A small percentage of influenza virus M1 protein contains zinc but zinc does not influence in vitro M1–RNA interaction

Christine Elster, Eric Fourest, Florence Baudin, Kjeld Larsen, Stephen Cusack and Rob W. H. Ruigrok

1 EMBL Grenoble Outstation, c/o ILL, BP 156, 38042 Grenoble Cedex 9 and 2 CEN-G/DBMS/TSV, BP 85X, 38041 Grenoble Cedex, France

A peptide containing the CCHH motif, the putative zinc-binding sequence of influenza virus M1 protein, was found to bind zinc in a one-to-one complex with the characteristics of a typical zinc-binding peptide. Intact influenza virus also contained zinc and we show that this zinc is bound to the M1 protein in the virus. However, only a small proportion of M1 contained zinc: 4% in virus and 6 to 9% in isolated protein. One strain, B/Yamagata/16/88, consistently contained more zinc: 15 to 20% both in virus and in isolated protein. We also determined the RNA binding and transcription inhibition activities of various M1 proteins and found that the zinc content of M1 had no influence on either activity. We suggest that the zinc in M1 has a structural role in the virion other than nucleic acid binding.

Introduction

Influenza viruses are enveloped viruses with a segmented negative strand RNA genome. Each vRNA segment is complexed with the major RNA-binding protein nucleoprotein (NP) and carries a copy of the polymerase complex. These structures, called ribonucleoprotein particles (RNPs), are independent active transcription units. The total genome consists of eight RNPs which are contained within a shell of M1 protein which lines the inside of the viral lipid bilayer. Embedded in this lipid bilayer are the viral glycoproteins which are essential for entry into and release from the host cell.

The structure of the virion suggests an important role for the M1 protein. M1 may interact with the cytoplasmic tails of the glycoproteins, the lipid membrane and the RNPs and so form the ‘glue’ between them. Interaction of M1 with lipid has been shown in intact virus using light-activated cross-linking (Gregoriades & Frangione, 1981) and also in vitro using isolated M1 and liposomes (Bucher et al., 1980). Contact between M1 and the glycoproteins has not yet been shown but interaction of M1 with RNP is suggested by the fact that purified M1, when added to transcribing RNPs, inhibits transcription (Zvonarjev & Ghendon, 1980; Ye et al., 1989). In vitro interaction of M1 with RNA has been shown with filter-binding assays and a blotting technique (Wakefield & Brownlee, 1989; Ye et al., 1989).

The primary sequence of all influenza A and B M1 proteins contains the putative zinc-binding sequence Cys–Xaa–Cys–Xaa–His–Xaa–His (CCHH motif) (residues 148 to 162 of A/PR/8/34 M1 protein; Allen et al., 1980), first remarked upon by Wakefield & Brownlee (1989), which could be involved in RNA binding. This CCHH motif is situated in a part of the M1 sequence that may be involved in transcription inhibition (Ye et al., 1989). Zinc-binding sequences have been implicated in specific protein–nucleic acid interactions and protein dimerization domains (see references in Discussion). In this paper we describe the zinc-binding activity of a 27 amino acid peptide containing the CCHH motif and have determined the zinc content of intact influenza virus and purified M1. We further show that the zinc content of M1 does not influence the in vitro RNA binding and transcription inhibition activities of M1.

Methods

Virus and M1 protein. Influenza viruses A/PR/8/34, B/Beijing/1/87 and B/Yamagata/16/88 were grown in embryonated hen's eggs and obtained in purified form from Pasteur-Mérieux, Marcy L'Etoile, France. M1 protein was isolated from spikeless virus. Spikes were removed by bromelain digestion (Brand & Skehel, 1972) which was stopped by addition of 100 mM-iodoacetamide. Spikeless virus was then purified by pelleting through 14% sucrose in PBS. The virus was then disrupted with 1% Triton X-100 in PBS and centrifuged on a 10 to 30% continuous glycerol gradient in PBS (SW41 rotor, 36000 r.p.m., 4°C, 16 h). M1 was collected from the upper fractions of the gradient and stored immediately at −20°C. The purity was judged to be higher than 90% by PAGE (Fig. 1).

Virus protein concentrations were determined by the Lowry method (Lowry et al., 1951) using BSA as a standard. The concentration of isolated M1 protein was determined with the Lowry and the Bradford
was determined using a least-square model fitting approach. The absorption spectrum of the Co\textsuperscript{2+}/peptide complex with various amounts of Co\textsuperscript{2+} is shown in Fig. 2(a). The spectrum is characteristic for tetrahedrally coordinated Co\textsuperscript{2+} with peak absorbances at 284 nm ($\varepsilon = 3680 \text{ M}^{-1} \cdot \text{cm}^{-1}$) and 620 nm ($\varepsilon = 570 \text{ M}^{-1} \cdot \text{cm}^{-1}$). When the absorbance at 284 nm is plotted against the Co\textsuperscript{2+} concentration (Fig. 2b), a linear increase in signal is observed until equimolar amounts of Co\textsuperscript{2+} and peptide are reached, suggesting a 1:1 Co\textsuperscript{2+}/peptide complex. Least-square curve-fitting suggests a $K_d$ of 0.2 \textmu M at pH 7.0. Addition of Zn\textsuperscript{2+} to the Co\textsuperscript{2+}/peptide complex leads to a decrease in the absorbance and since equimolar amounts of Zn\textsuperscript{2+} completely displace the CC\textsuperscript{2+}, the $K_d$ for Zn\textsuperscript{2+} must be considerably lower than that for Co\textsuperscript{2+}. The shape of the absorption spectrum, the molar absorbances and the $K_d$ are very similar to those of other Zn\textsuperscript{2+}-binding peptides, such as those derived from retroviral nucleic acid-binding proteins, alcohol dehydrogenase and alanin-tRNA synthetase (Green & Berg, 1989; Berg, 1993; Bergman et al., 1992; Miller et al., 1991).

Zinc content in virus and isolated M1

The zinc content of purified intact virus, spikeless virus, isolated M1 and RNP was determined by atomic absorption spectroscopy. The results (Table 1) clearly show that there is zinc in influenza virus. The zinc is not associated with purified RNP or with the spikes but was found associated with purified M1 protein. When expressed as moles of zinc per mole of M1 it becomes clear that only 4 to 6% of the M1 molecules contain a...
Fig. 2. Metal binding by peptide MP3. (a) Absorption scans of MP3 with various amounts of CoCl₂. The curves correspond to 60 μM-MP3 plus 0, 10, 20, 30, 40, 50, 60, 70, 80 and 90 μM-CoCl₂. (b) Absorbance at 284 nm of 60 μM-MP3 as a function of CoCl₂ concentration. (c) Absorbance of a 60 μM-Co²⁺/MP3 complex as a function of added concentration of ZnCl₂. The residual density after complete displacement of the cobalt by the zinc is probably due to light-scattering. The spectra showed a sloping, featureless line with none of the characteristic absorption peaks of the cobalt complex.

Table 1. Zinc content of influenza virus and isolated protein components

<table>
<thead>
<tr>
<th>Sample*</th>
<th>Zinc (μg per mg sample)†</th>
<th>Mole zinc per mole M1‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact virus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/PR/8/34</td>
<td>13.1 ± 1.0 (n = 7)</td>
<td>0.042 ± 0.003</td>
</tr>
<tr>
<td>B/Beijing/1/87</td>
<td>13.8 ± 0.7 (n = 3)</td>
<td>0.044 ± 0.002</td>
</tr>
<tr>
<td>B/Yamagata/16/88</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Batch 1</td>
<td>46.4 ± 1.3 (n = 3)</td>
<td>0.148 ± 0.004</td>
</tr>
<tr>
<td>Batch 2</td>
<td>63.5 (n = 1)</td>
<td>0.203</td>
</tr>
<tr>
<td>Spikeless virus†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/PR/8/34</td>
<td>70.5</td>
<td>0.059</td>
</tr>
<tr>
<td>B/Beijing/1/87</td>
<td>69</td>
<td>0.058</td>
</tr>
<tr>
<td>B/Yamagata/16/88</td>
<td>239</td>
<td>0.198</td>
</tr>
<tr>
<td>Isolated M1 protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/PR/8/34</td>
<td>221 ± 40 (n = 2)</td>
<td>0.092</td>
</tr>
<tr>
<td>B/Beijing/1/87</td>
<td>149 ± 11 (n = 2)</td>
<td>0.062</td>
</tr>
<tr>
<td>B/Yamagata/16/88</td>
<td>438 ± 101 (n = 2)</td>
<td>0.182</td>
</tr>
<tr>
<td>Isolated RNP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/PR/8/34</td>
<td>10 ± 18 (n = 5)§</td>
<td>–</td>
</tr>
</tbody>
</table>

* The seven determinations of intact A/PR/8 virus and three of intact B/Beijing virus were independent experiments using virus from three and two different batches respectively. The measurements on B/Yamagata virus were also done on virus from two separate batches, as indicated.
† Amounts of virus and spikeless virus are given as mg of protein.
‡ Assuming that all zinc is associated with M1 and that 13% of viral protein is M1. This amount of M1 is based on physical measurements on monodisperse, egg-grown virus (Ruigrok et al., 1984; Cusack et al., 1985). Virus contains 50% spikes, 25% lipid and 25% internal proteins (NP, M1 and polymerases) and spikeless-virus protein is assumed to be 50% M1.
§ This value is ng zinc per mg NP; this corresponds to 0.002 ions of zinc per molecule NP and to 0.15 zinc ions per RNP (assuming on average 75 NP per RNP) and consequently to 0.15 zinc ions per polymerase complex. The determined amount of Zn is at the limit of detection, hence the large relative standard deviation.

of zinc content in intact virus and purified M1 of A/PR/8 and B/Beijing virus on the one hand and B/Yamagata on the other, are strong arguments that zinc is indeed associated only with M1. We were surprised to find that B/Yamagata contained more zinc than B/Beijing and A/PR/8 (of which three different batches were tested always with the same result). We therefore tested a second batch of B/Yamagata that was produced a year later and again found a significantly higher zinc content, suggesting that this difference between B/Yamagata and the other two viruses is real.

Oxford & Perrin (1975) also detected zinc in a purified influenza virus preparation (3 ng zinc per mg viral protein) and suggested that it could be associated with the viral polymerases. However, the amount of zinc in purified RNP determined here is at or just below the reliable detection level and suggests that there cannot be more than 0.15 ions of zinc per polymerase complex (Table 1).

We tested for the presence of other divalent metal ions and found no significant amounts of copper, nickel or iron. The zinc in isolated M1 could not be removed by
RNA-binding activity of M1

The vRNA-binding activity of M1 was determined using a filter binding assay. We found that vRNA binding did not vary with the M1 zinc content (Fig. 3). RNA binding by B/Yamagata M1 was as strong as that by A/PR/8 and B/Beijing M1, although it contained four times more zinc. The average $K_d$ values for RNA binding by M1 are: 21 ± 6 nM ($n = 7$) for A/PR/8, 24 ± 5 nM ($n = 4$) for B/Beijing and 17 ± 6 nM ($n = 3$) for B/Yamagata. Wakefield & Brownlee (1989) found 50% retention of RNA at a concentration of 30 nM M1 from A/PR/8.

Dialysis of M1 against EDTA did not remove zinc (see above) and did not lead to a change in RNA binding. We also tested the RNA-binding activity of B/Beijing M1 that had been isolated and purified in the presence of 2 mM-DTT and 100 μM-ZnCl₂. In this type of M1 preparation it was found that after extensive dialysis against PBS between 40 and 100% of M1 molecules contained zinc (three experiments). Using this zinc-loaded M1 we still found a $K_d$ of 25 nM.

When RNA was incubated with the zinc-binding peptide MP3, in the presence or absence of zinc, the RNA was not retained on the filter. Gel retardation experiments also indicated no binding of this peptide to RNA (data not shown).

In vitro transcription inhibition

The transcription inhibition activities of purified M1 and peptide MP3 are shown in Table 2. As with the RNA-binding, the transcription inhibition activity of all three M1 preparations was very similar and apparently independent of the zinc content. MP3 did not inhibit transcription, either in the presence or absence of zinc. Interestingly, zinc by itself did inhibit transcription probably by poisoning the polymerase. This inhibition was overcome by adding the zinc-binding peptide, effectively removing the free zinc. This finding is a further indication that the zinc in influenza virus is associated with M1 and not with the polymerase as suggested by Oxford & Perrin (1975). We found that several divalent cations could inhibit the polymerase activity to different extents in the order ZnCl₂ > MnCl₂ > CaCl₂ > CoCl₂. RNP that had been dialysed against EDTA lost all its polymerase activity, which could be completely restored by the addition of 1.5 mM-MgCl₂ or to a lesser extent by MnCl₂ (25% restoration with 50 μM but inhibition at 1 mM).

Discussion

Generally, zinc-binding sequences in proteins are involved in specific nucleic acid recognition, protein dimerization or otherwise holding together protein loops; zinc can also have a catalytic function in enzymes (Schwabe et al., 1990; Cunningham et al., 1991; Kaptein, 1991; Vallee & Auld, 1990). We show here that influenza virus M1 protein does contain zinc but that its zinc content is not stoichiometric and does not influence its interaction with vRNA as measured by filter binding assays and in vitro transcription inhibition. The peptide containing the zinc-binding sequence did not bind to RNA or inhibit transcription. Therefore, we think that the zinc in M1 is not involved in an RNA-binding structure and must have some other function.
Retrovirus nucleocapsid proteins (NC) contain two stretches of a conserved sequence Cys–Xaa4–Cys–Xaa4–His–Xaa3–Cys which have been shown to bind zinc both in the isolated peptide and in the protein (Green & Berg, 1989, 1990). Some purified retroviruses contain zinc at a stoichiometry of two zinc ions per copy of NC (Bess et al., 1992). However, in one retrovirus, avian myeloblastosis virus, only 2% of NC molecules contain zinc (Jentoft et al., 1988), analogous to the case of the zinc in influenza virus M1.

Only a small proportion of M1 in influenza virus contains zinc, about 4% in A/PR/8 and B/Beijing and between 15 and 20% in B/Yamagata. This could equally mean that 4% (15%) of the virions have fully zinc-occupied M1 (Zn/M1) or that every virion has 4% (15%) of its M1 occupied with zinc. If zinc has an influence on the conformation of M1, one could imagine that a virus particle with 100% Zn/M1 might appear different from a particle with no Zn/M1. In fact, electron microscopy of negatively stained influenza virus has shown two separate types of virus with different polymerization forms of M1: virus with ‘fingerprints’ which are arrays of single lines of protein with units spaced 4 nm apart, and virus with ‘coils’ which are paired lines of protein with the same spacing between the lines within a pair but with a larger spacing between adjacent pairs (Ruigrok et al., 1989). We examined the A/PR/8 virus used in the present study and found that less than 0.1% of particles had coils, suggesting that the particles with coils are not the hypothetical virions with 100% Zn/M1. The two type B virus strains did not contain any particles with coils. Further, we found no morphological differences between B/Yamagata and B/Beijing virus, both showing only one type of virus having ‘fingerprints’ with similar spacings between the lines and the subunits. Therefore, we propose that, with respect to zinc content, two different types of virus particles do not exist and that every virion contains M1 both with and without zinc.

We suggest that Zn/M1 may occupy a position in the virus different from that of M1 without zinc. Electron microscopy of the fine structure of the M1 layer in the virus suggests that it is not homogeneous (Ruigrok et al., 1989). An exciting hypothesis is that the molecules of M1 that surround the few copies of M2 present in the viral membrane (Zebedee & Lamb, 1988) are different from the rest of the M1. Interaction between M1 and M2 has been suggested by Zebedee & Lamb (1989) who found that virus growth restriction by anti-M2 antibodies could be overcome by mutations in M1.

At present we can only speculate why there is relatively more of the zinc-binding form of M1 in B/Yamagata than in A/PR/8 and B/Beijing. The M1 amino acid sequences of B/Yamagata and B/Texas/88, a B/Beijing-like strain, differ by only one residue (Paul Rota, personal communication) which is not near the CCHH motif. The two type B viruses are in two separate co-circulating lineages (Rota et al., 1990) and the difference between them lies largely in the sequence of the haemagglutinin HA1 region and in the sequence of the part of RNA segment 6 that codes for both the stalk of neuraminidase and for NB, the type B virus homologue of type A virus M2 (Burmeister et al., 1993). If Zn/M1 does surround M2 (or NB), then differences in NB could possibly lead to a requirement for more Zn/M1. To test this, more sequence information on segment 6 and zinc determinations of type B strains would be needed.

The presence of two different forms of M1 in influenza virus would not be unique in the negative-strand RNA virus group. Two biochemically different kinds of vesicular stomatitis virus M protein, one membranous and one cytoplasmic, were recently found after expression of M in HeLa cells (Chong & Rose, 1993). Two different kinds of M in rabies virus also exist, one palmitoylated and one unmodified (Gaudin et al., 1991). Recently, two antigenically different types of M were found in Sendai virus (de Melo et al., 1992) and functional differences between M proteins were found in subacute sclerosing panencephalitis virus and its mesiales parental virus (Hirano et al., 1992).

We thank Dominique Nalis (EMBL, Heidelberg) for peptide synthesis, Jean Gagnon (IBS, Grenoble) for amino acid analysis, Jean Roux (CEN-Grenoble) for help with atomic absorption, Paul Rota (CDC, Atlanta) for the M1 sequences of B/Yamagata and B/Texas, Mark Krystal (Mount Sinai School of Medicine, New York) for the plasmid containing the segment 8 sequence and Reuben Leberman for advice and comments on the text.

References


(Received 14 April 1993; Accepted 1 September 1993)