Intermediates in influenza virus PR/8 haemagglutinin-induced membrane fusion

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The fusion kinetics with erythrocyte ghosts of two influenza A virus strains, A/Aichi/2/68 (X:31) and A/PR/8/34 (PR/8), were compared and correlated with the kinetics of haemagglutinin (HA) conformational change. Previously it had been shown that X:31 fuses with liposomes or erythrocytes at 4 °C, pH 5 after a lag time of 5 to 10 min whereas PR/8 displayed no fusion with liposomes at that temperature. We have confirmed the absence of cold fusion by PR/8 with erythrocyte ghosts. In contrast to X:31, PR/8 could not be committed to fuse at neutral pH and 37 °C by a preincubation at low pH and 4 °C. To examine whether the lack of commitment and cold fusion were due to a failure of PR/8 HA to undergo conformational changes at low temperature and pH, we analysed susceptibility of HA to proteinase K digestion, liposome binding to the virus, and immunoprecipitations of HA with conformation-specific antibodies. Although there was little binding of PR/8 to liposomes at 4 °C and pH 5, we did observe exposure of the fusion peptide. This study reveals a low temperature intermediate in membrane fusion exhibited by the HA of influenza virus strain PR/8, which involves low pH-induced conformational changes including exposure of the fusion peptide with little interaction of HA with the target membrane.

Influenza virus fusion is triggered by low pH-induced conformational change(s) of the viral haemagglutinin (HA) (Skehel et al., 1982). Based on reactivity to a panel of conformation-specific anti-peptide antibodies to various epitopes of the X:31 strain HA1 molecule, White & Wilson (1987) proposed a two-stage model for the low pH-induced conformational transition, which consists of a partial dissociation of the globular head domains and exposure of the fusion peptide, followed by complete dissociation of the headgroups which leads to exposure of trimer interface epitopes. The conformational change of A/Aichi/2/68 (X:31) HA has been tracked by its susceptibility to protease digestion and by attachment of the virus to zwitterionic liposomes, presumably mediated by insertion of the hydrophobic fusion peptide into the liposome membrane.

The onset of low pH-induced fusion measured by redistribution of fluorescent dyes is preceded by a time delay which is pH- and temperature-dependent (Morris et al., 1989; Sarkar et al., 1989; Stegmann et al., 1990; Kaplan et al., 1991; Clague et al., 1991). In the case of X:31, fusion of virus with liposomes at 4 °C was first demonstrated by White et al. (1982) and subsequently characterized by Stegmann et al. (1990) as a multi-step event. Using conformation-specific antibodies to X:31 HA, Stegmann et al. (1990) described a fusion-competent intermediate associated with stage I of the low pH-induced conformational transitions of HA, whereas conformational stage II of HA leads to the loss of fusion. Subsequently, Stegmann et al. (1991) showed by photochemical labelling that the 4 °C/pH 5 intermediate was associated with insertion of the fusion peptide into the target membrane prior to the actual merging of membranes. This intermediate was examined further by Schoch et al. (1992) who characterized it as a long-lived state for influenza virus-erythrocyte complexes committed to fusion at neutral pH following brief exposure to low pH and temperature. The longevity of this ‘fusion-committed’ state made it amenable to biochemical analysis. By probing its sensitivity to neuraminidase, DTT and various proteolytic enzymes, Schoch et al. (1992) showed that this fusion-committed state is mediated by HA2-lipid interactions, consistent with the photolabelling results of Stegmann et al. (1991).

In contrast to X:31, no fusion could be detected even after prolonged incubations of A/PR/8/34 (PR/8) at 4 °C and pH 5 with target membranes (Tsurudome et al., 1992). We examined the ‘cold fusion’ of the X:31 and PR/8 influenza A virus strains using erythrocytes as targets. The temperature and pH dependence of X:31 and PR/8 fusion to red blood cell (RBC) ghosts was compared by octadecylrhodamine B chloride (R18)
fluorescence dequenching as described previously (Hoekstra et al., 1984; Krumbiegel et al., 1992). R18-labelled virus bound to RBC ghosts, when added to buffer pre-adjusted for pH or temperature, displayed maximal fusion kinetics at pH 4.7 to 5.0 for both virus strains. Fusion was temperature-dependent, with optimal fusion rates and extents observed at 25 °C to 37 °C. As the incubation temperature was decreased, differences between the X:31 and PR/8 strains emerged. X:31 underwent cold fusion at 4 °C with a lag of approximately 6 min whereas PR/8 did not exhibit any fusion at 4 °C, in agreement with the literature (Stegmann et al., 1990; Tsurudome et al., 1992).

The ability of X:31 and PR/8 strains to commit to fusion was monitored as described (Schoch et al., 1992). Viruses prebound to RBC ghosts were exposed to pH 5 buffer at various temperatures to induce commitment, then added directly to neutral pH buffer at 37 °C. Fluorescence dequenching was initiated at this point. Fig. 1(a) shows that PR/8 HA did not commit to fuse after a low pH preincubation at 4 °C for 60 min or 10 °C for 2 to 15 min. Preincubation at 15 °C for 5 to 10 min or 10 °C for 30 min still did not lead to commitment, although under these conditions R18 dequenching had already begun as indicated by non-zero dequenching observed at time zero. In contrast, the X:31 virus could be committed to fuse at pH 5 to 6 and 37 °C by preincubating the virus-RBC complexes at pH 5 and 4 °C for > 2 min (Fig. 1b). The PR/8 virus was not inactivated during the preincubation since subsequent acidification of the buffer resulted in rapid fusion of virus and RBC ghosts at 37 °C (results not shown).

The absence of cold fusion by PR/8 as well as the failure to commit to fuse indicates that at 4 °C this virus strain either does not undergo exposure of the fusion peptide or that there are obstacles at later steps of the cascade leading to fusion. To distinguish between these possibilities we examined conformational changes by monitoring susceptibility to proteinase K digestion, liposome binding, and immunoprecipitations with conformation-specific antibodies.

Susceptibility of X:31 and PR/8 to proteinase K (Sigma) digestion was tested as described (Puri et al., 1990). Fig. 2(a) shows that X:31 HA was cleaved by proteinase K following pretreatment to pH 5 at 37 °C, 15 °C and 4 °C. PR/8 virus, on the other hand, showed no proteolytic cleavage of HA at any temperature below 37 °C. In the case of X:31, the rapid susceptibility of HA to proteinase K, occurring well before the onset of membrane fusion (Schoch et al., 1992), permits this assay to be used to observe the extent of the intermediate conformational change (Sato et al., 1983; Doms et al., 1985). The lack of exposure of proteinase K-sensitive sites for PR/8 HA at the lower temperatures precluded the use of this method to observe the kinetics of the conformational change relevant for fusion. This finding is not unprecedented. The HA analogue HEF (HA, esterase, and fusion protein) of influenza C viruses also resists proteinase K digestion until well after membrane fusion has begun (Formanowski et al., 1990). HEF does undergo a conformational change, detectable by tryp-
Liposomes composed of egg lecithin/egg PE/NBD-PE (molar ratio 2/1/0.6) (PE, phosphatidylethanolamine; NBD, N-4-nitrobenz-2-oxa-1,3-diazole) were prepared by freeze-thaw extrusion (Rafalski et al., 1991). Liposome binding was performed as described (Stegmann et al., 1987). The binding of liposomes to PR/8 virus at 37 °C was maximal at the earliest time measured (2 min), whereas the interaction of liposomes was minimal at 4 °C (Fig. 3a). This was not the case for X:31 virus, which rapidly bound liposomes even at the low temperature (Fig. 3b). At 15 °C the pH-dependent binding of liposomes by PR/8 was slow, with a t½ of 20 min and a maximum observed after 75 min. The temperature-dependent time course of liposome binding by PR/8 virus closely reflected the kinetics of RBC ghost fusion, e.g. at 4 °C, at which there was no binding or fusion. Interestingly, X:31 virus appears to insert its fusion peptide into liposomes prior to the onset of membrane fusion at 4 °C (Stegmann et al., 1991), whereas at the same temperature little of the PR/8 fusion peptide inserts into model membranes (Brunner et al., 1991). These results and the correlation of liposome binding with RBC ghost fusion suggest that the major barrier for PR/8 virus fusion involves either exposure of the fusion peptide and/or subsequent interactions with lipid.

To determine whether the fusion peptide of PR/8 was exposed under the conditions of pH 5 and 4 °C, we used a conformation-specific antibody, 24C, raised against the sequence of HA2 residues 1 to 29 (which includes the fusion peptide) from X:31. Although the 24C antibody was generated against X:31, the conserved nature of amino acids 1 to 29 between X:31, PR/8 and Japan strains (GenBank) permits the use of this antibody to detect fusion peptide exposure for PR/8. This is evident by the successful immunoprecipitation of Japan strain HA (Puri et al., 1990). In addition, an antibody generated against a PR/8 HA trimer interface epitope (Yewdell et al., 1993) was used to confirm the low pH-induced conformational change. Immunoprecipitations of HA were performed as described by Kemble et al. (1992). Table 1 shows immunoprecipitation of PR/8 HA with conformation-specific anti-HA antibodies (24C and Y8-10C2) and liposome binding to the virus following exposure for 30 min at pH 5 and three different temperatures. Both the 24C and Y8-10C2 antibodies immunoprecipitated PR/8 HA following a low pH incubation at 4 °C, albeit with an efficiency of approximately 60 % compared to immunoprecipitations following incubation at 37 °C, pH 5. Although the total amount of immunoprecipitate was low, even at optimal pH and temperature, the 75 % immunoprecipitation with the 24C antibody was comparable to that measured for X:31 HA at pH 5 and 4 °C (approx. 8 %) (Stegmann et al., 1990). Moreover, two- to threefold less X:31 HA appears precipitable following treatment at low pH at 4 °C compared to 37 °C (J. White, personal communication). Therefore, our data

Fig. 2. Susceptibility to proteinase K digestion following low pH treatment. Influenza virus (a) X:31 or (b) PR/8 was exposed to pH 5 or pH 7 buffer for 30 min, re-neutralized, and incubated with proteinase K (PK). Proteins were precipitated with TCA and separated by SDS-PAGE. Protein bands were visualized by Coomassie blue staining. Lanes 1, pH 7, 37 °C and PK; lanes 2, pH 5, 4 °C and PK; lanes 3, pH 5, 15 °C and PK; lanes 4, pH 5, 37 °C and PK; lanes 5, untreated virus. NP, nucleoprotein; M, matrix protein.

Fig. 3. Influenza virus binding of liposomes at different temperatures. Virus (25 nmol) and liposomes (5 nmol) consisting of phosphatidylcholine/PE/NBD-PE (molar ratio of 1/1/0.6) were mixed and incubated at pH 5 at various temperatures for the times given. Samples were neutralized and centrifuged. Liposome binding (%) was determined as the percentage fluorescence in pellet relative to fluorescence in the pellet plus supernatant. Percentage of large unilamellar vesicles (LUV) bound was normalized to the maximal fluorescence level observed. (a) PR/8 virus plus liposomes were incubated at the following temperatures: 37 °C (□), 15 °C (○) and 4 °C (△). (b) X:31 virus plus liposomes were incubated at the following temperatures: 37 °C (○), 15 °C (□) and 4 °C (△). Note the different time scales for PR/8 and X:31.

tophan fluorescence and visual morphological changes, that coincides with the kinetics of membrane fusion (Formanowski et al., 1990). Binding of liposomes to HA requires the exposure of previously hidden hydrophobic sites (e.g. the fusion peptide) and accessibility of these sites to the liposomes. Liposomes composed of egg lecithin/egg PE/NBD-PE (molar ratio 2/1/0.6) (PE, phosphatidylethanolamine;...
immunoprecipitated (IP) according to: 100 × [IP at pH 5 (c.p.m.)−IP PR/8 HA]/protein A-agarose was added to all samples for 1 h to precipitate temperatures for 30 to 60 min at pH 5. Samples were re-neutralized and incubated with 24C (1:5 dilution) and Y8-10C2 (1:1 dilution) radioactivity was about 240 000 c.p.m./sample. Means and s.o. from a indicate that the commitment failure was not due to lack exposure of PR/8 HA at low temperature and pH, but that subsequent interaction of the fusion peptide with the target membrane appears to be a major barrier under these conditions.

What could the reason be for the apparent failure of PR/8 HA to interact with the target membrane at 4 °C and pH 5? The sequence of the N-terminal HA2 of X:31 (GFLGAIAGFIEKGEMIGDGYGF−) differs from that of PR/8 only in positions 12 (N → G) and 15 (E → T). Since the substitutions are on the polar surface of the fusion peptide amphipathic helix (White, 1990), they are not likely to affect liposome binding or insertion.

The observations of Wharton et al. (1988) and Rafalski et al. (1991) that a polar mutation in position 4 (E → G) of a synthetic peptide of the above sequence did not affect its binding and insertion into lipid is consistent with this notion. It seems unlikely that the fusion peptide of PR/8 per se is responsible for this block. The competition for association of the fusion peptide with either the viral or the target membrane surface may play an important role in the insertion process. Alternatively, portions of the PR/8 HA outside of the fusion peptide which differ from X:31 HA may prevent insertion of the latter region despite its exposure. Studies are underway to examine the insertion of the HA1 of the X:31 and PR/8 strains into biological membranes.

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### Table 1. Low pH-induced conformational changes of PR/8 HA

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>24C</th>
<th>Y8-10C2</th>
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<tbody>
<tr>
<td>4</td>
<td>7.5 ± 21 (2)</td>
<td>10.5 ± 31 (4)</td>
</tr>
<tr>
<td>15</td>
<td>9.3 ± 04 (2)</td>
<td>11.8 ± 21 (4)</td>
</tr>
<tr>
<td>37</td>
<td>13.5 ± 52 (2)</td>
<td>17.5 ± 13 (4)</td>
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</tbody>
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* Samples of 125I-labelled PR/8 HA were incubated at the indicated temperatures for 30 to 60 min at pH 5. Samples were re-neutralized and incubated with 24C (1:5 dilution) and Y8-10C2 (1:1 dilution) antibodies for 2 to 3 h at room temperature or 4 °C, respectively. Protein A-agarose was added to all samples for 1 h to precipitate antibodies, washed twice, and radioactivity was counted. Input radioactivity was about 240000 c.p.m./sample. Means and s.d. from a number of experiments (in parentheses) were calculated as percentage immunoprecipitated (IP) according to: 100 × [IP at pH 5 (c.p.m.)−IP at pH 7.4 (c.p.m.)/input c.p.m.].

† PR/8 virus binding to liposomes was measured following exposure to pH 5 for 30 to 60 min under conditions described in the legend to Fig. 3. Means and s.d. from a number of experiments (in parentheses) were calculated as percentage bound according to: [100 × (fluorescence in pellet at pH 5−fluorescence in pellet at pH 7.4)/total fluorescence (pellet + supernatant)].

References


Tsurudome, M., Gluck, R., Graf, R., Falchetto, R., Schaller, U.


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