Proteolytic Activation of the Haemagglutinin–Neuraminidase of Newcastle Disease Virus Involves Loss of a Glycopeptide

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SUMMARY

The uncleaved (HN₀) and the cleaved (HN) forms of the haemagglutinin–neuraminidase glycoprotein of Newcastle disease virus (NDV), strain Ulster, were analysed by polyacrylamide gel electrophoresis under reducing and non-reducing conditions. When HN₀ is converted into HN, a glycopeptide with an apparent mol. wt. of about 8000, which is not found in the mature spike, is removed.

Activation of biological function by limited proteolysis is an important step in the biosynthesis of both surface glycoproteins of paramyxoviruses. Cleavage is not necessary for particle formation and depends on the presence of an appropriate protease in the host cell. Thus, virions with uncleaved glycoproteins that can be cleaved in vitro by protease treatment, may be obtained. In the case of the fusion protein, a precursor F₀ (mol. wt. 54 × 10⁶ to 68 × 10⁶) is cleaved into fragments F₁ (mol. wt. 48 × 10⁶ to 56 × 10⁶) and F₂ (mol. wt. 10 × 10⁶ to 16 × 10⁶) which are linked by disulphide bonds (Scheid & Choppin, 1977). With strains Ulster and Queensland of Newcastle disease virus (NDV) a precursor HN₀ of the haemagglutinin–neuraminidase glycoprotein (Nagai et al. 1976a; Nagai & Klenk, 1977) has also been detected. Cleavage of HN₀ (mol. wt. 82 × 10⁶) in vitro or in vivo yields a large fragment HN (mol. wt. 74 × 10⁶) that constitutes the spike. In the present study we have analysed the problem of whether a small cleavage fragment of HN₀, analogous to F₂, is also retained in the spike or whether it is eliminated in the cleavage reaction. The principal virus used was strain Ulster. Some experiments were also conducted on strain Italien where the precursor HN₀ has not yet been identified.

HN₀ and HN are glycoproteins, but it was not known whether or not the small cleavage fragment is glycosylated. To answer this question we have determined the carbohydrate–protein ratio in HN₀ and HN. Virions of strain Ulster were grown in MDBK cells in the presence of ¹⁴C-amino acids and ³H-glucosamine (Nagai & Klenk, 1977). The glycoproteins that were either uncleaved or cleaved by trypsin treatment were isolated as described in Fig. 1 and analysed by polyacrylamide gel electrophoresis (PAGE) under reducing conditions (gels not shown). The ratio ³H/¹⁴C was determined as a measure for the relative carbohydrate content of the glycoproteins and found to be 1.46 for HN₀, and 1.03 for HN, thus indicating that the small cleavage fragment must be relatively rich in carbohydrate.

It was now of interest to find out whether this glycopeptide is retained in the HN spike after cleavage. Evidence for the existence of glycoprotein F₂ was first obtained when the disulphide-bonded F₁₂ complex was identified by gel electrophoresis under non-reducing conditions (Nagai et al. 1976b; Scheid & Choppin, 1977). We have, therefore, used this procedure to analyse the cleaved and the uncleaved form of the haemagglutinin–neuraminidase protein of strain Ulster. When the virus was grown in the absence of trypsin, the glycoprotein profile shown in Fig. 1(a) was observed. The mol. wt. of these proteins were determined by co-electrophoresis with marker proteins, and by re-electrophoresis under reducing conditions they were identified as (i) the dimer of HN₀, (ii) the dimer of HN which
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Fig. 1. Analysis of the cleaved and uncleaved glycoproteins by PAGE under reducing and non-reducing conditions. The NDV strain Ulster was grown in MDBK cells, in the absence or presence of trypsin labelled with $^3$H-glucosamine and purified as described before (Nagai et al. 1976a). The viral glycoproteins were separated from the other viral proteins (Scheid & Choppin, 1973) and subjected to PAGE (Laemmli, 1970) in the absence of mercaptoethanol. Column gels containing 10% acrylamide and 0.27% bisacrylamide were used. Glycoprotein bands eluted from these gels were subjected to re-electrophoresis in the presence of mercaptoethanol on gels containing 15% acrylamide, 0.4% bisacrylamide and 8 M-urea. Marker proteins for determination of mol. wt. were γ-globulin (153 × 10^3), RNA-polymerase subunits (165 × 10^3; 155 × 10^3; 39 × 10^3), phosphorylase a (96 × 10^3), catalase (60 × 10^3), and cytochrome c (12 × 10^3). (a) Electrophoresis of uncleaved glycoproteins under non-reducing conditions. HN, 150K; F0, 115K; F0, 65K. (b) Electrophoresis of cleaved glycoproteins under non-reducing conditions. HN, 126K; F1, 2, 110K; F0, 65K. (c) Re-electrophoresis of the HN dimer (126K) of (b) under reducing conditions. (d) Re-electrophoresis of the F1,2 monomer (65K) of (b) under reducing conditions.

was always present in small amounts in virus grown in the absence of trypsin (Nagai & Klenk, 1977), (iii) the dimer of F0 and (iv) the monomer of F0. The glycoproteins obtained from virus grown in the presence of trypsin were analysed by the same procedure (Fig. 1b) and identified as (i) the dimer of HN, (ii) the dimer of the F1,2 complex and (iii) the monomer of F1,2. Comparison of the unreduced forms of the uncleaved and of the cleaved glycoproteins allows several conclusions. The observations that the monomers of F0 and F1,2 always exceeded the respective dimers and that the amount of dimers varied depending on the glycoprotein preparation, suggest that the dimers are artefacts resulting from oxidation of the free SH-groups of monomers. In contrast, HN is present only in the dimeric form and it has been suggested before that this is the natural configuration of this glycoprotein in the native spike (Ozawa et al. 1976). By the same line of evidence the data presented here suggest that HN0 is also present in a dimeric form in the spikes. The observation that the HN dimers obtained from trypsin-treated and from untreated virus differ in their apparent
mol. wt. by 7000 is difficult to explain, whereas the monomers analysed under reducing conditions are indistinguishable (Nagai et al. 1976a). It is conceivable that there are conformational differences between in vivo and in vitro cleaved HN which affect the electrophoretic mobility of the protein only when it is present in a non-reduced state. It should also be pointed out that mol. wt. determinations under non-reducing conditions allow only rough estimates. This may explain the observation that the dimers never have exactly twice the mol. wt. of the respective monomers. Nevertheless, the data shown in Fig. 1(a) demonstrate that there is a distinct difference in mol. wt. of about $17 \times 10^3$ between the dimeric forms of HN$_0$ and HN which agrees fairly well with a difference of about 8000 mol. wt. between the respective monomers obtained under reducing conditions (Nagai et al. 1976a).

In contrast, the F$_{1,2}$ complex, at least in the monomeric form, is indistinguishable in mol. wt. from the precursor F$_0$. These observations suggest that there is no small cleavage fragment linked by disulphide bonds to HN. This concept was confirmed when the unreduced proteins were examined by re-electrophoresis under reducing conditions. The HN dimer yields exclusively the HN monomer (Fig. 1c), whereas, by the same procedure, the monomeric and the dimeric forms of the F$_{1,2}$ complex are split into the large fragment F$_1$ and the small fragment F$_2$ (Fig. 1d).

Since in the experiment described in Fig. 1 only glycosylated proteins are detected, we have carried out the same type of analysis with $^3$H-amino acids. The gel patterns obtained under reducing and non-reducing conditions corresponded to those shown in Fig. 1 (data not shown). Thus, the possibility can be ruled out that an unglycosylated peptide is linked to HN by disulphide bonds.

The possibility still existed that the small fragment might be present in the biologically active haemagglutinin–neuraminidase spike as an essential constituent, yet in non-covalent linkage. We have, therefore, isolated intact spikes from virions of strain Ulster grown in the presence of trypsin and of strain Italien, both labelled with $^3$H-amino acids. The isolation procedure involved detergent solubilization and separation by isoelectric focusing (T. Kohama and H.-D. Klenk, unpublished data). Again, only HN could be detected in the haemagglutinin–neuraminidase spike by gel electrophoresis under reducing conditions, whereas F$_1$ and F$_2$ were present in the other type of spike (data not shown).

Proteolytic activation of HN$_0$ can be accomplished by a variety of proteases with substrate specificities different from trypsin. Although HN obtained by cleavage with these enzymes is indistinguishable by mol. wt. from HN obtained by trypsin cleavage, it has to be concluded that different peptide bonds are cleaved (Nagai & Klenk, 1977). It was, therefore, of interest to find out whether a peptide is also lost after cleavage of HN$_0$ with non-trypsin-like enzymes. Since F$_0$ is not susceptible to these enzymes (Nagai & Klenk, 1977), all cleavage fragments generated under these conditions on the surface of the virion should be derived from HN$_0$. It was, therefore, possible in this experiment to analyse directly intact virions which had not undergone any detergent treatment before gel electrophoresis. Fig. 2 shows that cleavage with chymotrypsin, thermolysin and elastase yielded only HN and that a small fragment of HN comparable in size to F$_2$ was not generated by these enzymes.

The results presented here provide additional support for the concept that proteolytic activation of the haemagglutinin–neuraminidase glycoprotein of NDV differs in several respects from that of the paramyxovirus fusion protein and the influenza virus haemagglutinin. Previously, it has been shown that activation of the latter two glycoproteins specifically requires cleavage by trypsin (Klenk et al. 1975; Lazarowitz & Choppin, 1975; Scheid & Choppin, 1976), whereas the former protein is activated by proteases of various specificities (Nagai & Klenk, 1977). It is also well-established that cleavage of F$_0$ and of the influenza haemagglutinin precursor yields two fragments which, linked by disulphide bonds, are both constituents of the spike (Compans & Klenk, 1979). In contrast, we show here
Fig. 2. Cleavage of viral glycoproteins with various proteases. Purified virions of strain Ulster grown in MDBK cells and labelled with $^3$H-glucosamine were subjected to in vitro incubation with various proteases (Nagai & Klenk, 1977). The proteins were analysed by electrophoresis in slab gels containing a 10 to 15% acrylamide gradient with an acrylamide–bisacrylamide ratio of 37.5:1. Glycoprotein bands were visualized by fluorography (Ronner & Laskey, 1974). 1, Control virus, no protease; 2, TPCK-trypsin treatment; 3, chymotrypsin treatment; 4, thermolysin treatment; 5, elastase treatment. The following marker proteins have been used: alcohol dehydrogenase (35K), ferritin (24K), myoglobin (17K), cytochrome c (12.5K), aprotinin (6.5K) and insulin B chain (3.4K).

that a glycopeptide with an apparent mol. wt. of about 8000 is eliminated when $\text{HN}_0$ is converted to HN. Since HN is incorporated into the lipid layer by its carboxy-terminus (Scheid et al. 1978), it appears that the glycopeptide is removed from the amino-terminal end of $\text{HN}_0$.

When this study was initiated, it was hoped that a small cleavage fragment might perhaps occur as a constituent of the mature haemagglutinin–neuraminidase spike, not only with strain Ulster but also with strain Italien, thus indicating that HN of Italien is also derived from $\text{HN}_0$. The failure to detect such a fragment with either strain leaves the question open as to whether synthesis of $\text{HN}_0$ is a general phenomenon with all paramyxoviruses.
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REFERENCES


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