Intracellular Oligomerization of Influenza Virus Nucleoprotein

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It has previously been shown that the purified influenza virus nucleoprotein (NP) forms the oligomers in vitro in NP preparations obtained from virions (Wiley et al., 1977, Virology, 79, 446-448; Ruigrok and Baudin, 1995, J. Gen. Virol., 76, 1009-1014) and infected cells (Becht and Weiss, 1991, Behring Inst. Mitt., Justus-Liebig Universitat, Giessen, 89, 1-11). We have shown in this report that boiling-sensitive NP oligomers (di- and trimers) are formed in vivo in the course of intracellular influenza virus replication. They are detected by PAGE about 10 min after monomeric 56-kDa NP molecules are synthesized. NP oligomers are formed by different strains of influenza virus in different cell lines. Some influenza virus strains are characterized by complete conversion of NP monomers into oligomers and others by only partial conversion. In the Triton X-114 phase partitioning system NP oligomers show more hydrophobicity than NP monomers. NP oligomers are detected in the sedimentable and soluble fractions of both cell lysate and extracellular medium. The possibility is discussed that oligomeric NP is a native and functionally significant form of influenza virus NP.

INTRODUCTION

Influenza virus nucleoprotein (NP) attracts the attention of virologists because it is intimately connected with the virus genome and affects its structure and function. In comparison with glycoproteins NP is antigenically conservative (van Wyke et al., 1980). Some interesting properties of this protein were reported recently. Specifically, it has been shown that in addition to 56-kDa NP, the form of NP with the smaller molecular weight appears in infected cells (Becht and Weiss, 1991; Zhimov and Bukrinskaya, 1984).

It is also known that influenza virus NP in the course of infection can be associated with the cell membranes, exposed at the cell surface, and secreted in extracellular medium (Yewdell et al., 1981; Cook et al., 1988; Stitz et al., 1990; Prokudina and Semenova, 1991). These data suggest the possibility that in vivo in the course of infection influenza virus NP can be modified from the hydrophylic form to a hydrophobic form. One of the means of such modifications may be oligomerization.

There are some publications about in vivo oligomerization of different viruses membrane proteins (Gething et al., 1986; Doms et al., 1993; Oomens et al., 1995) and flavivirus NS 1 protein (Winkler et al., 1988, 1989; Flaman et al., 1992).

As for influenza virus NP it has been shown that the purified NP forms in vitro the oligomers in NP preparations obtained from virions and infected cells. Specifically in the presence of crosslinking agents influenza virus internal protein was detected in purified virus cores in di- and trimeric forms (Wiley et al., 1977). Recently Ruigrok and Baudin (1995) showed by electron microscopy that purified influenza virus NP forms in vitro self-oligomers. The abundance of different kinds of boiling-sensitive NP oligomers was detected in purified NP preparations from infected cells (Becht and Weiss, 1991).

It was tempting to suppose that the NP is oligomerized in vivo in the course of influenza virus infection and as a result of oligomerization NP acquires the hydrophobic properties.

The present report is dedicated to the detection of the intracellular in vivo formation of influenza virus NP oligomers and the investigation of some of their properties.

MATERIALS AND METHODS

Cells and viruses

Primary chick embryo fibroblasts (CEF) and the continuous cell lines canine kidney cells (MDCK), human cells (HeLa), and porcine kidney cells (SPEV) were used throughout the study.

Most of the experiments were carried out using human influenza virus: A/WSN/33 (H1N1). In some experiments the following other strains of influenza viruses kindly provided by Professor N. V. Kaverin were used; human strains: A/FM/1/47 (H1N1), A/Singapore/1/57 (H2N2), A/Flamand/1/1977 (H3N3), A/FPV/Weybridge (H7N7); and nonhuman mammalian strain Seal/Massachusetts/1/80/ (H7N7). All the viruses used were propagated in the embryonated chicken eggs.

Analysis of [35S]methionine-labeled proteins in influenza virus-infected cells

The cells were infected with influenza viruses at 10 PFU/cell. After the appropriate periods of time the cells
were labeled with $^{35}$S-methionine (50 μCi/ml). The cells were then dissociated in Laemmli sample buffer (Laemmli, 1970). Each sample was divided before PAGE into two equal portions, one of which was boiled for 3 min and the other was not. The analysis was carried out by PAGE with a 10% acrylamide concentration.

The separation of the cell lysate and culture medium into sedimentable and soluble fractions

The influenza virus-infected cells were mechanically disrupted with a Dounce homogenizer and the homogenate was centrifuged at 10,000 rpm for 1 hr. The pellet and supernatant of the homogenate were lysed with buffer containing 0.5% Triton X-100 and then precipitated with acetone (Winkler et al., 1988).

The culture medium was clarified and then centrifuged at 75000 g for 2 hr. The lysed high-speed pellet and the supernatant from the culture medium were precipitated with acetone.

All acetone precipitates from cell lysate and culture medium were dissolved in Laemmli sample buffer, divided into boiled and unboiled portions, and analyzed in PAGE with a 10% acrylamide concentration.

Radioimmunoprecipitation (RIPA)

The cell lysate from infected and labeled cells was preliminarily incubated with Protein-A-Sepharose to decrease the background, and the supernatant was then incubated with pooled anti-NP MAbs, which were kindly provided by Dr. R. G. Webster (St. Jude Children’s Research Hospital, Memphis, TN) and Dr. H. Becht (Justus-Liebig University, Giessen, Germany). 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 analyzed in PAGE.

Phase partitioning of NP molecules in Triton X-114

Detergent phase partitioning was performed as described previously (Flamand et al., 1992; Bordier, 1981). Briefly, influenza virus-infected MDCK cells were labeled with $^{35}$S-methionine (50 μCi/ml) for 20 min at 6 hr after infection.

Cell extracts were prepared with 2% Triton X-114 in PBS. The mixture was left on ice for 1 hr. Triton X-114 and supernatant phases were separated by heating the samples for 2 min at 37°C and centrifugation at 6500 rpm at 30°C for 5 min. The supernatant was harvested, heated, and recentrifuged before collecting the final aqueous phase. The detergent phase was washed twice and finally diluted to give the same volume as the aqueous phase. Proteins from both phases were analyzed by PAGE with a 15% acrylamide concentration.

FIG. 1. Detection of high-molecular-weight NP in A/WSN/33 influenza virus-infected MDCK cells and its identification by RIPA. MDCK cells were labeled with $^{35}$S-methionine between 4 and 18 hr after infection. Each sample was divided before PAGE into two equal parts, one of which was boiled and the other was not. A 10% acrylamide concentration was used. Lanes: 1 and 2, uninfected cell lysate; 3 and 4, cell-associated soluble material; 5 and 6, cell-associated pellet; 7 and 8, extracellular soluble material after centrifugation at 75000 g; 9 and 10, extracellular 75000 g pellet; 11 and 12, RIPA of nonfractionated cell lysate. The positions of marker proteins are indicated on the left. Unboiled (C); boiled (Φ).

RESULTS

Detection and identification of NP oligomers in A/WSN/33 influenza virus-infected cells

MDCK cells were infected with A/WSN/33 influenza virus and labeled with $^{35}$S-methionine (50 μCi/ml) from 4 to 18 hr after infection. The separating of the cell lysates and culture medium into sedimentable and soluble fractions was carried out as described under Materials and Methods. Each sample was divided before PAGE into two equal portions, one of which was boiled and the other was not.

It is seen from Fig. 1 that all A/WSN/33-infected unboiled samples in addition to 56-kDa NP contain two clear high-molecular-weight bands: about 100-kDa “I” and about 150-kDa “II.” After boiling, both high-molecular-weight proteins disappear.

For identification of the nature of the boiling-sensitive high-molecular-weight proteins RIPA with anti-NP MAbs was carried out. The specificity of these MAbs was studied earlier (van Wyke et al., 1980; Becht and Weiss, 1991; Prokudina and Semenova, 1991). It is seen from Fig. 1 (lanes 11, 12) that the high-molecular-weight proteins react with anti-NP MAbs. The autoradiograms without the intentional overexposure (Fig. 1, lanes 11 and 12; Figs. 2, 3, 4, and 5) reveal that after boiling, simultaneously with the disappearance of high-molecular-weight bands the radioactivity in the position of 56-kDa NP is correspondingly increased.

These data suggest that high-molecular-weight pro-
teins dissociate after boiling into monomeric 56-kDa NP. The use of the anti-HA MAb did not reveal the above-described high-molecular-weight proteins (not shown). The molecular weights, the characteristic of dissociation, and the ability to bind anti-NP MAb suggest that high-molecular-weight proteins are NP oligomers: di- and trimers.

It is also seen from Fig. 1 that NP oligomers are present in both cell-associated soluble (lane 3) and sedimentable (lane 5) cell materials. The same situation is seen from the distribution in SDS – PAGE of the extracellular medium: the supernatant (lane 7) and pellet (lane 9) after high-speed (75000 g) centrifugation contains both forms of NP oligomers in addition to monomeric NP. The high-speed pellet of extracellular medium of A/WSN/33-infected cells was layered on a discontinuous 15–60% sucrose gradient and centrifuged at 75000 g for 2 hr. The viral fraction obtained contained two NP oligomers as well as the initial unpurified material (not shown).

The data presented suggest that in vivo in the course of A/WSN/33 virus infection part of intracellular viral NP is oligomerized; NP oligomers are included into virions and part of them is secreted into the culture medium together with monomeric NP. The same NP oligomers were detected in other cell lines infected with A/WSN/33 influenza virus: MDCK cells, HeLa, and SPEV (not shown).

Analysis of hydrophobic and hydrophilic properties of monomeric and oligomeric NP

It was previously shown that influenza virus NP is able to expose on the cell surface, which contradicts the idea that NP is a typical hydrophilic protein (Yewdell et al.,...
1981; Cook et al., 1988; Stitz et al., 1990; Prokudina and Semenova 1991). At the same time it is known that oligomerization is a kind of modification which causes an increase in the hydrophobic properties of proteins (Get-thing et al., 1986; Winkler et al., 1988; Flamand et al., 1992; Doms et al., 1993; Oomens et al., 1995). Thus, it was interesting to study the hydrophobicity of the oligomeric influenza virus NP in comparison with that of the monomeric one.

It is known that in a two-phase system consisting of Triton X-114 and aqueous buffer the hydrophobic proteins are extracted into the detergent phase and hydrophobic ones into the aqueous phase (Bordier, 1981). To analyze the behavior of oligomeric forms of influenza virus NP in a solution of Triton X-114, MDCK infected with A/WSN/33 were labeled for 20 min with $[^{35}S]$-methionine at 6 hr p.i. The results of partitioning experiments which were carried out as described under Materials and Methods are presented in Fig. 2. It is seen from Fig. 2 that approximately similar quantities of NP oligomers are extracted in the aqueous phase (lane 1) and the detergent phase (lane 3). In contrast to oligomers, NP monomers are almost quantitatively extracted into the aqueous phase (lane 1) together with HA and the great majority of cellular proteins, indicating that all these proteins are hydrophobic and water soluble. The data presented suggest that NP oligomers are more hydrophobic than NP monomers. One may suppose that the hydrophobicity allows the oligomeric fraction of NP to bind with membranes and expose on the cell surface. In special experiments it was shown that in comparison with NP monomers the oligomers are markedly more resistant to protease (not shown).

Formation of NP oligomers by different strains of influenza viruses in different cells

MDCK cells were infected with different strains of influenza viruses and labeled from 4 to 10 hr p.i. with $[^{35}S]$-methionine (50 $\mu$Ci/ml). It is seen from Fig. 3 that A/WSN/33 (H1N1), A/USSR/90/77 (H1N1), A/FM/1/47 (H1N1), A/Aichi/2/ (H3N8), and A/Duck/Ukraine/63 (H3N8) influenza viruses form similar boiling-sensitive NP oligomers. The same oligomers are detected with other influenza virus strains: A/Singapore/1/57 (H2N2), A/FPV/Weybridge (H7N7), and Seal/Massachusetts/1/80 (not shown).

Interestingly, different influenza virus strains are characterized by different efficiencies of NP oligomerization. Specifically, A/WSN/33 reveals only partial oligomerization of 56-kDa NP (Fig. 3, lanes 1, 2). These data are confirmed by the results presented above in Figs. 1 and 2 and by the results obtained in pulse-chase kinetics analysis (not shown). In comparison with A/WSN/33 the other influenza virus strains presented in Fig. 3: [A/USSR/90/77 (H1N1) (lanes 3, 4), A/FM/1/47 (H1N1) (lanes 5, 6), A/Aichi/2/ (H3N8) (lanes 7, 8)] reveal a higher efficiency of oligomerization (Fig. 3 lanes 3–8). In contrast to all these viruses A/Duck/Ukraine/63 (Fig. 3, lanes 9, 10) reveals complete oligomerization of NP: NP oligomers only are seen in the unboiled sample (Fig. 3, lane 9), whereas NP monomers in this sample are practically absent and appear only after boiling (Fig. 3, lane 10). The same complete NP oligomerization was detected in MDCK infected with influenza virus Seal/Massachusetts/1/80 (not shown).

The comparison of different cells infected with the A/Duck/Ukraine/63 strain of influenza virus (Fig. 4) have shown that in all of the cell lines tested labeled from 4 to 18 hr p.i. there is no monomeric 56-kDa NP in unboiled samples, and irrespective of the type of cells all molecules of A/Duck/Ukraine/63 strain NP monomers are practically completely oligomerized. These data also suggest that influenza virus NP oligomerization does not depend on the type of cell line.

For the analysis of the relationships between monomeric and polymeric NP in the cells infected with strain A/Duck/Ukraine/63, pulse-chase experiments were carried out. MDCK cells were infected with A/Duck/Ukraine/63 virus and labeled with $[^{35}S]$-methionine (10 $\mu$Ci/ml) at 6 hr p.i. for 10 min. Then the cells were incubated with label-free medium. It is seen in Fig. 5 (lane 1) that a 10-min period of labeling of A/Duck/Ukraine/63 virus-infected cells reveals the presence of both monomeric and oligomeric NP in unboiled samples. As a result of the chase (lanes 3, 4, 5, and 6) the radioactivity of the 56-kDa NP is gradually decreased and the radioactivity of oligomeric NP increased. These data support the idea that monomeric NP is a primary product converted afterward into NP oligomers. In addition to this suggestion the presence of NP oligomers in the 10-min labeled sample (Fig. 5, lane 1) suggests that a 10-min period is enough for a partial conversion of NP monomers to oligomers. The data also confirm the above observations that in the case of the A/Duck/Ukraine/63 strain practically all molecules of in vivo synthesized NP monomers are converted into oligomers. Only a rather short period of labeling permits detection of the presence of A/Duck/Ukraine/63 NP monomers (Fig. 5, lane 1). The chase (Fig. 5, lanes 3, 4, 5, 6) or a long period of labeling (Fig. 3, lane 10 and Fig. 4) reveal practically complete oligomerization of the A/Duck/Ukraine/63 strain NP.

**DISCUSSION**

The data presented in our report demonstrate that in vivo in the course of infection of different strains of influenza virus, newly synthesized monomeric 56-kDa NP undergoes posttranslational oligomerization. We determined constantly only two forms of NP oligomers in in-
fected cells: about 100- and 150-kDa, which probably correspond to the dimer and trimer of 56-kDa NP. The molecular weight of NP oligomers is suggestive of homo-oligomers. This suggestion may also be confirmed by the fact that after boiling oligomers dissociate only into NP monomers without the appearance of other than 56-kDa proteins. At the same time it is difficult to exclude completely a partial complex formation of NP with some unlabeled cellular proteins. Sometimes we observed boiling-sensitive polymers with a molecular weight more than 150 kDa, which also transited to monomeric 56-kDa NP after boiling and could be a result of further NP polymerization. The indicated in vivo synthesized oligomers resemble NP oligomers obtained in vitro by Becht and Weiss (1991) in unlabeled NP preparations purified by ion-exchange chromatography and preparative isoelectric focusing from influenza virus-infected chorion allantoic membranes.

The data presented of in vivo kinetic analysis suggest that the conversion of NP monomers to oligomers happens very quickly (about 10 min) after de novo monomer synthesis.

Interestingly, different influenza virus strains are characterized by different types of NP oligomerization: some of them demonstrate only a partial oligomerization of NP monomers and others a complete one. For example, only a part of A/WSN/33 NP monomers is oligomerized, and practically all A/Duck/Ukraine/63 NP monomers undergo oligomerization. In the latter case, only the short period of labeling permits us “to catch” the intracellular presence of NP monomers. During the chase or the long-period labeling of A/Duck/Ukraine/63-infected cells, the NP monomers disappear and only NP oligomers are present. It is possible that the NP oligomerization efficiency really differs among the different influenza virus strains. On the other hand it is difficult to exclude that these differences are connected with the difference in NP oligomer stability during PAGE, which may be due to the peculiarities in NP’s primary structure.

The comparative analysis in the Triton X-114 phase partitioning system showed that NP oligomers are more hydrophobic than NP monomers. These data are consistent with the idea that as a result of translation influenza virus NP appears as a monomeric hydrophilic protein and then approximately 10 min after translation, NP undergoes partial or complete dimerization and trimerization. As a result of oligomerization NP becomes partially hydrophobic, probably because of a conformational modification. Such hydrophobic NP oligomers may acquire the ability to associate with cell membranes, and therefore it has probably been detected on the cell surface (Yewdell et al., 1981; Stitz et al., 1990; Prokudina and Semenova, 1991).

It is known that the types of the bonds stabilizing different viral protein oligomers are variable (Doms et al., 1993). For example, influenza virus M2 homotetramers (Holsinger and Lamb, 1991) are stabilized by disulfide bonds. The nature of the bonds stabilizing NP oligomers is not clear. On the one hand the resistance of NP oligomers to β-mercaptoethanol (without boiling) as shown in the present report, as well as the published data about a small number of cysteines in influenza virus NP (Winter and Fields, 1981), permit us to suggest that these bonds are not disulfide and may be noncovalent. On the other hand it is clear that these bonds are rather strong, because they are stable under the gel conditions including the presence not only of mercaptoethanol (in the sample buffer) but also of SDS. Only boiling in the presence of mercaptoethanol and SDS destabilizes NP oligomer bonds.

There are at least two reports about in vitro detection of NP oligomers in influenza virions. Rather long ago Wiley et al. (1977) published a report that in DMS-cross-linked proteins from influenza virus cores a part of internal proteins was detected as di- and trimers. Recently Ruigrok and Baudin (1995) demonstrated self-polymers (trimers, tetramers, and small rings) in influenza virus NP purified from virions. Both reports confirm the fact that influenza virus NP is able to be polymerized in vitro. It is difficult, however, to decide for the present if NP oligomers detected in vitro and in vivo are exactly the same. For example, Ruigrok and Baudin (1995) did not observe NP dimers in in vitro formed polymers; in vitro polymerization depends on the protein concentration, in vitro polymerization being much more extensive at 4° than at 37°.

In contrast to the in vitro self-polymers described by Ruigrok and Baudin, the in vivo formed NP oligomers described in the present report include dimers. In vivo oligomerization probably does not depend on concentration, because the oligomers were detected in labeled nonconcentrated materials. In vivo polymerization was much more extensive at 37° than 4° (unpublished data).

The in vivo oligomerization of NP is probably a general feature of influenza virus infection. This suggestion is based on the presented facts that NP oligomers were detected in different cells and with different strains of influenza viruses belonging to different serogroups and host species. These findings together with the facts that some viruses (for example A/Duck/Ukraine/33 and Seal/Massachusetts/1/80) contain only oligomeric NP without the monomeric one and NP oligomers are included into the virion, the data about the relative hydrophobicity of NP oligomers, and unpublished data about the relative resistance of NP oligomers to protease suggest that the NP oligomers are a functionally significant form of influenza virus NP.

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