptides obtained from ts⁺-infected and group VI mutant (ts-61S and ts-134)-infected cells at the permissive and non-permissive temperature were analysed by SDS–PAGE (Fig. 1). Whereas two polypeptides were resolved with ts⁺-infected cells at either temperature and mutual-infected cells at the permissive temperature around the position where the HA poly-
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Fig. 1. PAGE of viral polypeptides synthesized in infected cells. Cells infected with ts-61S or ts-134 were incubated at 34 °C or 39.5 °C for 5 h. Mock- or ts+ infected cells were incubated at 39.5 °C. All cultures were labelled with [35S]cysteine for 1 h in cysteine-free medium at the respective temperatures. Cell lysates were analysed by electrophoresis. Lane 1, mock-infected cells at 39.5 °C; lane 2, cells infected with ts+ at 34 °C; lane 3, ts+ at 39.5 °C; lane 4, ts-61S at 34 °C; lane 5, ts-61S at 39.5 °C; lane 6, ts-134 at 34 °C; lane 7, ts-134 at 39.5 °C. The arrow indicates the band corresponding to the HA' polypeptide.

peptide was expected to migrate, the slower migrating band was missing in mutant-infected cells at the non-permissive temperature. The pattern of synthesis of other polypeptides, i.e. NP, M and NS, did not differ substantially between ts+ and mutants or between permissive and non-permissive temperatures. Subsequent attention was, therefore, focused on the above two polypeptides. By immunoprecipitation with anti-HA serum, both the slower and faster migrating
polypeptides were found to be HA polypeptides and were, therefore, designated HA and HA', respectively (Fig. 2). The pulse-chase experiment presented in Fig. 5 showed that there was a definite precursor-product relationship between HA and HA'. Labelling with radioactive mannose (Fig. 3) and glucosamine (data not shown) showed that both the HA and HA' polypeptides were glycosylated. A previous study has shown that the band corresponding to polypeptide HA' was devoid of fucose (Ueda & Kilbourne, 1976).

The first step in the glycosylation of many viral envelope glycoproteins involves the attachment of mannose-rich oligosaccharides to the nascent polypeptide via an N-glycosidic linkage. The oligosaccharide chains are subjected to further modifications as the glycoproteins move along the intracellular membrane toward the cell surface. Mannose-rich oligosaccharides are known to be susceptible to enzymic cleavage with endo H (Trentino & Maley, 1974). We tested whether the carbohydrates of HA' consisted of mannose-rich oligosaccharides. Cells infected with ts-61S were incubated at either permissive or non-permissive temperature and labelled with $[^{14}C]$mannose. Cell lysates treated or untreated with endo H were analysed by SDS-PAGE. Fig. 3 shows that mannose was incorporated into both HA and HA'. Endo H treatment resulted in the selective disappearance of HA' synthesized at either temperature. With the gel system employed in this study, it was not possible to resolve the neuraminidase (NA) polypeptide, probably due to its co-migration with the NP polypeptide. However, whether the band migrating to the position corresponding to NP was indeed NP labelled by radioactive amino acids which might have been metabolically converted from $[^{14}C]$mannose or the NA has not been determined.

The results obtained thus far were interpreted in the following way. In the labelling condition employed, HA was synthesized first as the HA' form with predominantly mannose-rich and, therefore, endo H-sensitive oligosaccharides, which was subsequently processed into the slower migrating, endo H-resistant, mature HA form. Both the HA' and HA forms were precipitated by anti-HA serum. At the non-permissive temperature, the processing of HA' into HA was blocked resulting in the accumulation of immature HA'.
Influenza virus HA mutants

Fig. 4. Immunofluorescent staining. Cells on coverslips were infected with ts-61S or mock-infected at 34 °C or 39.5 °C. At 6 h after infection, cells were fixed with 4% paraformaldehyde. The surface of the cells was stained after the paraformaldehyde fixation (a, b), while the inside of the cells was stained after treatment of cells with 0.05% Triton X-100 following paraformaldehyde fixation (c, d). For staining, infected cells (a, b, c) were treated successively with rabbit anti-HA serum, biotinylated anti-rabbit IgG, and fluorescein-conjugated avidin. (a) Surface staining of cells infected at 34 °C; (b) surface staining of cells infected at 39.5 °C; (c) staining of the inside of cells infected at 39.5 °C; (d) staining of Golgi apparatus of mock-infected cells at 39.5 °C.

Localization of HA by immunofluorescence

The intracellular localization of the immature HA polypeptide of ts-61S was examined by indirect immunofluorescence. Infected cells fixed with paraformaldehyde were treated successively with rabbit anti-HA serum, biotinylated anti-rabbit IgG and fluorescein-conjugated avidin. Fine granular fluorescence was seen to cover the entire surface of cells infected with ts+ at either temperature (data not shown) or with ts-61S at the permissive temperature (Fig. 4a). No surface fluorescence was observed in cells infected with ts-61S at the non-permissive temperature (Fig. 4b). The surface immunofluorescence with anti-neuraminidase serum was similar at permissive and non-permissive temperatures (data not shown). When made permeable with Triton X-100 after paraformaldehyde fixation, or when fixed with acetone, ts-61S-infected cells at the non-permissive temperature were found to contain HA antigen within the cytoplasm (Fig. 4c). To locate the HA antigen within the cytoplasm, cells were stained with fluorescein-conjugated lectins and the pattern of distribution of lectins was compared to that of HA antigen. Wheat germ agglutinin was located in a pattern most closely resembling that of HA antigen (Fig. 4d), indicating the localization of the latter in the Golgi apparatus. Immunofluorescent findings were similar for ts-134 HA antigen, i.e. absence at the cell surface and predominant localization at the perinuclear zone at the non-permissive temperature (data not shown).

Reversal of the block in processing by the temperature shift-down

It was of interest to determine whether the immature HA polypeptide synthesized at the non-permissive temperature could be processed into mature, functional HA when cells were transferred to the permissive temperature. Cells infected with the mutants were labelled with [35S]cysteine at the non-permissive temperature and then chased at either permissive or non-permissive temperature. Fig. 5 shows the conversion of HA' to HA after shift-down, indicating reversibility of the processing defect. Reversal of the maturation defect was shown also by the rapid production of infectious virus in the temperature shift-down experiment (Fig. 6). Three
sets of duplicate cultures were infected with ts-61S. One set was incubated at the permissive temperature, the second at the non-permissive temperature throughout, while the third set was maintained at the non-permissive temperature for the first 6 h and then transferred to the permissive temperature. All cultures were thoroughly washed at 6 h and the subsequent release of infectious virus into the culture fluid was monitored. In 15 to 30 min after the temperature shift-down, a significantly higher infectivity appeared in the fluid than at the non-permissive temperature. In similar experiments, mutants defective in RNA synthesis took 2 h or more for the infectious progeny to emerge (data not shown). The pre-existing HA polypeptide in its immature form appeared to be utilized for the formation of infectious virus once the block was removed.

**Heat stability of HA activity**

As reported earlier, ts-61S grown at the permissive temperature was unusually heat-labile, its HA activity being rapidly inactivated at 51 °C, at which temperature ts+ HA was stable (Ueda & Kilbourne, 1976). Mutant ts-134 was similar to ts-61S in this respect (Table 1). Because the association of two phenotypes, i.e. temperature sensitivity and heat lability, in the two mutants appeared to be more than coincidental, an analysis of revertants was undertaken. The reversion frequencies were 0.00067 for ts-61S and 0.00052 for ts-134, as shown in Table 1. All nine revertant clones derived from ts-61S had HA with heat stability comparable to ts+. Among eight revertant clones from ts-134, heat stability had been restored to the level of ts+ in five (revertants 3, 4, 5, 6 and 8) and to an intermediate level in two (1 and 2), while one clone (7) remained as
Fig. 6. Infectious virus production after temperature shift-down. Mutant ts-61S-infected cells were incubated at 34 °C or 39.5 °C. At 6 h after infection (arrow), cultures were washed, and one set of cultures which had been incubated at 39.5 °C was transferred to 34 °C. At the indicated time, a 0.05 ml portion of culture fluid was removed for determination of infectivity by plaque assay. Each point represents the average of values from duplicate cultures. O, 34 °C throughout; ●, 39.5 °C throughout; ■, shift-down from 39.5 °C to 34 °C.

Table 1. Thermal stability of haemagglutinating activity of ts⁺, ts-61S, ts-134, and revertant clones derived from the mutants

<table>
<thead>
<tr>
<th>Virus</th>
<th>E.o.p.*</th>
<th>HA titre†</th>
<th>Not heated</th>
<th>Heated</th>
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<td>ts⁺</td>
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<td>256</td>
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<td>64</td>
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<td>2</td>
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<td>128</td>
<td>128</td>
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<td>3</td>
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<tr>
<td>4</td>
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<td>64</td>
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<td>64</td>
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<tr>
<td>7</td>
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<tr>
<td>9</td>
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<td>64</td>
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</tr>
<tr>
<td>ts-134</td>
<td>0.00052</td>
<td>256</td>
<td>&lt;2</td>
<td></td>
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<tr>
<td>ts-134 revertant 1</td>
<td>0.65</td>
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<td>8</td>
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<tr>
<td>2</td>
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<td>8</td>
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* The ratio of the efficiency of plaquing at 39.5 °C to that at 34 °C.
† Viruses grown at 34 °C were diluted 1:5 in PBS (pH 7.2) containing 0.2% bovine serum albumin and incubated for 15 min either at 4 °C or 51 °C. HA titration was done using 0.5% chicken red blood cells at room temperature.
heat-labile as the parent ts-134. The concomitant reversion in two phenotypes suggests that these phenotypes were two pleiotropic manifestations of mutation at a single site of the HA gene. The restoration of wild-type phenotype could have been caused by reversion at the original site of mutation or could be the result of suppressor mutation(s) at other sites within the HA gene.

**DISCUSSION**

One of us (M.U.) has reported earlier that no HA polypeptide was present in ts-61S-infected cells at the non-permissive temperature (Ueda & Kilbourne, 1976). In fact, however, the bands corresponding to those designated as HA and HA' in this study had been clearly resolved on the electropherogram in the previous study (Ueda & Kilbourne, 1976, Fig. 3 and 4). The reason which led us to this erroneous conclusion was our assumption at the time that the faster migrating HA' was the NA polypeptide. The use of specific anti-HA serum in this study has dispelled the confusion.

Despite the presence of immunologically reactive HA polypeptide, no HA activity was found in the extract of cells infected with ts-61S or ts-134 at the non-permissive temperature. The proper glycosylation or the resulting proper configuration of the molecule appeared to be essential for functional activity, as evidenced by the aberrant virus formed in the presence of tunicamycin. While unglycosylated HA and NA synthesized in the presence of tunicamycin were expressed at the cell surface and even incorporated into virions, released virions had markedly reduced HA and NA activities (Basak & Compans, 1983). Since in the above study totally unglycosylated HA reached the cell surface, the lack of proper glycosylation per se does not seem to be responsible for the accumulation of HA polypeptide of the group VI mutants in the Golgi area, but the failure of migration is the likely cause of impaired glycosylation.

By manipulating cloned DNA of an influenza HA gene, Sveda et al. (1982, 1984) were able to construct a series of HA-SV40 mutants coding for HA in which stretches of hydrophobic amino acid sequence near the carboxy terminus of HA were either deleted or radically altered. Whereas the product of the complete HA gene expressed in monkey kidney cells was normally glycosylated and transported to the cell surface, the HA polypeptide produced by mutants was processed abnormally. With one group of mutants, HA polypeptides present in the cell were all endo H-sensitive. Instead of being processed into an endo H-resistant form and transported to the cell surface, they were secreted into the surrounding medium. The HA polypeptide produced by another mutant became endo H-resistant and acquired HA activity but was confined to the Golgi apparatus and was not transported to the cell surface. Apparently, the lack of, or modification of the membrane anchorage signal was responsible for their aberrant processing. From ts-61S-infected cells, shedding of HA polypeptide into the medium did not occur (data not shown). The HA polypeptides produced by our mutants appeared to be more immature than that of the second HA-SV40 mutant in terms of endo H sensitivity and the lack of HA activity. In view of the phenotypic similarity, it is tempting to speculate that the defect in our mutants was also caused by an anomalous association of their HA polypeptides with the membrane structure in which they were inserted.

Apart from the mutants described here, the only ts mutant defective in the HA gene so far characterized is ts227 derived from fowl plague virus (Lohmeyer & Klenk, 1979). This mutant has many features in common with ours. Although the HA polypeptide was synthesized and incorporated into the rough endoplasmic reticulum, its transport was blocked. As a result, glycosylation was incomplete, mannose and glucosamine but not galactose and fucose being attached to the polypeptide. Trimming of mannose-rich carbohydrate did not occur. The immature HA had no haemagglutinating activity, yet reacted with anti-HA antibody. It is noteworthy that all three HA mutants, two derived from the WSN strain and one from fowl plague virus, isolated independently of each other shared a block in similar step(s) of post-translational processing. Certain regions of the HA polypeptide, possibly those including the region involved in the interaction with the membrane, may be particularly prone to ts mutation. Changes of amino acid residues in other regions may be relatively tolerated in the integrity of the HA molecule, though some of them may give rise to antigenic variants.
We thank Dr K. Nakajima for making ts-134 available to us. This work was supported partially by a Grant-in-Aid of Scientific Research from Ministry of Education, Japan.

Note added in proof. After the submission of this manuscript, it was learnt that the report by Rodriguez-Boulan et al. (1984) had dealt with the defect of influenza WSN, ts-61 (Rodriguez-Boulan, E., Paskiet, K. T., Salas, P. J. I. & Bard, E. (1984). Intracellular transport of influenza virus haemagglutinin to the apical surface of Madin–Darby canine kidney cells. *Journal of Cell Biology* **98**, 309–319). In our original nomenclature (Ueda & Kilbourne, 1976), ts-61 was a double mutant defective in HA and NP genes, while ts-61S was a segregant derived from ts-61 and a single mutant defective in HA gene only. It is not entirely clear whether Rodriguez-Boulan et al. used ts-61 or ts-61S. If they used the former, it could partly explain the minor discrepancy between their results and ours.

REFERENCES


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