Nucleoproteins of Animal Influenza Viruses, in Contrast to Those of Human Strains, Are Not Cleaved in Infected Cells

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SUMMARY

We previously reported that nucleoproteins (NPs) of human influenza viruses are cleaved in infected cells and, as a result, two forms of NP, uncleaved (mol. wt. 56000) and cleaved (mol. wt. 53000) were accumulated late in infection. Here, we report that NPs of animal influenza viruses of non-human origin (isolated from pigs, equids, seals, whales, birds) exhibited proteolytic resistance in infected cells and did not undergo a change in mol. wt. in the course of infection. The resistance of the animal virus NPs to proteolytic cleavage was shown to be a virus-specific property and not the consequence of a low level of proteolysis in infected cells. Influenza A/H3N2 viruses isolated from swine in Hong Kong in 1976 were found to have a cleavable NP like that of 'human' viruses, supporting the hypothesis concerning the 'human' origin of these strains. The NP of human influenza virus (A/Aichi/2/68) adapted to an animal host (mouse) retained susceptibility to limited intracellular proteolysis. Thus, NP resistance to cleavage seems to be a stable viral characteristic enabling the NP56→NP53 modification to be used as an indication of the origin of influenza viruses.

The major nucleocapsid protein (NP) of human influenza viruses is cleaved in infected cells. As a result of cleavage, the NP of mol. wt. 56000 (NP56) is converted into an NP of mol. wt. 53000 (NP53). From NP gene sequencing data, the excised 3000 mol. wt. peptide is most probably the N-terminal part of the NP molecule (Winter & Fields, 1981; Huddleston & Brownlee, 1982), this peptide being involved in the virus budding process (Zhirnov & Bukrinskaya, 1981). The proteolytic modification of NP is a host-dependent event: proteolytic cleavage is more effective in chick (CEF) and porcine (SPEV) cells than in canine (MDCK) cells and is greatly reduced in human (HeLa) cells (Zhirnov & Bukrinskaya, 1981). It follows that host cell factors are involved in the NP56→NP53 modification.

In this paper we show that the NP of avian and non-human mammalian influenza virus strains (which are operationally termed 'animal viruses') are resistant to proteolytic cleavage and are not cleaved in infected cells. Thus, NP cleavage is not only a host-dependent but also a virus-dependent process.

The following human influenza viruses were used: A/WSN (H1N1), A/PR/8/34 (H1N1), A/USSR/90/77 (H1N1), A/USSR/5/80 (H3N2), A/Udorn/72 (H3N2), B/Lee/40. Non-human mammalian influenza A viruses were: swine/Iowa/30 (H1N1), whale/Pacific Ocean/76 (H1N3), seal/Massachusetts/1/80 (H7N7), equi/Miami/63 (H7N8). Avian influenza A viruses were: FPV/Weybridge (H7N1), tern/South Africa/61 (H5N3), turkey/California/1/71 (H5N2), chicken/Germany/49 (H10N7), duck/Ukraine/63 (H3N8), shearwater/Australia/72 (H6N5). Additionally, we analysed several H3N2 influenza A viruses: human influenza Aichi/2/68 virus adapted for mouse lungs, and swine/Hong Kong/3/76, 4/76 and 6/76 viruses which were isolated from pigs in Hong Kong and kindly provided by Dr A. P. Kendal. All the viruses used were propagated in chicken eggs. The allantoic fluid usually contained about 10^3 haemagglutinating units/ml and 10^7 to 10^8 p.f.u./ml and was used as inoculum for infecting cell monolayers. Primary cultures of chicken embryo cells (CEF) and a porcine embryo kidney cell line (SPEV) were used because they provided the greatest proteolysis of human virus NP (Zhirnov &
Bukrinskaya, 1981). Intracellular proteolytic modification of NP was studied by electrophoretic analysis of pulse–chase-labelled infected cell polypeptides. In the event of cleavage, NP was detected after the chase in two forms, NP56 and NP53.

As can be seen in Fig. 1, the pulse-labelled NP of all the strains investigated was present in CEF in its uncleaved form. After a 5 h chase (lanes 2 in Fig. 1) and more distinctly after an 11 or 18 h chase, the NP of human influenza viruses was detected in two forms, NP56 and NP53, indicative of extensive proteolytic modification in CEF. Scanning of autoradiographs (14 to 16 h post-infection) showed that the degree of cleavage varied among the different human virus strains from 30 to 90%. On the other hand, NPs of animal viruses under the same conditions were not cleaved, remaining as an intact NP56 band even after a prolonged chase. Similar results were obtained in the porcine (SPEV) cells (see Fig. 1), canine kidney (MDCK) cells and primary mouse kidney cells (not shown). It follows from these data that the NPs of animal influenza viruses, unlike those of human viruses, are resistant to proteolytic cleavage in infected cells.

The differences in cleavage could be either a genuine virus-specific property or a consequence of an insufficient activation of intracellular proteolysis by animal virus infection. To discriminate between these possibilities, double-infection experiments were carried out in CEF cells with B/Lee and different animal influenza A viruses. Since the electrophoretic mobility of B/Lee virus NP was clearly distinct from that of the NP of influenza A viruses, it was possible to examine the NP cleavage of each virus in mixedly infected cells. Fig. 2 shows that the NP of B/Lee virus is intracellularly cleaved and detected after chase in three forms, uncleaved NP64, cleaved NP62 and NP55. Additionally, a 13K (mol. wt. 12,500) polypeptide appeared after the chase in B/Lee virus-infected cells in parallel with a decrease in the amount of NS1. NP55 was present as a minor component in total cell lysate and was clearly visible in viral ribonucleoproteins (RNPs) prepared from infected cells (not shown). A similar cleaved product of B/Lee virus NP has also been reported by Nakamura & Homma (1981). B/Lee virus NP, from this observation, seems to be cleaved in infected cells in two stages, NP64→NP62→NP55 or alternatively NP62→NP64→NP55.

After double infection of CEF cells the NP of B/Lee virus underwent cleavage and NP64 and NP62 polypeptides were present in infected cells, whereas the NPs of animal viruses were not cleaved and were detected only as the intact NP56 form (see Fig. 2). Similar results were obtained with all 12 strains of animal influenza viruses studied. Thus, co-infection with B/Lee did not permit cleavage of the NP protein of avian and non-human animal influenza viruses. This observation suggests that the relative resistance of the NP of animal influenza viruses to proteolytic cleavage in infected cells is most probably a genuine viral property rather than the consequence of a low activation of intracellular proteolysis by animal virus infection.

The resistance of animal influenza virus NP to proteolysis during chase, i.e. post-translational resistance, did not exclude co-translational NP cleavage. To analyse this possibility, virus polypeptide synthesis in the presence of protease inhibitors was studied. The following inhibitors were used: TLCK, a trypsin inhibitor (Shaw et al., 1965), TPCK, a chymotrypsin inhibitor (Schoellmann & Shaw, 1963) and inhibitors of trypsin- and chymotrypsin-like proteases, phenylmethylsulphonyl fluoride (PMSF; Gold, 1965) and aprotinin [Gordox®, Gedeon Richter, Hungary (Trautschold et al., 1967)]. The intracellular cleavage of human influenza virus NP was found to be effectively inhibited by TPCK and less by TLCK. The NP56→NP53 conversion was prevented by addition of TPCK to culture medium at a final concentration as low as 10 μM (Fig. 3a, lanes 3 to 7), whereas only high concentrations of TLCK (200 μM and more) and aprotinin (1000 kallikrein inhibition units/ml) markedly inhibited NP cleavage (Fig. 3a, lanes 8 to 11 and 12 to 15 respectively). These observations suggest that in infected cells NP56→NP53 processing is probably mediated by the coupled action of trypsin- and chymotrypsin-like proteases, and is more sensitive to deficiency of the latter.

Further experiments were carried out to study the possible co-translational cleavage of animal influenza virus NPs. For this purpose, we examined the NP products synthesized in infected cells when proteolysis was inhibited. Of most interest were the influenza viruses A/swine/Iowa/30 (H1N1), and A/equi/Miami/63 (H7N8) because their NPs had fast electrophoretic
Fig. 1. Polypeptide patterns in SPEV and CEF cells infected with different influenza viruses. Cells were infected with different influenza viruses: WSN, PR/8/34, Aichi/2/68 (Ach), USSR/90/77 (USSR/77), USSR/5/80 (USSR/80), whale/Pacific Ocean (W/PO), seal/Massachusetts/1/80 (S/M), swine/Iowa/30 (S/I), equi/Miami/63 (E/M), chicken/Germany/49 (C/G), tern/South Africa/61 (T/SA), turkey/California/71 (T/C), FPV/Weybridge and duck/Ukraine/63 (D/U) at 10 to 50 p.f.u./cell. Infected cells were pulse-labelled with $^{14}$C-algal hydrolysate for 30 min at 5-5 h post-infection (lanes 1), washed with Medium 199 containing 5% lactalbumin hydrolysate and chased with unlabelled Medium 199 for 5 (lanes 2), 11 (lanes 3) or 18.5 (lanes 4) h. The cells were fixed with 10% trichloroacetic acid and washed with ethanol. Cellular polypeptides were treated in dissociation buffer (2% SDS, 0.01 M-dithiothreitol, 0.2 M-Tris-HCl pH 6.8) for 1 min at 100 °C and analysed by electrophoresis in 10 to 15% gradient polyacrylamide gels using Tris–glycine–SDS buffer followed by autoradiography as previously described (Zhirnov et al., 1982a). Positions of major viral polypeptides NP, M and NS1 are designated in accordance with their mol. wt.; HA polypeptides are scarcely seen in SPEV cells and therefore are indicated only in the CEF panel.
mobilities, similar to the cleaved NP53 of human virus strains (Fig. 1). The results of a typical experiment are shown in Fig. 3(b). It can be seen that in cells infected with these virus strains in the absence or presence of a protease inhibitor (TPCK, TLCK or PMSF) NPs were synthesized which had the same electrophoretic migration. Similarly, these protease inhibitors failed to cause an increase in the mol. wt. of NPs synthesized in cells infected with other animal virus strains (not shown). Based on these findings, the possibility of intracellular co-translational cleavage of animal virus NP by chymotrypsin- or trypsin-like protease seems unlikely.

Of special interest is the NP cleavability of 'human' influenza viruses which had been passaged in animals. To study this, we examined influenza virus strain A/Aichi/2/68 which had been laboratory-adapted for mouse lungs by mouse to mouse intranasal passaging. After adaptation, this virus strain replicates effectively in mouse lungs and induces fatal haemorrhagic pneumonia (Zhirnov et al., 1982b, 1984). Analysis of the protease resistance of the NP indicated that this mouse-adapted A/Aichi/2/68 strain retained the cleavable form; in infected cells the NP of this strain was clearly resolved as two forms: cleaved (NP53) and uncleaved (NP56) (Fig. 4).

Following the appearance of H3N2 influenza viruses in 1968 in man, these viruses were isolated from several other species, but particularly from pigs (Webster & Laver, 1975). The cleavability of the NPs of Hong Kong-like influenza (H3N2) viruses isolated in 1976 (swine/Hong Kong 3/76, 4/76 and 6/76) was also analysed. By oligonucleotide mapping of viral RNA (Nakajima et al., 1982) and antigenic analysis of haemagglutinin and neuraminidase (Shortridge et al., 1979), A/swine/Hong Kong/3/76 and A/swine/Hong Kong/6/76 viruses were
Fig. 3. Effect of protease inhibitors on the electrophoretic profiles of viral NPs in CEF cells infected with different influenza viruses. CEF cells were infected with (a) A/WSN (left) or Udorn/72 (right) and (b) swine/Iowa/30, equi/Miami/63 at 10 to 50 p.f.u./cell. (a) Cells were pulse-labelled at 7.5 h post-infection for 30 min (lanes 1), washed, and chased for 7 h with unlabelled Medium 199 alone (lanes 2), or Medium 199 containing 2 μM (lanes 3), 5 μM (lanes 4), 10 μM (lanes 5), 20 μM (lanes 6), or 50 μM TPCK (lanes 7); 50 μM (lanes 8), 100 μM (lanes 9), 200 μM (lanes 10), or 300 μM (lanes 11) TLCK; 250 (lane 12), 500 (lane 13), 1000 (lane 14), or 2500 (lane 15) kallikrein inhibition units/ml of aprotinin (Ap). (b) Infected CEF cells were pulse-labelled at 5.5 h post-infection for 30 min followed by electrophoretic analysis of polypeptides. For labelling, 14C-amino acids were dissolved in Hanks' balanced salt solution (HBSS) alone (lanes 9), or in HBSS containing 50 μM-PMSF (lanes 1), 100 μM-PMSF (lanes 2), 20 μM-TPCK (lanes 3), 50 μM-TPCK (lanes 4), 50 μM-TLCK (lanes 5), 100 μM-TLCK (lanes 6), 250 μM-TLCK (lanes 7) or 500 μM-TLCK (lanes 8). Lanes 10, proteins from infected cells after pulse-labelling and a 14 h chase. Before labelling, the cells were pre-incubated for 30 to 40 min with protease inhibitors. In all experiments, 100 mM-TPCK and 30 mM-PMSF dissolved in methanol and 2-propanol, respectively, were used as stock solutions; TLCK was prepared in aqueous solution.
Fig. 4. Electrophoretic profiles of NPs of mouse-adapted (m.a.) Aichi/2/68 and swine/Hong Kong viruses in infected CEF cells. CEF cells were infected with swine/Iowa/30, swine/HK/3/76, 4/76, 6/76 and m.a. Aichi viruses at 10 to 50 p.f.u./cell. Pulse-labelling (lanes 1), 15 h chasing (lanes 2) and polypeptide analysis were performed as described for Fig. 1.

found to be closely related to early ‘human’ A/Hong Kong/68, and A/swine/Hong Kong/4/76 closely related to contemporary prevalent A/Victoria/75 viruses. Analysis of their NPs demonstrated that these swine viruses, like ‘human’ viruses, contained cleavable proteins. In infected cells, NPs of these virus strains were markedly cleaved and NP53 was clearly resolved (see Fig. 4, lanes 2). Thus, the data obtained support the ‘human’ origin of Hong Kong-like viruses isolated from swine in 1976 and additionally support the concept (Webster & Laver, 1975) that animals, in particular pigs, may be a reservoir of human influenza viruses. So far, virus strains with cleavable NP have been only occasionally isolated from non-human populations.

It has been shown previously that the NP56→NP53 proteolytic modification of human influenza viruses occurs predominantly in the late stages of infection and markedly depends on the host cell type. It was suggested that NP cleavage involved host cell factors, which are preferentially activated in virus-damaged cells (Zhirnov & Bukrinskaya, 1981). As described above, the NP56→NP53 processing can be effectively prevented by TPCK and TLCK, inhibitors of chymotrypsin- and trypsin-like proteases respectively. Therefore, the host factors that can induce virus NP cleavage are possibly cellular serine proteases. On the other hand, it can not be excluded that the NP56→NP53 proteolysis is directed by a virus-specified enzyme.
Short communication
1133

(e.g. one of the virus nucleocapsid proteins, PB1, PB2, PA or NP itself) whose function is activated by host cell factors. The intracellular proteolytic processing of viral proteins by virus-specific proteases has been reported for many viruses, including togaviruses (Scupham et al., 1977; Aliperti & Schlesinger, 1978), picornaviruses (Pelham, 1978; Palmenberg et al., 1979), retroviruses (von der Helm, 1977; Dittmar et al., 1980) and probably adenoviruses (Bhatti & Weber, 1979) and comoviruses (Pelham, 1979).

The avian and non-human mammalian influenza viruses, as shown here, failed to exhibit intracellular cleavage of newly synthesized NP. This difference between human and animal influenza virus NPs, confirmed by double-infection experiments, is most probably a property of the virus strain itself. Two explanations of the virus-specific nature of this phenomenon are possible. First, it is likely that the NPs of animal influenza viruses either have different amino acid sequences at the proteolytic site, or perhaps the intracellular viral RNPs have different conformation states and hence protect the proteolytic sites in the NP molecule. The second explanation, which was discussed above for human viruses, concerns the viral or cellular origin of the protease responsible for NP cleavage. The possibility is that animal influenza viruses either do not have a virus-associated proteolytic activity or, if they have one, that its function is controlled by host factors and is not activated in the host cell types used. Non-human animal viruses may have a specific intracellular pathway and as a result their RNPs fail to come into contact with the cellular compartments responsible for viral NP cleavage.

The biological significance of NP cleavage is obscure. Virtually no intracellular viral RNPs with cleaved NP53 are incorporated into mature virions and therefore they accumulate in infected cells (Zhirnov & Bukrinskaya, 1981). It seems likely that persistence of viral RNPs could be developed in those cells which are able to support NP cleavage. This hypothesis specifically concerns human influenza viruses since they possess cleavable NPs.

These data indicate that the cleavability of human virus NPs is a stable viral characteristic. This property of human viruses is not lost after laboratory adaptation of virus to animals, or by their natural circulation in pigs. Thus, the phenomenon of the NP56→NP53 proteolytic modification and its strain-specific variations can be used as a marker of virus origin during investigation of recombinants and study of influenza virus evolution.

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REFERENCES


Short communication


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