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Extracellular truncated influenza virus nucleoprotein

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Abstract

In the culture medium of MDCK cells infected with influenza A/Duck/Ukraine/1/63(H3N8) virus two kinds of virus nucleoprotein (NP) are detected: full-length 56 kDa NP and truncated 53 kDa NP. However, in infected cells 53 kDa NP may be detected only at short pulse and after 10 min chase it becomes nondetectable. The extracellular truncated 53 kDa NP is detected in free RNP, and not in the virions. Both extracellular free 53 and 56 kDa NP in the virions are completely oligomerized. Several data argue against the possibility of extracellular 53 kDa NP formation being a result of extracellular 56 kDa NP proteolytic degradation. Thus, the accumulation of extracellular 53 kDa NP takes place only in the course of infection, and the amount of 53 kDa NP is not increased during prolonged storage of cell-free culture medium at + 37°C. Moreover, all extracellular 56 kDa NP of A/Duck/Ukraine/1/63 influenza virus is present in the oligomeric form, and the latter, in contrast to the mononeric form, is highly resistant to proteases. The possibility is discussed that in the course of A/Duck/Ukraine/1/63 (H3N8) influenza virus infection a fraction of the synthesized 56 kDa monomeric NP undergoes the proteolytic cleavage in the infected cells before oligomerization and forms the 53 kDa NP. This 53 kDa NP is then oligomerized, enters the RNP and is quickly secreted from the cells.

Keywords: Influenza virus; Nucleoprotein; Secreted proteins

The published data indicate that the intracellular influenza virus nucleoprotein (NP) in infected cells is heterogeneous. Thus, Zhirnov and Bukrinskaya (1981, 1984) reported that the intracellular proteolytic processes cause the appearance of cell-associated 53 kDa NP. This truncated NP, in accordance with the data of these authors, is accumulated only in the cells infected with human but not with nonhuman influenza virus strains. Other authors (Stitz et al., 1990; Becht and Weiss, 1991) observed that cleaved cell-associated NP molecules are detected also in the cells infected with avian A/FPV/Rostock influenza virus. These cleaved NP-molecules are represented by 42 kDa NP and also by different NP domains and NP fragments exposed at the cell surface. The subsequent fate of the restricted NP domains exposed at the cell surface and the possibility of their extracellular secretion have not been studied.

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It has been known for a long time that the culture medium and allantoic fluid contain extravirion free NP antigenic determinants named complement fixation antigen (Henle and Wiener, 1944). Later studies using anti-NP mAbs confirmed the presence of free NP-determinants not only in the culture medium and allantoic fluid, but also in the nasal secretions of infected animals (Flawith, 1978; Yewdell et al., 1981; Townsend et al., 1986; Cook et al., 1988; Siebinga and de Boer, 1988).

It was known that the truncation of the hydrophobic region of viral *trans*-membrane glycoproteins may result in a soluble secreted protein (Doms et al., 1993). However a fraction of influenza virus NP is cell-membrane associated and behaves as membrane protein (Stitz et al., 1990; Becht and Weiss, 1991). Since the fragments of the influenza virus NP are detected at the cell membrane surface (Stitz et al., 1990; Wraith and Askonas, 1985), one may suggest that a fraction of such NP molecules may be secreted.

We have reported previously that in the course of human and avian influenza virus infection the immunoreactive NP antigenic determinants nonassociated with virions are detected not only at the cell surface but also in the culture medium (Prokudina and Semenova, 1991). In this report we have detected an extracellular truncated form of influenza virus NP and studied some of its properties.

A/Duck/Ukraine/1/63(H3N8) influenza virus and continuous cell line of canine kidney cells MDCK were used in this study. First of all we tried to detect the structures containing the free antigenic determinants of NP in the culture medium. For this the culture medium of the infected MDCK cells was collected after 18 h p.i. and analysed in 15–30% sucrose gradient and in equilibrium CsCL gradient. The fractions of both gradients were analysed by RIA. 96-well plates were coated with rabbit anti-NP IgG, and the material from each fraction was introduced in the wells without the addition of detergent. The immune complexes were detected with pooled 125-I anti-NP Mabs that was kindly provided by Dr R.G. Webster and Dr L. Stitz. It is seen from Fig. 1A that without previous treatment of fractions with detergent the immunoreactive NP is detected in the sucrose gradient in the virion-containing fractions (fraction 13) and in the upper part of the gradient (fraction 3). In CsCl gradient (Fig. 1B) the immunoreactive NP is detected in fractions corresponding to the position of virions ($P = 1.24$ g/ml) and in the fractions corresponding to the position of RNP ($P = 1.31$ g/ml). The treatment of the fractions with 0.01% Tween before RIA markedly increased the amount of NP detected by the RIA in virus fractions and did not influence the amount of NP detected in the upper fractions of the sucrose gradient and in the RNP fractions of CsCl gradient (not shown). The data presented suggests that free NP antigenic determinants in the culture medium are located in virus RNP.

We have shown previously that at the surface of cells infected with some human and avian influenza viruses and in the virus free culture medium the NP antigenic determinants can be detected by radioimmunoassay (Prokudina and Semenova, 1991). In cells infected with A/Duck/Ukraine/1/63 influenza virus after long-term labelling only 56 kDa NP is detected (Semenova et al., 1999). However at 5 min pulse labelling of the infected cells with [^{35}S] methionine (50 μCi/ml) the cleaved 53 kDa NP is sometimes detected in PAGE. In Fig. 2A the example of such result obtained after 6 h p.i. is presented. It is seen from Fig. 2A that 53 kDa NP detected at 5 min labelling (lane 1) disappears after 10 min chase (lane 3).

Free NP in the culture medium of the infected cells has been studied by PAGE. For this MDCK cells have been infected with A/Duck/Ukraine/1/63 influenza virus and labelled with [^{35}S] methionine from 4 to 18 h p.i. After 18 h the culture medium has been clarified and centrifuged at 70000*g*. The pellets and supernatants have been precipitated with acetone and analysed in PAGE. It is seen in Fig. 2B (lane 3) that only 56 kDa NP is detected in the sedimentable virion pellet. In the initial culture medium analysed before high speed centrifugation (lane 1) in addition to the predominant 56 kDa NP, the band of 53 kDa is seen (the identification of 53 kDA band as A/Duck/Ukraine NP will be presented below at Fig. 3 in the description of the results of radioimmuno-

sorbtion). It is also seen in Fig. 2B that in the supernatant obtained after the removal of virus by centrifugation (lane 2) the 53 kDa NP becomes predominant.

To exclude the possibility that the detected cultural free 53 kDa NPs are a result of the extracellular 56 kDa NP proteolysis, a part of the initial noncentrifuged culture medium has been additionally incubated at +37°C for 72 h and then centrifuged to remove the virus. It is seen in Fig. 2B (lane 4) that after such additional incubation at +37°C the amount of the free extracellular 53 kDa NPs in the supernatant is the same as before the incubation (compare lane 4 and lane 2). At the same time the amount of the extracellular free 56 kDa NPs has somewhat increased, probably because of a partial destruction of the extracellular virus. The kinetics of accumulation of 53 kDa NP in the culture medium is correlated with the accumulation of the mature virions (not shown). Thus the appearance of the free extracellular cleaved 53 kDa NP in the culture medium is rather the result of the secretion from the cells than the result of extracellular proteolysis.

In further experiments the culture medium of the infected MDCK cells (18 h p.i.) has been centrifuged in equilibrium CsCL gradient, and the fractions corresponding to the positions of virions ($P = 1.24$ g/ml) and RNP ($P = 1.31$ g/ml) were analysed by PAGE. As a result of these experiments, only 56 kDa NP was detected in the virion's fractions. RNP fractions contained about 2/3 of 53 kDa NP and about 1/3 of 56 kDa NP (not shown). These data suggest that 53 kDa NP is associated with free RNP.

It was previously shown that 56 kDa NP of influenza virus is oligomerized (Prokudina-Kantorovich and Semenova, 1996; Elton et al., 1999). Therefore it was interesting to study the ability to form such boiling sensitive oligomers by extracellular truncated 53 kDa NP. For this the culture medium of the infected MDCK cells has been collected after 18 h p.i. and precipitated with acetone. The precipitates have been dissolved in

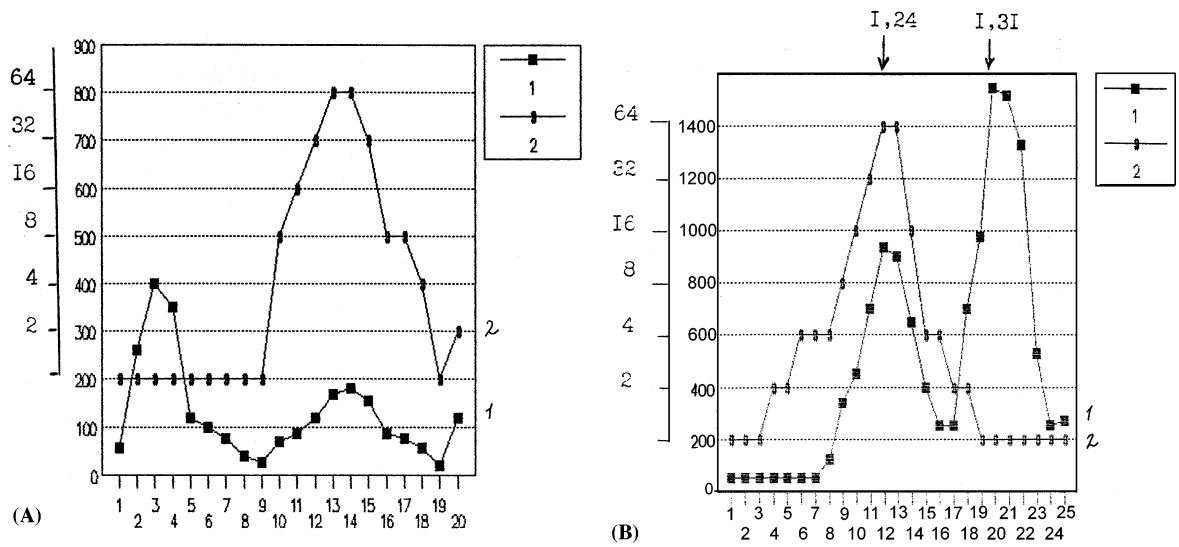

Fig. 1. The distribution of the immunoreactive NP from the culture medium in the velocity sucrose gradient (A) and equilibrium CsCL gradient (B). (A) The culture medium of the MDCK cells infected with A/Duck/Ukraine/1/63 influenza virus was collected after 18 h p.i. About 1 ml of the culture medium was placed on top of the preformed 15–30% sucrose gradient and centrifuged in SW-41 rotor at 24000 rpm for 1 h. Fractions of 0.5 ml were collected and analysed in RIA. (B) Culture medium (5 ml) was mixed with dry CsCL to $P = 1.29$ g/ml and centrifuged in SW-50 rotor at 24000 rpm for 24 h. Fractions of 0.2 ml were collected and analysed in RIA. For RIA 96-well plates were coated with rabbit anti-NP IgG, and the material from each fraction was introduced in the wells without the treatment with detergent. The immune complexes were detected with 125-I Mabs. Abcissa-fraction number, Ordinate 1-Haemagglutinin titres, Ordinate 2-cpm. Indications: 1-immunireactive NP; 2-HA titres.

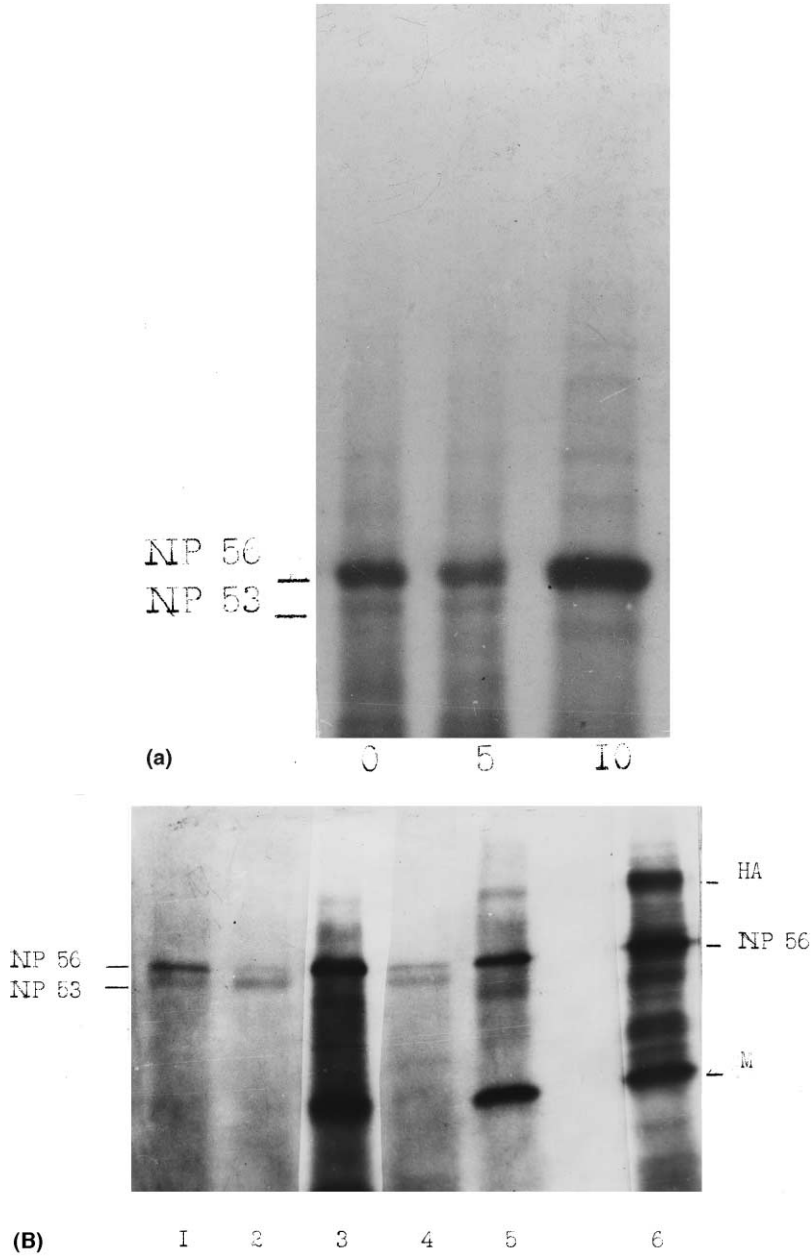

Fig. 2. (*Continued*)

RIPA buffer and incubated with pooled anti-NP Mabs The immune complexes were adsorbed on Protein-A Sepharose, dissolved in Laemmli sample buffer (Laemmli, 1970) and divided into two parts. One of them has been boiled for 3 min before PAGE and another was not.

It can be seen in Fig. 3 that the 56 kDa NP of Duck/Ukraine/1/63 influenza virus in cell lysate (lanes 1,2) and in virions (lanes 7,8) is completely oligomerized. After boiling the NP-oligomers from cell lysate (lanes 1,2) and virions (lanes 7,8) dissociate and form a single band of monomeric

56 kDa NP. In contrast to intracellular and virion NP, the NP oligomers from the initial total culture medium (lanes 3,4) are dissociated in two types of monomeric NP with the molecular weight 56 and 53 kDA. The NP oligomers from virus-free supernatant (lanes 5,6) are dissociated predominantly in 53 kDa monomeric NP. The possible difference in the mobility in PAGE of the oligomers of 56 and 53 kDA NP probably cannot be revealed at the top of the gel at the used conditions of PAGE. The data obtained suggest that the cleaved 53 kDa NP is oligomerized in a way similar to the native NP molecules. It was shown earlier that in contrast to NP monomers the 56 kDa NP oligomers are highly resistant to protease (Prokudina and Semenova, 1991; Prokudina-Kantorovich and Semenova, 1996; Semenova et al., 1999). The treatment of the culture medium with the commercial protease carried out in this study has not increased the amount of the 53 kDa NP (not shown).

It was described earlier that the accumulation of the cell- associated cleaved NP occurs in the cells infected with human influenza viruses, and no such accumulation of the cleaved NP takes place in the avian influenza virus-infected cells (Zhirnov and Bukrinskaya, 1984). In this report we also did not detect the accumulation of cleaved NP in cells infected with avian A/Duck/Ukraine/1/63 influenza virus. The 53 kDa NP was detected in infected cells irregularly after a short pulse and disappeared from the cells after 10 min chase. Accumulation of 53 kDa NP was detected in the culture medium.

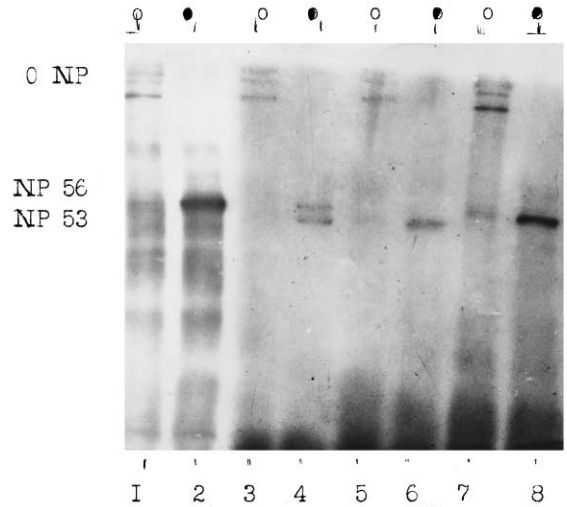

Fig. 3. The boiling-sensitive oligomeric forms of the A/Duck/Ukraine (H3N8) extracellular 53 kDa cleaved NPs. MDCK cells were infected with A/Duck/Ukraine virus and labelled with [^{35}S] methionine from 4 to 18 h. The culture medium was centrifuged at 70000*g* for 1 h. The initial culture medium, supernatant and pellet were precipitated with acetone. The precipitates were dissolved in a RIPA buffer and incubated with pooled anti-NP MAbs. The immune complexes were adsorbed on Protein-A Sepharose. After RIPA the samples were dissolved in Laemmli sample buffer, divided into boiled and unboiled parts and analysed in PAGE. 1,2-Lysate of the infected cells; 3,4-Initial culture medium before centrifugation; 5,6-Supernatant; and 7,8-virus pellet after centrifugation at 70000*g*. Indications: o-NP-oligomeric NP. ○ unboiled; ● boiled.

Several data argue against the possibility of extracellular 53 kDa NP formation being a result of extracellular 56 kDa NP proteolytic degradation. Thus, the accumulation of extracellular 53

Fig. 2. The cleaved 53 kDa NP in A/Duck/Ukraine/63 (H3N8) virus infection. (A) The detection of intraclellular 53 kDa NP by pulse-chase analysis. MDCK cells were infected with A/Duck/Ukraine/1/63 influenza virus and at 6 h p.i were pulse-labelled for 5 min with [^{35}S] methionine (50 μCi/ml). The labelled cultures were then washed, covered with labelled-free medium and incubated at indicated periods (min). The cell lysate was preliminarily incubated with Protein-A-Sepharose to decrease the background, and the supernatant was then incubated with pooled anti-NP MAbs. The immune complexes were adsorbed on Protein-A Sepharose. After radioimmunoprecipitation (RIPA) the samples were dissolved in Laemmli sample buffer and analysed in PAGE. (B) The extracellular 53 kDa NP. MDCK cells were infected with A/Duck/Ukraine (H3N8) influenza virus and labelled with [^{35}S] methionine from 4 to 18 h p.i. The culture medium was clarified with low speed centrifugation and divided into two portions. One of them was centrifuged at 70000*g* immediately after collecting (2,3) and the other after additional incubation for 72 h at 37°C (4,5). All samples were precipitated with acetone before PAGE. The samples analysed immediately after collection: 1-initial culture medium before high-speed centrifugation. 2-Supernatant after centrifugation at 70000*g*. 3-The pellet after centrifugation at 70000*g*. The samples analysed after additional incubation of the culture medium at + 37°C for 72 h. 4-Supernatant after centrifugation at 70000*g*. 5-The corresponding pellet. 6-Lysate of the infected cells labelled from 4 to 18 h p.i.

kDa NP takes place only in the course of infection, and the amount of 53 kDa NP is not increased during prolonged storage of cell-free culture medium at +37°C (Fig. 2B). Moreover, all extracellular 56 kDa NP of A/Duck/Ukraine/1/63 influenza virus is present in the oligomeric form, and the latter, in contrast to the mononeric form, is highly resistant to proteases (Semenova et al., 1999).

The extracellular truncated 53 kDa NP is located in free RNP together with full-length 56 kDa NP, and 53 kDa NP is not located in virions. However it is difficult to exclude the extracellular presence of both types of NP as a free molecules. All extracellular NP are completely oligomerized.

The biochemical pathways of 53 kDa NP production and extracellular secretion is not completely clear. Zhirnov et al. (1999) have shown the absence of the caspase-dependent site in NP of avian strains of influenza virus. Therefore, most likely other types of intracellular proteases that take part in the cleavage of A/Duck/Ukraine/1/63 influenza virus NP are described here. Moreover the production of 53 kDa NP is detected in infected cells at the early stages of infection (6 h p.i.) before apoptosis (Fig. 2A). These results probably preclude the possibility that 53 kDa NP of A/Duck/ Ukraine/1/63 influenza virus arises from cell lysis associated with apoptosis. Some data indicated that the patches of NP are associated with the membranes of influenza virus infected cells (Stitz et al., 1990; Virelizier et al., 1977). It may be suggested that some structural or conformation changes may increase this NP patches hydrophylicity and lead to its secretion.

Intracellular cleavage takes place probably before 56 kDa NP oligomerization, since only monomeric NP is sensitive to proteases (Semenova et al., 1999). The formed 53 kDa NP is then oligomerized, enters the RNP, and is quickly secreted from the cells.

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