Low pH-induced Conformational Change of Rubella Virus Envelope Proteins

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SUMMARY

Fusion of rubella virus-infected cells was induced by their brief treatment at pH below 6.0. Exposure of rubella virus to pH 5 caused an irreversible conformational change of the viral envelope glycoproteins, E1 and E2. The change was manifested in the marked reduction in both infectivity and haemagglutinating activity of the virus, the increased resistance of E1 and decreased resistance of E2 polypeptides to proteolytic digestion with trypsin, and the acquisition of liposome-binding activity of the virus. The above changes are presumed to mimic the events occurring in the acidic environment within endosomes following endocytosis of the virus.

INTRODUCTION

Rubella virus has been shown to be composed of three polypeptides, two envelope glycoproteins E1 and E2, and one capsid protein (C) (Oker-Blom et al., 1983; Waxham & Wolinsky, 1983) and its structure closely resembles that of the alphaviruses, another group of Togaviridae, which include Semliki Forest virus (SFV) (Ziemiecki & Garoff, 1978) and Sindbis virus (Dalrymple et al., 1976).

Upon binding to cell surface receptors SFV is internalized in coated pits and coated vesicles, and transported to the endosomal compartment. The exposure to acidic pH in the endosomes triggers a conformational change in the spike glycoproteins of the incoming virus particles, and these altered glycoproteins mediate the fusion of the viral membrane with the endosomal membrane. As a result of the fusion reaction the nucleocapsid is released into the cytoplasm to initiate infection. The virus spikes that are integrated into the endosomal membrane during fusion, and any unfused virus particles, are subsequently transported to the lysosomes and degraded (Helenius et al., 1980).

When SFV was exposed to low pH, the E1 polypeptide became resistant to, and the E2 sensitive to, trypsin in the presence of Triton, both in vitro and in the endosomes of infected cells (Kielian & Helenius, 1984, 1985). In Sindbis virus, at pH 7, a certain hidden epitope of E1 became reactive with a number of monoclonal antibodies (MAbs) after low pH treatment (Schmaljohn et al., 1983), and E2 became sensitive to trypsin digestion (Edwards et al., 1983).

The penetration and uncoating processes of rubella virus, however, have not been studied. The similarity of the penetration process of rubella virus to that of SFV was suggested when it was found that the fusion and haemolytic activities of rubella virus are induced by acidic pH (Kobayashi, 1978; Väänänen & Kääriäinen, 1978).

In this paper we report that fusion of rubella virus-infected cells and an irreversible conformational change of viral envelope polypeptides are induced by low pH and during the process the virus acquires liposome-binding activity. These results suggest that rubella virions fuse with host cell membrane in an acidic environment in the same way as alphaviruses do.

METHODS

Virus and cells. The M33 strain of rubella virus and the baby hamster kidney cell line BHK-21 were used. BHK-21 cells were propagated in Eagle's MEM supplemented with 5% calf serum and 10% tryptose phosphate broth.
Confluent monolayers of BHK-21 cells were infected with approximately 1 p.f.u. of rubella virus per cell and were overlaid with Eagle's MEM containing 2% calf serum and 10% tryptose phosphate broth. Infected culture fluid was harvested daily from day 2 to day 7 and replaced with fresh MEM containing 0-2% bovine serum albumin (BSA). Purification of virus was done as described previously (Katow & Matsuno, 1980), with a slight modification. Briefly, after a low speed centrifugation to remove the cell debris, virus in the culture fluid was precipitated with 10% polyethylene glycol and purified by centrifugation through a 15 to 50% (w/w) linear sucrose gradient at 25000 r.p.m. for 16 h in a Beckman SW28 rotor. Purified virus was suspended in TES (10 mM-Tris-HCl pH 7.5, 1 mM-EDTA, 100 mM-NaCl). Haemagglutination (HA) activity of the virus was assayed by a microtitre method using goose red blood cells at 4°C (Inouye et al., 1978).

**Radiolabelling.** Metabolic labelling of virus was done with $^{35}$S-methionine (1134 Ci/mmol; DuPont/New England Nuclear) in BHK-21 cells, and virus was purified as previously described (Katow & Sugiura, 1985). For trypsin digestion, to be described below, $^{35}$S-methionine-labelled virus was mixed with approximately 512 HA units of unlabelled virus, so that the digestion was carried out at a fixed ratio of virus protein to trypsin concentrations.

Purified virus was labelled *in vitro* with Na$^{125}$I (15.0 mCi/ml of 0.01 M-NaOH; ICN Radiochemicals), using Iodogen (Pierce Chemicals, Rockford, Ill., U.S.A.) according to Markwell & Fox (1978). Labelled virus was separated from non-incorporated Na$^{125}$I by passing the mixture through an Excellulose GF-5 desalting column (Pierce Chemicals) using TES as the eluant (Laemmli, 1970). After autoradiography, bands corresponding to viral polypeptides were excised from the gel and radioactivity was determined by the auto well gamma system ARC-500 (Aloka, Tokyo, Japan). Approximately 75% of the radioactivity was associated with the E1 polypeptide. Aliquots of labelled virus were stored at -80°C until use.

To prevent the non-specific adsorption of labelled viral materials, all plastic tubes, pipette tips and ultracentrifuge tubes were pre-coated with silicone and BSA.

**Antiserum.** MAb 85, 103, 104 (Umino et al., 1985) and 48B1 (Y. Umino, unpublished data) against the E1 polypeptide were used. They all reacted with distinct non-overlapping antigenic sites of the E1 polypeptide. The MAb against the E2 polypeptide (E2-1) (Waxham & Wolinsky, 1985) was a gift from Dr J. S. Wolinsky. Rabbit anti-E1 serum was prepared by immunizing a rabbit with the E1 polypeptide recovered from a slab gel after SDS-PAGE of purified rubella virus, M33 strain, according to Kalkkinen et al. (1984). Human rubella convalescent serum with a haemagglutination inhibition titre of 1:1024 was also used.

**Cell fusion.** BHK-21 cells in 12-well plates (Costar) were infected with rubella virus at an m.o.i. of approximately 10 p.f.u./ml and cultured for 3 days. The infected cells were exposed for 15 min at 37°C to the fusion medium, i.e. Eagle's MEM containing 0-2% heat-inactivated foetal bovine serum containing either 10 mM-MES (for pH 4-5 to 6-5) or 10 mM-HEPES (for pH 7-0) instead of sodium bicarbonate. The pH was adjusted by the addition of sodium hydroxide (White et al., 1981). After exposure to a desired pH, the fusion medium was replaced with medium at pH 7 and cells were incubated for an additional 3 h at 37°C. Cells were examined and photographed under a phase-contrast microscope; the extent of cell fusion was evaluated as described by Edwards & Brown (1986).

**Treatment at pH 5.** An aliquot of radiolabelled or unlabelled virus was acidified to pH 5 with 0.1 M-citric acid and kept at 37°C for 15 min, unless stated otherwise. The virus was then neutralized with 0.5 M-Tris-HCl pH 7.4. As a control for pH 5 treatment, the same volume of a pre-neutralized mixture of 0.1 M-citric acid and 0.5 M-Tris-HCl pH 7-4 was added to the virus.

**Trypsin digestion.** TPCK-trypsin (type XIII; Sigma) was dissolved in phosphate-buffered saline (PBS) pH 7-4. One-fourth volume of trypsin of an appropriate concentration was added to each virus sample. (Trypsin concentration in the text and figures is given as its final concentration in a reaction mixture.) The virus–trypsin mixture was kept at 37°C for 30 min and the reaction was stopped by the addition of one-fourth volume of trypsin inhibitor mixture. The concentration of trypsin inhibitors was proportional to the concentration of trypsin; for 1 mg/ml trypsin, soybean trypsin inhibitor was 1 mg/ml, aprotinin was 5 trypsin inhibition units/ml, and PMSF (Sigma) was 2.5 mM. Trypsin-treated virus in a volume of 100 μl was layered on the top of a discontinuous sucrose gradient consisting of 450 μl of 15%, and 50 μl of 50% sucrose (w/v in TES) in a 700 μl ultracentrifuge tube (Beckman no. 344090). After centrifugation at 38000 r.p.m. for 2 h at 4°C in a Beckman SW50.1 rotor, six 100 μl fractions were taken from the bottom and counted for radioactivity. Virions were recovered in the bottom fraction and released components in the top.

**Immunoprecipitation and SDS-PAGE.** Immunoprecipitation of detergent-disrupted virions and SDS-PAGE for detection of virus-specific polypeptides were done as previously described (Katow & Sugiura, 1985), Antiserum at a dilution of 1:10 in PBS were used for immunoprecipitation. After electrophoresis gels were dried and fluorographed (Bonner & Laskey, 1974). The fluorogram of trypsin-treated $^{35}$S-methionine-labelled virions was scanned in a Model 620 video densitometer (Bio-Rad). The areas under the absorbance peaks of individual specific bands of E1, E2 and C polypeptides and a cleavage product of E1 polypeptide were determined by densitometric tracing.
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**Liposome-binding assay.** Liposomes were freshly prepared from phosphatidylcholine for each experiment as described by Doms *et al.* (1985). Five μl of pH 7- or pH 5-treated 125I-labelled virus was mixed with 45 μl of liposome suspension in TES and kept at 37 °C for 15 min. The virus–liposome mixture was placed in a 700 μl tube and the solution was brought to 50% sucrose by mixing with 150 μl of 67% sucrose (w/w in TES). This was then overlaid with 200 μl each of 25% and 10% sucrose (w/w in TES). After centrifugation at 38000 r.p.m. for 2.5 h at 4 °C in an SW50.1 rotor, six 100 μl fractions were taken from the top by careful pipetting from the liquid–air interface. Fractions were counted for 125I content in the auto well gamma system. Radioactivity in the top half of the tube was defined as bound to liposomes, and that in the lower half unbound. For the liposome flotation marker, liposomes containing 1-3-phosphatidylcholine, 1,2-di[14C]palmitoyl (117 mCi/mmol; Amersham) were centrifuged in parallel. 14C content was determined in a Beckman LSC 100 liquid scintillation counter.

**RESULTS**

Low pH-induced cell fusion of rubella virus-infected cells

Fusion activity of Sindbis virus is reported to be maximal at pH 4.0 to 5.3 (Edwards & Brown, 1986). Haemolysis by rubella virus was studied at pH 6-4 (Kobayashi, 1978), but was found to increase at lower pH (Väänänen & Kääriäinen, 1980). Unlike the cell fusion by Sindbis virus, which occurs within 1 h after addition of the virus to cells (fusion from without) and exposure to low pH, cell fusion by rubella virus was very inefficient, observed only as fusion from within, i.e. in low pH-exposed virus-infected cells, and it took 3 h to be visualized after exposure to low pH (Fig. 1). Fusion occurred at pH 6 or below, its extent increasing with decreasing pH until a plateau was reached at pH 5 (Fig. 2).

Effects of pH 5 treatment and trypsin on HA activity

Treatment of rubella virus at pH 5 resulted in a rapid decline in HA activity; a four- or eightfold reduction was observed in less than 2 min (Fig. 3). The initial rapid drop was followed by a gradual further fourfold decline during 30 min. In all subsequent experiments, the duration of pH 5 exposure was 15 min. The exposure of the virus to pH 5 for 15 min caused a 500-fold drop in infectivity and a slight decrease in the sedimentation velocity (data not shown). However, pH 5-treated virions sedimented in a single peak, indicating that the reduced HA activity and infectivity were not due to aggregation of the treated virions.

HA activity of pH 7- and pH 5-exposed virus was equally sensitive to trypsin (Fig. 4).

Change in trypsin sensitivity of E1 and E2 polypeptides

Unlike the internal protein protected by the viral envelope, envelope proteins are subject to the degradative effect of externally added proteolytic enzymes, depending upon their conformation. If a low pH-induced irreversible conformational change occurs in the spike protein composed of heterodimers of E1 and E2 polypeptides, altered sensitivity of E1 and E2 polypeptides to proteolytic enzymes might follow the exposure of virions to low pH, as was found with alphaviruses (Edwards *et al.*, 1983; Kielian & Helenius, 1985). The effect of trypsin on [35S]methionine-labelled rubella virions was thus studied.

As expected, the C polypeptide was resistant up to 5 mg/ml of trypsin, whether or not virions were exposed to pH 5 (Fig. 5 and 6). On the other hand, the E1 polypeptide of control (pH 7-exposed) virions almost completely disappeared after the treatment with 50 μg/ml of trypsin, while the E2 polypeptide was slightly more resistant (Fig. 5a and 6a). It should be noted that, as will be described below, the polypeptide band of M, 41K derived from the E1 polypeptide overlapped with the faster migrating part of the E2 polypeptide, increasing the apparent density of the latter. The differential susceptibility of E1 and E2 polypeptides under neutral conditions had been reported previously (Ho-Terry & Cohen, 1980). The exposure to pH 5 markedly changed the trypsin digestion pattern. The E1 polypeptide became as resistant as the C polypeptide, withstanding up to 5 mg/ml of trypsin (Fig. 5b and 6b). By contrast, the E2 polypeptide became more susceptible, with more than 50% being removed by 0.8 μg/ml of trypsin (Fig. 6b). As the E2 polypeptide band partially overlapped with the 41K E1 cleavage band, the area of the lane which corresponded to the latter (Fig. 5) was excluded from the E2 band in densitometric tracing.
Fig. 1. Fusion of rubella virus-infected cells. BHK-21 cells were infected with rubella virus and cultured for 3 days. The infected cells were exposed to fusion medium pH 5 or pH 7 for 15 min at 37 °C. The fusion medium was then replaced with pH 7 medium and cultures were incubated for 3 h at 37 °C. Monolayers were photographed under a phase contrast light microscope. (a) No fusion after exposure to pH 7 medium. (b) Cell fusion after exposure to pH 5 medium. Bar markers represent 100 μm.
Polypeptide species, whether virion-associated or released into the supernatant, generated from control and low pH-exposed virions by trypsin digestion were essentially similar, indicating that trypsin cleaves at the same sites of E1 and E2 of either virion preparation. Among newly generated polypeptides, the virion-associated 41K polypeptide was found to be derived from the E1 polypeptide by its precipitation by rabbit anti-E1 serum (Fig. 7a, lane 3) and different anti-E1 MAbs [Fig. 7a, lane 4 for MAb 85 (data not shown for the other three)]. A released polypeptide migrating as a broad band with an \( M_r \) ranging from 43K to 55K was also
Fig. 5. SDS–PAGE of trypsin-treated rubella virus. 

$[^{35}S]$Methionine-labelled rubella virus was exposed to either pH 7 (a) or pH 5 (b) and then treated with different concentrations (µg/ml) of trypsin (lane 1, 0; lanes 2 and 7, 0.8; lanes 3 and 8, 3.2; lanes 4 and 9, 12.8; lanes 5 and 10, 51.2; lanes 6 and 11, 200). Trypsin-treated virus was separated into pellet (lanes 1 to 6) and supernatant (lanes 7 to 11) fractions by centrifugation through a sucrose cushion as described in Methods. The pellet and supernatant were analysed by SDS–PAGE. The number on right denotes $M_r$ ($\times 10^{-3}$).

E1-derived (Fig. 7a, lane 7). No E2-derived polypeptide was identified by use of the single anti-E2 MAb (E2-1) available (Fig. 7a, lane 5).

The 105K polypeptide (Fig. 5) corresponded to a heterodimer of E1 and E2 (Waxham & Wolinsky, 1983). The newly generated 87K polypeptide (Fig. 5a; Fig. 7, lane 3; Fig. 8, lanes 2 and 3) was thought to be derived from the heterodimer of E1 and E2 and/or the homodimer of E1, because of its precipitation with anti-E1 serum (Fig. 7, lane 3) and its intense $^{125}$I-labelling (Fig. 8, lanes 2 and 3, see below).

**Digestion of $^{125}$I-labelled rubella virus by trypsin**

That the same species of cleavage products are generated from the E1 polypeptide by trypsin digestion was also shown with $^{125}$I-labelled rubella virus (Fig. 8). When intact rubella virions were labelled with Na$^{125}$I, radioactivity was incorporated predominantly into the E1 polypeptide (Fig. 8, lane 1). $^{125}$I-labelled virions were exposed to either pH 7 or pH 5 and then treated with trypsin. The most abundant products were the 41K virion-associated polypeptide.
Fig. 6. Susceptibility of rubella virus polypeptides to trypsin after pH 7 or pH 5 treatment. [35S]Methionine-labelled rubella virus was exposed to either pH 7 (a) or pH 5 (b) and treated with the indicated final concentrations of trypsin. The pellet separated as described in Methods was analysed by SDS-PAGE. Individual polypeptides and the 41K cleavage product of E1 polypeptide shown in Fig. 7(b) were scanned with a densitometer on fluorograms. The areas under the respective absorbance peaks were expressed in an arbitrary scale. As the E2 polypeptide band partially overlapped with the 41K E1 cleavage product band, the area corresponding to the latter was excluded from the E2 band for densitometry. The results from two experiments were combined. ○, E1; △, E2; □, C; ●, 41K cleavage product of E1.

Fig. 7. Immunoprecipitation of trypsin-treated rubella virus. (a) pH 7-exposed [35S]methionine-labelled rubella virus was treated with trypsin at a final concentration of 200 μg/ml for 30 min. Trypsin-treated virus was separated into pellet and supernatant fractions as described in Methods. The supernatant and detergent-disrupted pellet were then subjected to immunoprecipitation. Lane 1, rubella virus without trypsin treatment; lane 2, pellet fraction after trypsin treatment; lanes 3 to 5, detergent-disrupted pellet fraction was immunoprecipitated with rabbit anti-E1 serum (lane 3), anti-E1 MAb 85 (lane 4) or anti-E2 MAb E2-1 (lane 5); lane 6, supernatant fraction after trypsin treatment; lane 7, supernatant fraction immunoprecipitated with rabbit anti-E1 serum; lane 8, M markers. (b) Schematic drawing of the electrophoretic pattern of E1-related polypeptide bands. The horizontal line at the top indicates the origin. Solid bands indicate polypeptide bands with high radioactivity and open bands indicate those with low radioactivity. Lane 1 was constructed from lane 1 in (a), lane 2 from lane 3 in (a) and lane 3 from lane 7 in (a).
Fig. 8. SDS-PAGE of $^{125}$I-labelled rubella virus. Rubella virus radiolabelled in vitro with $^{125}$I was exposed to either pH 7 or pH 5 and treated with trypsin at a final concentration of 100 μg/ml for 30 min. Trypsin-treated virus was separated into pellet and supernatant fractions as described in Methods. The detergent-disrupted pellet and the supernatant to which detergents were added were subjected to immunoprecipitation with human convalescent serum. Lane 1, $^{125}$I-labelled virus; lanes 2 to 5, detergent-disrupted immunoprecipitated materials from pellet (lane 2) and supernatant (lane 4) of pH 5-exposed and trypsin-treated virus and pellet (lane 3) and supernatant (lane 5) of pH 7-exposed and trypsin-treated virus.

and the approximately 50K released polypeptide fragment, irrespective of whether the virions were exposed to pH 7 or pH 5, as in the metabolically labelled virions described in the preceding section.

Liposome-binding activity of pH 5-exposed virus

Low pH-induced conformational change of the spike proteins of rubella virus would be expected to reflect the fusion mechanism of the virus with the cell membrane during its entry into the host cell, in the same manner as Sindbis, Semliki Forest and influenza viruses. We therefore studied liposome-binding activity of low pH-exposed rubella virus.

When the pH 5-exposed virions were mixed with liposomes, approximately 40% became adsorbed to them (Fig. 9c). No liposome-binding activity was detected in control virions (Fig. 9b). When control virions and liposomes were mixed and then exposed to pH 5 for 15 min at 37 °C, 47% of the virions adsorbed to liposomes. As well as the liposome-binding activity, pH 5-exposed virion showed enhanced binding to plastic tubes (data not shown). These activities may indicate that the conformational change caused by pH 5 treatment renders the virus amphiphilic and capable of fusing with the cell membrane.

DISCUSSION

Acid-induced conformational change in the spike proteins of enveloped viruses is believed to be part of the mechanism by which viral genetic material enters the cells via the acidic environment of intracellular vesicles. The change in the haemagglutinin protein of influenza
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Rubella virus has so far been the most intensively studied. Among phenomena associated with the acid-induced conformational change of the bromelain-cleaved ectodomain of a haemagglutinin spike are the increased susceptibility to proteolysis with trypsin and proteinase K, acquisition of a liposome-binding property, and a change in antigenic properties (Skehel et al., 1982; Doms et al., 1985). The alphaviruses, Sindbis virus and SFV, are known to undergo an acid-induced conformational change, which is manifested in altered trypsin sensitivity of their E1 and E2 envelope glycoproteins (Edwards et al., 1983; Kielian & Helenius, 1985). With Sindbis virus this antigenic change, which can be recognized with some MAbs, was reported to occur on the E1 polypeptide after a low pH treatment (Schmaljohn et al., 1983). Rubella virus, like the above-mentioned viruses, also acquires haemolytic and fusion activity during a brief exposure to low pH (Kobayashi, 1978; Väänänen & Kääriäinen, 1980). It is expected, therefore, that the spike proteins of the virus are subject to a similar conformational change during this process.

The present study has shown for the first time for rubella virus that an irreversible change in the glycoproteins occurs after exposure to low pH, at which fusion of virus-infected cells is induced, and that virions become amphiphilic during acid exposure. The change was manifested in the reduced infectivity and HA activity and in the altered susceptibility of E1 and E2 glycoproteins to tryptic cleavage. Normally the E1 polypeptide is more readily degraded by trypsin than the E2 polypeptide (Ho-Terry & Cohen, 1980). Higher trypsin sensitivity and preferential iodination of the E1 compared with the E2 polypeptide (Fig. 8) may indicate the former is outermost in the spike. Acid treatment reversed the relative trypsin sensitivity, making

Fig. 9. Liposome-binding of pH 7- or pH 5-exposed rubella virus. 125I-labelled rubella virus exposed to pH 7 (b) or pH 5 (c) was mixed with phosphatidylcholine liposomes and separated into liposome-bound and non-bound fractions by floating through a discontinuous sucrose gradient during centrifugation as described in Methods. Radioactivity in the fractions was determined in a γ-counter. Liposomes containing 14C-labelled phosphatidylcholine alone (a) were centrifuged in the same way as the liposome flotation marker. ●, Virions without liposomes; ○, virions mixed with liposomes. Arrows 1 and 2 indicate the bottom and top of the centrifuge tube respectively (see Methods).
the E1 polypeptide very resistant and the E2 polypeptide very susceptible. This finding suggests that the conformational change makes the E1 polypeptide less exposed and the E2 polypeptide more exposed on the surface of a spike. The decrease in trypsin sensitivity of the E1 and increase in sensitivity of the E2 polypeptide are also features of acid-induced conformational change in both Sindbis virus and SFV (Edwards et al., 1983; Kielian & Helenius, 1985). In the haemagglutinin protein of influenza virus, acid-induced conformational change uncovered hitherto inaccessible sites for trypsin cleavage in the HA1 subunit (Skehel et al., 1982). In contrast, no new cleavage sites appeared to be exposed in the E1 polypeptide of rubella virus as a result of conformational change, because the same cleavage products, though in different amounts, were generated from control and low pH-exposed virions. The change in trypsin sensitivity is therefore thought to be quantitative rather than qualitative.

The 41K polypeptide derived from E1 is likely to have resulted from the cleavage at lysine 110 in the reported amino acid sequence (Frey et al., 1986) because it remained anchored in the envelope after cleavage.

Liposome-binding activity acquired by pH 5-exposed rubella virus, and the fusion of rubella virus-infected cells induced by exposure to the same pH, are thought to be as relevant to the rubella virus entry mechanism through membrane fusion in the acidic endosomal compartment as similar changes in SFV and influenza virus are to their respective modes of entry (White & Helenius, 1980; Doms et al., 1985). Although E1 and E2 polypeptides are tightly associated within a spike and are both subject to the acid-induced conformational change, the E1 polypeptide appears to play a dominant role, since a preliminary experiment showed that 38% of pH 5-exposed, trypsin (40 μg/ml)-treated virions, from which most E2 polypeptides should have been removed, still bound to liposomes.

By immunoprecipitation studies, at least with the limited number of antisera and MAbs available, we found that there was no indication that rubella virions had been antigenically altered by acid treatment (data not shown).

REFERENCES


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